

Fig. 42

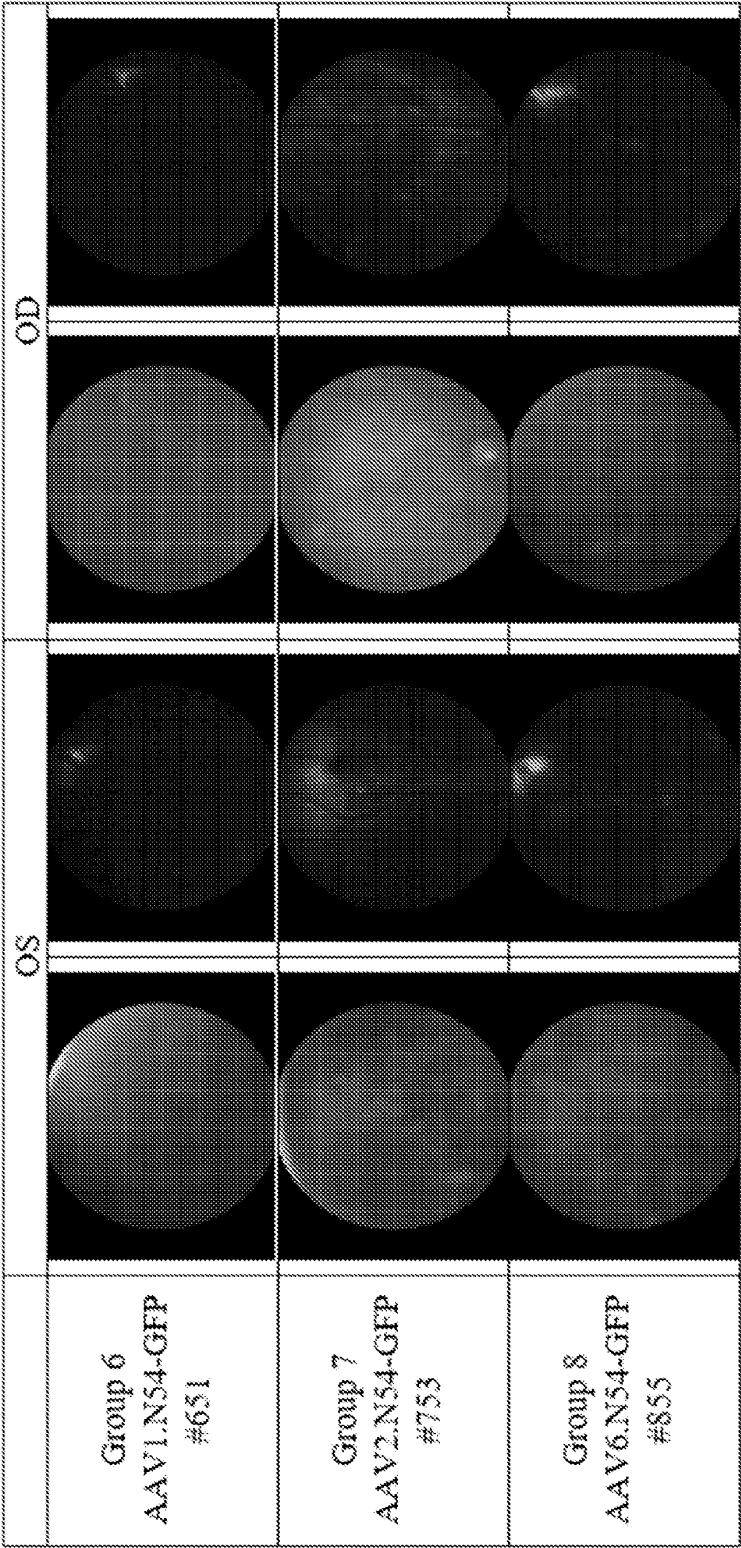


Fig. 43

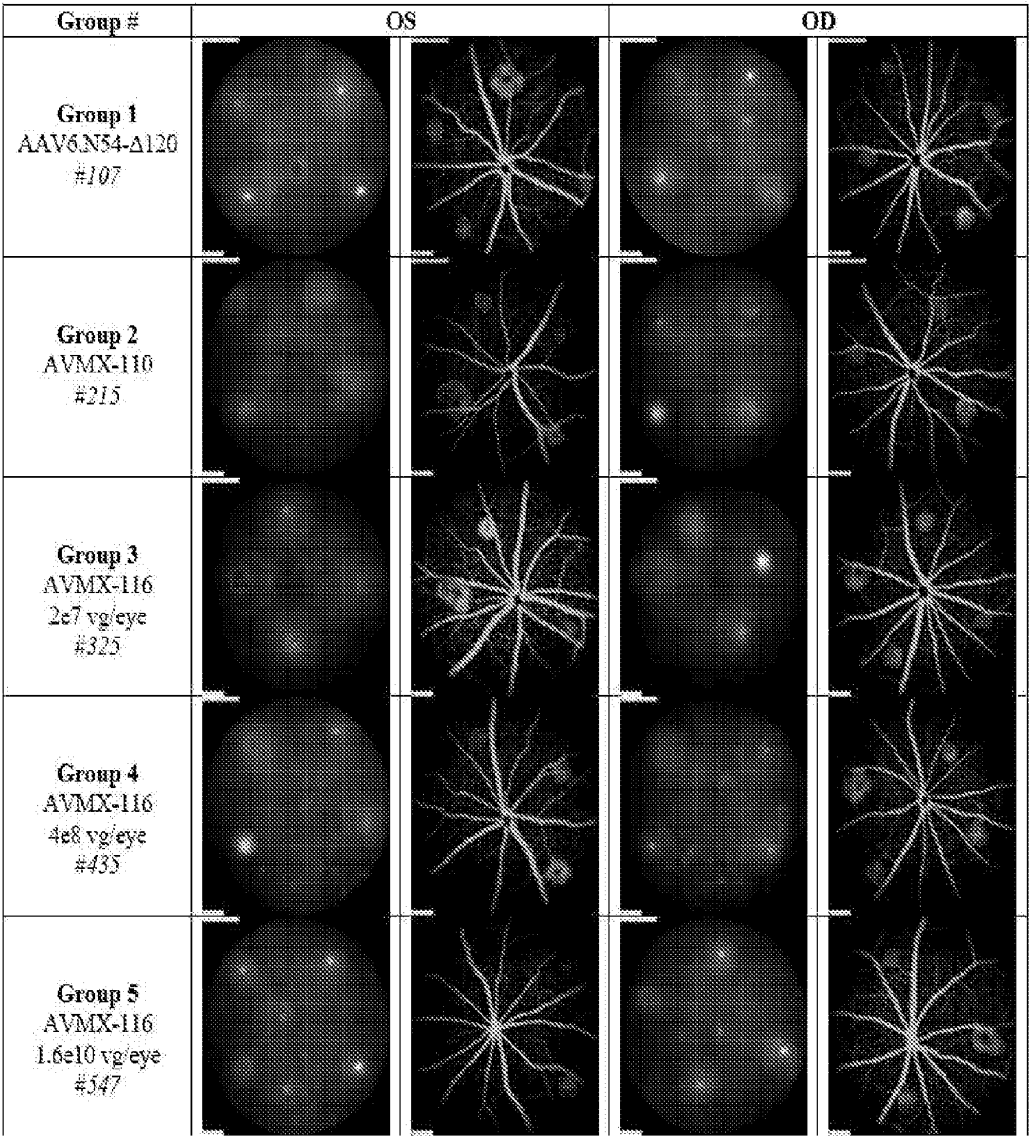


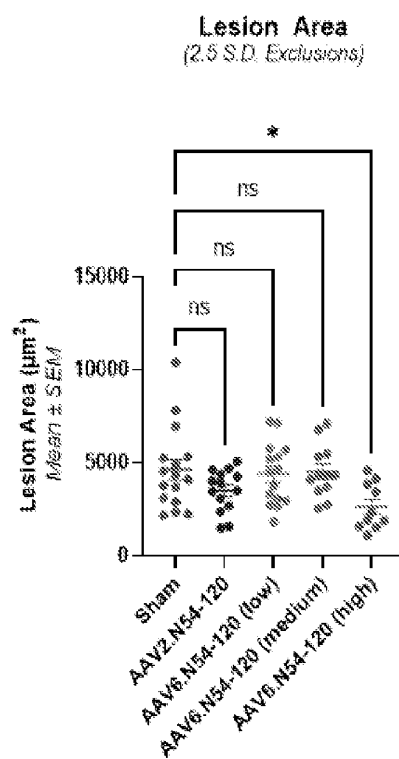
Fig. 44

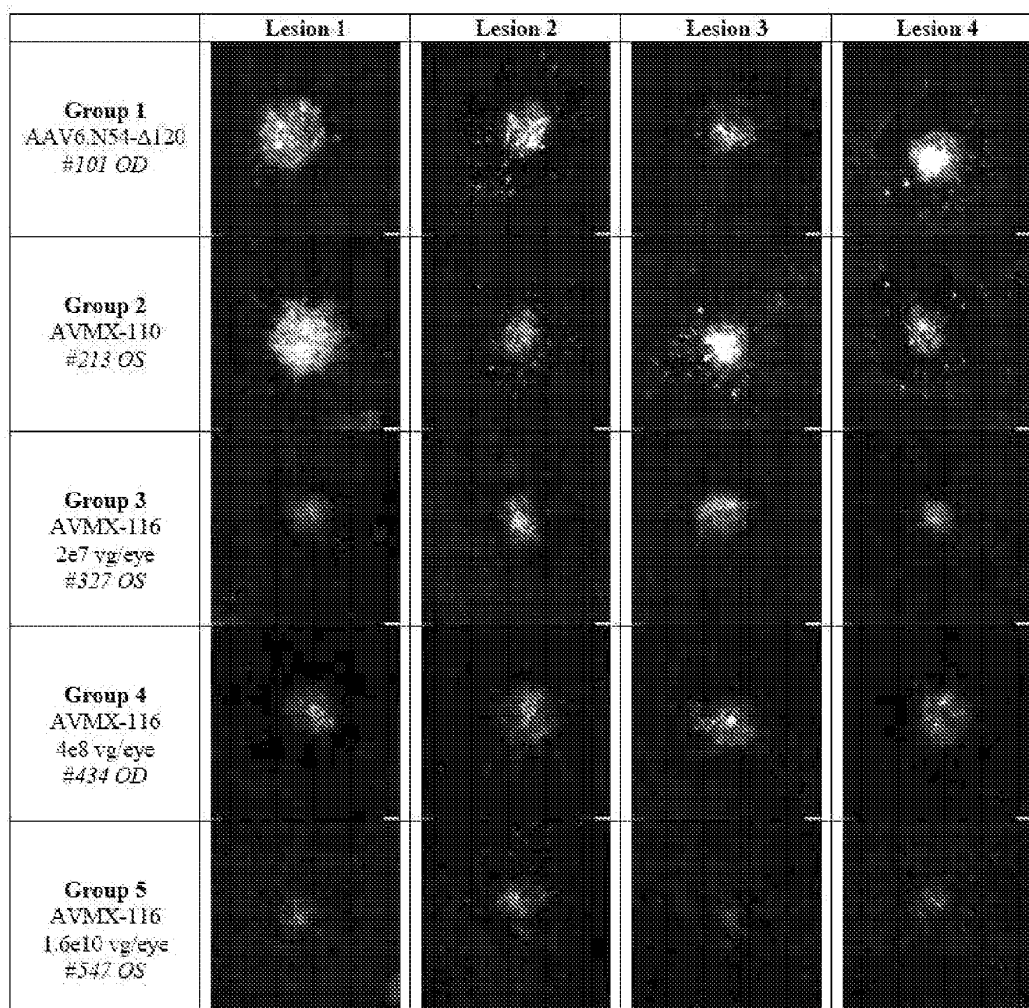
Fig. 45

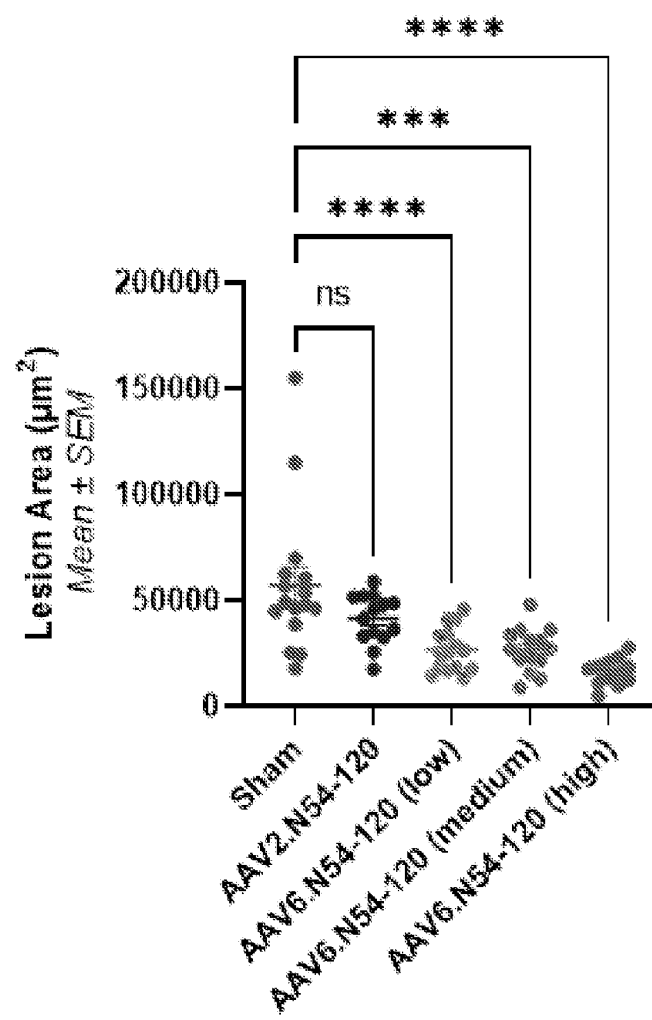
Fig. 46

Fig. 47

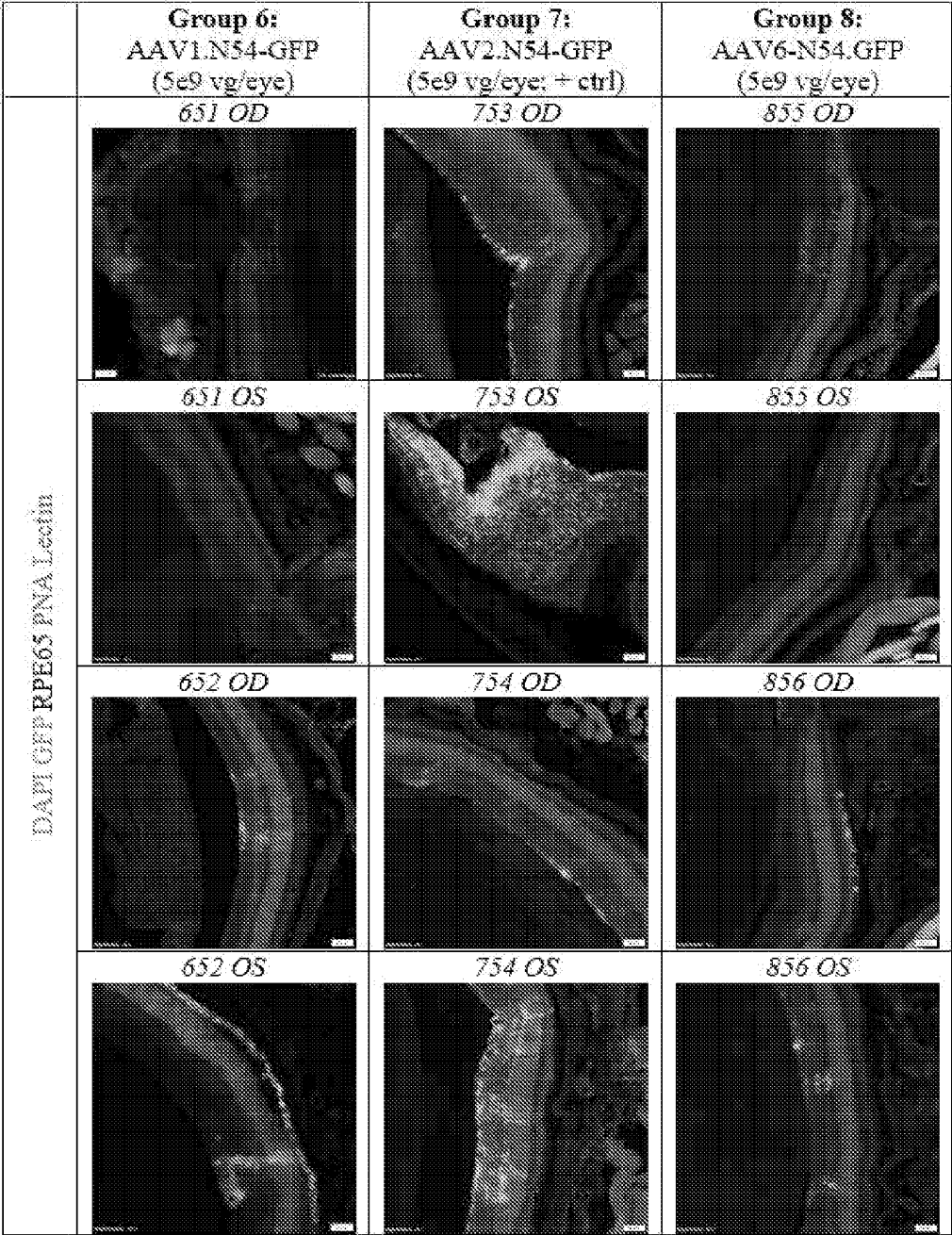


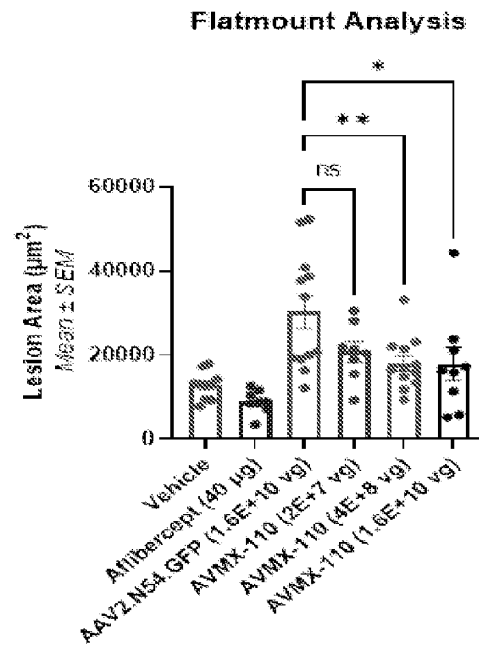
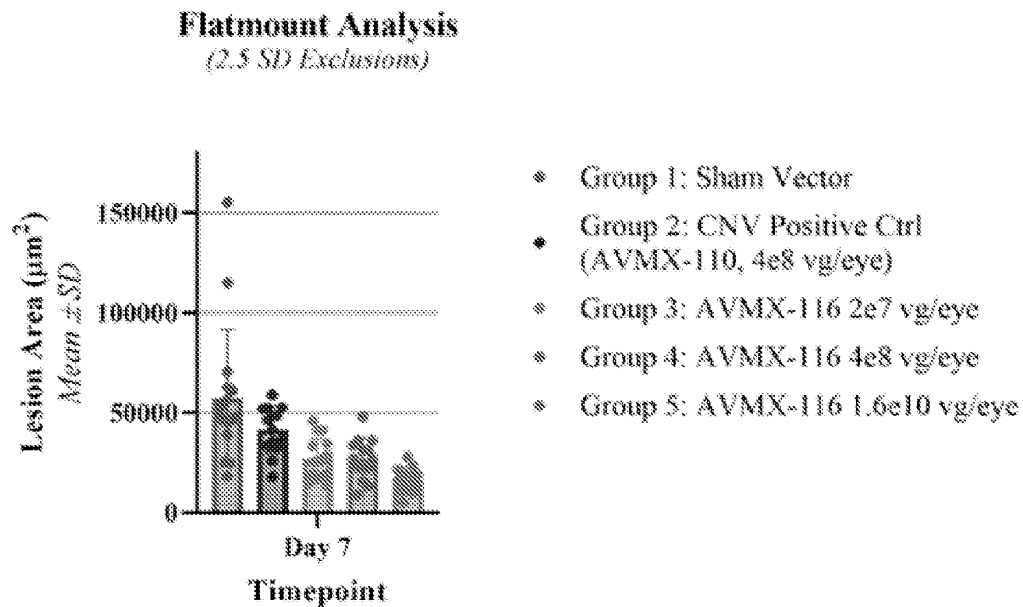
Fig. 48A**Fig. 48B**

Fig. 49A

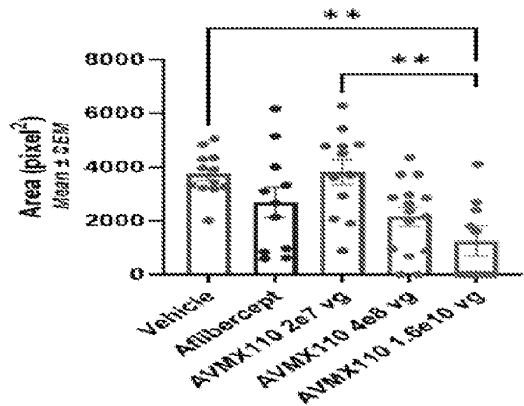


Fig. 49B

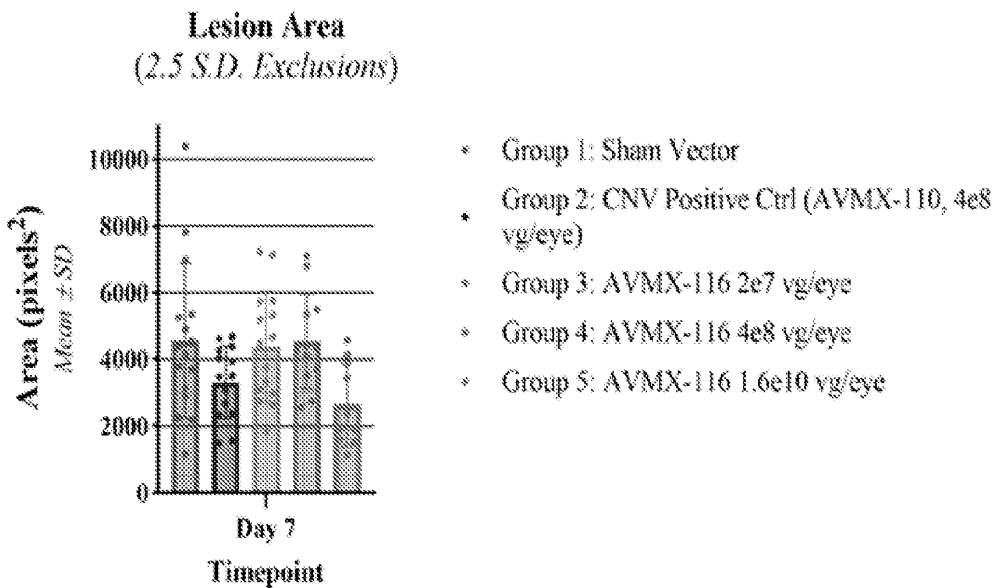


Fig. 50

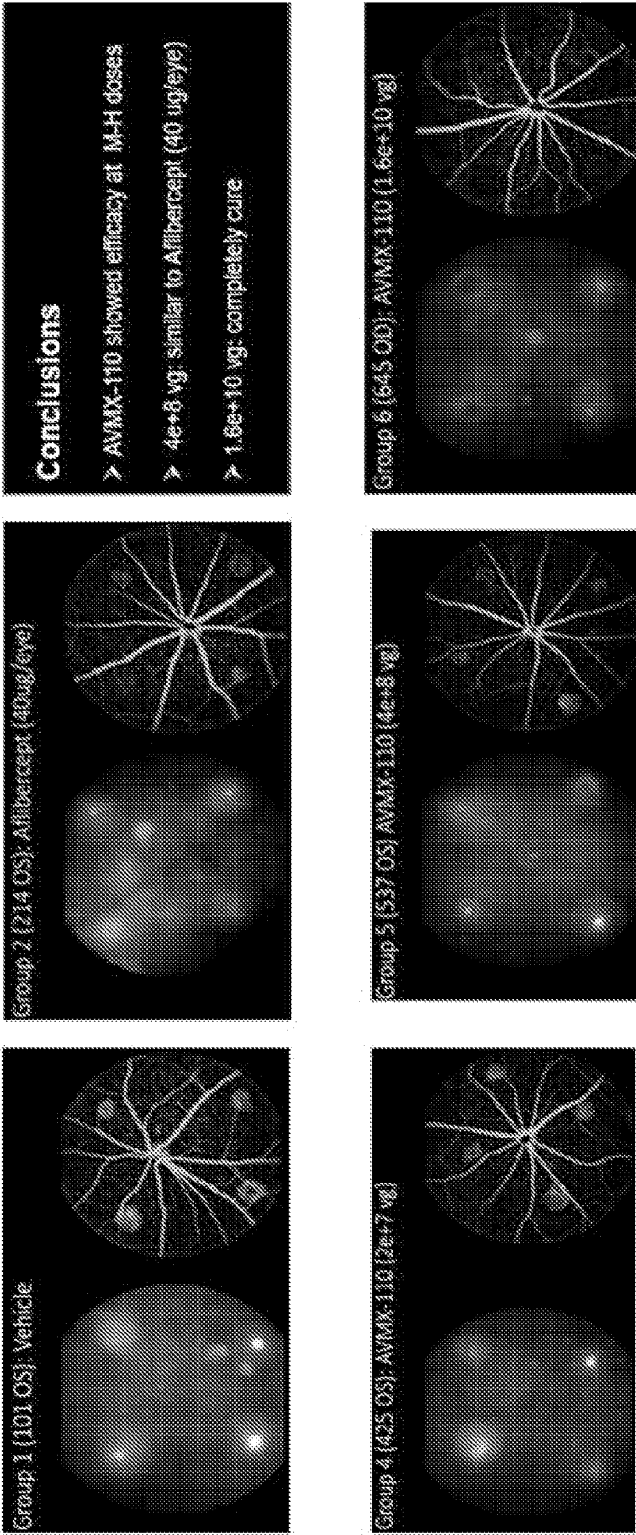
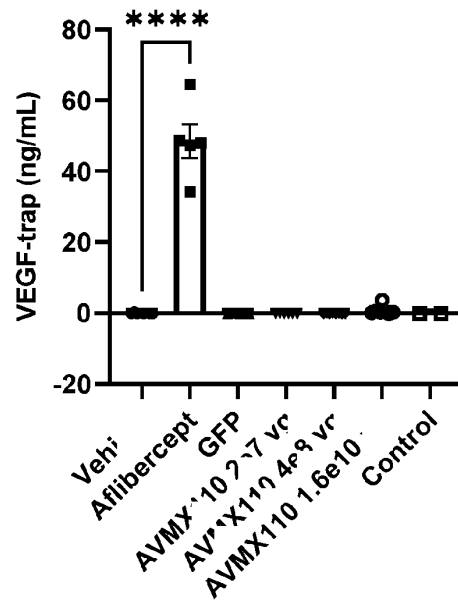
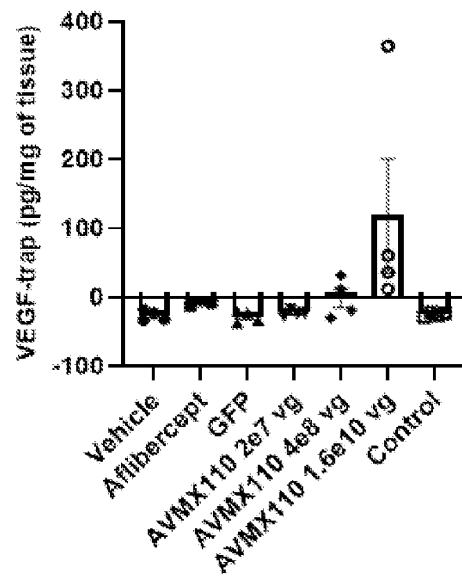


Fig. 51A**Fig. 51B**



(11)

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(12)

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(54) **ENTACAPONE-RELATED COMPOUNDS TO TREAT MACULAR DEGENERATION**

ENTACAPON-ASSOZIIERTE VERBINDUNGEN ZUR BEHANDLUNG VON
MAKULADEGENERATION

COMPOSÉS APPARENTÉS À L'ENTACAPONE POUR TRAITER LA DÉGÉNÉRESCENCE
MACULAIRE

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- **YONEKAWA, YOSHIHIRO et al.:** "Clinical Characteristics and Current Treatment of Age-Related Macular Degeneration", COLD SPRING HARB PERSPECTMED, vol. 5, no. 1, 3 October 2014 (2014-10-03), XP055591946, ISSN: 2157-1422, DOI: 10.1101/cshperspect.a017178
- **Anonymous:** "Levodopa Advanced Patient Information - Drugs.com", , 29 June 2021 (2021-06-29), Retrieved from the Internet: URL:<https://www.drugs.com/cons/levodopa.html> [retrieved on 2022-01-26]

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- BRILLIANT MURRAY H. ET AL: "Mining Retrospective Data for Virtual Prospective Drug Repurposing: L-DOPA and Age-related Macular Degeneration", AMERICAN JOURNAL OF MEDICINE., vol. 129, no. 3, 1 March 2016 (2016-03-01) , pages 292-298, United States ISSN: 0002-9343, DOI: 10.1016/j.amjmed.2015.10.015

(52) Cooperative Patent Classification (CPC): (Cont.)

C-Sets
A61K 31/277

Description

Introduction

5 [0001] Macular degeneration is a disease which can lead to loss of central vision due to damage in macula of the retina. It affects millions of people globally, and typically occurs in older people (so-called age-related macular degeneration, AMD). Genetic factors may play a role in this disease, as well as lifestyle: smoking, exposure to sunlight and unhealthy energy intake are the common risk factors. In 2010, there were 23.5 million patients globally, and it is the fourth most common cause of blindness after cataracts, preterm birth, and glaucoma. In the United States, AMD is the most common cause of vision loss in people over the age of fifty. See, e.g. Mehta, S., Age-Related Macular Degeneration. Prim Care 2015, 42 (3), 377-91; Velez-Montoya, et al., Current knowledge and trends in age-related macular degeneration: genetics, epidemiology, and prevention. Retina 2014, 34 (3), 423-41. There is no cure for AMD, though it is treated with laser coagulation, and more commonly with drugs that inhibit the growth of blood vessels, e.g. de Jong PT (2006). "Age-related macular degeneration". N Engl J Med. 355 (14): 1474-1485.

10 [0002] We previously disclosed in US2014/0148383A1 identification of a known FDA approved drug - entacapone ((2E)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)-N,N-diethylprop-2-enamide), as an FTO inhibitor using structure-based virtual screening method in combination with biological activity measurements, including enzymatic activity, cellular activity and in high-fat diet induced obesity (DIO) animal model. We also previously disclosed in PCT/CN2015/082052 derivatives of entacapone having related activities.

15 [0003] Entacapone is a catechol-O-methyltransferase (COMT) inhibitor used for treating Parkinson disease, typically administered in conjunction with dopamine derivatives levodopa (L-DOPA) or carbidopa, see Comtan Full Prescribing Information-Novartis. WO2015095257 discloses treating diabetes or diabetic retinopathy with dopamine, and in embodiments the dopamine is administered in combination with a COMT inhibitor, such as entacapone, tolcapone, or nitecapone.

20 [0004] Here we disclose the use of entacapone and related formulations to treat macular degeneration or age-related macular degeneration.

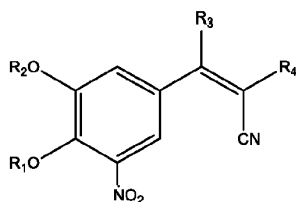
25 [0005] WO 2015/095257 A2, discloses the use of a combination levodopa/entacapone for treating macular degeneration. The combination can be in the form of an ophthalmic composition which is administered by intravitreal injection or as an implant. The compounds can also be administered in a liquid or gel composition into the vitreous cavity of the eye.

30 Summary of the Invention

[0006] The invention provides a pharmaceutical composition or formulation comprising entacapone-related compounds, as defined in the claims to treat or inhibit macular degeneration, particularly age-related macular degeneration, provided that the composition or formulation does not include another anti-Parkinsons medicament, neuroactive agent, anti-obesity, and/or anti-diabetes.

35 [0007] In an aspect the invention provides a pharmaceutical composition or formulation comprising entacapone, an entacapone derivative as defined in the claims, or pharmaceutically-acceptable salt thereof, in a person in need thereof, to treat or inhibit macular degeneration or age-related macular degeneration, preferably wherein the person does not have Parkinson's disease, obesity, diabetes or diabetic retinopathy.

40 [0008] In a particular embodiment the entacapone derivative comprises a structure of formula I of WO/2016/206573, a stereoisomer thereof, or a pharmaceutically-acceptable salt thereof:



2 (Ia) 2 1

wherein :

R1 and R2 are independently H or Me;

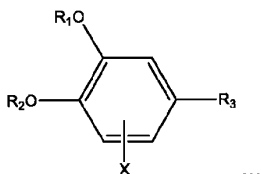
55 R3 is H, OH or NHR, wherein R is H or an optionally substituted, optionally hetero-, optionally cyclic C1-C18 hydrocarbyl; and

R4 is optionally substituted, optionally hetero-, optionally cyclic C1-C18 hydrocarbyl.

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[0009] In a particular embodiment the entacapone derivative comprises a structure of formula I of US5112861, here named (Ib), a stereoisomer thereof, or a pharmaceutically-acceptable salt thereof:

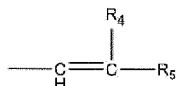
5



10

wherein R₁ and R₂ independently represent hydrogen, alkylcarbonyl of 2 to 5 carbon atoms or alkylcarbonyl of 2 to 5 carbon atoms, X represents nitro or cyano and R₃ represents

15

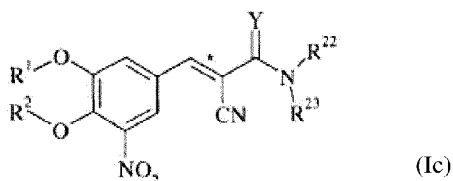


20

wherein R₄ represents cyano or alkylcarbonyl of 2 to 5 carbon atoms and R₅ represents cyano; alkylcarbonyl of 2 to 5 carbon atoms; or carbamoyl which is unsubstituted or substituted with alkyl of 1 to 8 carbon atoms, or hydroxyalkyl of 1 to 8 carbon atoms.

[0010] In a particular embodiment the entacapone derivative comprises a structure of formula I of WO2007144169, here named (Ic), a stereoisomer thereof, or a pharmaceutically-acceptable salt thereof:

25



30

(Ic)

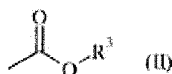
wherein

35

Y is sulfur or oxygen,

R¹ is a group of the following formula II

40



or when Y is S, R¹ can be in addition H,

R² is H or a group of formula II which may be the same as or different from R¹,

45

each R³ is independently (C₁-C₂₀)-alkyl, (C⁴R⁵)_X-R⁶, (C₁-C₂₀)-alkylene-(C₁-C₂₀)-alkoxy, (C₂-C₂₀)-alkenyl, (C₁-C₂₀)-alkynyl, (C₀-C₂₀)-alkylene-(C₃-C₁₈)-cycloalkyl, (C₀-C₂₀)-alkylene-(3-18-membered)-heterocycloalkyl, (C₁-C₂₀)-alkylene-(C₃-C₁₈)-cycloalkenyl, (C₀-C₂₀)-alkylene-(3-18-membered)-heterocycloalkenyl, (C₀-C₂₀)-alkylene-(C₆-C₁₈)-aryl, (C₀-C₂₀)-alkylene-(5-18-membered)-heteroaryl, (C₂-C₂₀)-alkenylene-(C₃-C₁₈)-cycloalkyl, (C₂-C₂₀)-alkenylene-(3-18-membered)-heterocycloalkyl, (C₂-C₂₀)-alkenylene-(C₃-C₁₈)-cycloalkenyl, (C₂-C₂₀)-alkenylene-(3-18-membered)-heterocycloalkenyl, (C₂-C₂₀)-alkenylene-(C₆-C₁₈)-aryl, or (C₂-C₂₀)-alkenylene-(5-18-membered)-heteroaryl,

50

wherein the total number of carbon atoms of R³ is at most 30,

each R⁴ and R⁵ are independently of one another selected from the group consisting of H, (C₁-C₂₀)-alkyl, (C₁-C₂₀)-alkylene-hydroxy, (C₀-C₂₀)-alkylene-(C₁-C₂₀)-alkoxy, OH, (C₀-C₂₀)-alkylene-N(R⁷)CO-(C₁-C₂₀)-alkyl, (C₀-C₂₀)-alkylene-CON(R⁸)(R⁹), (C₀-C₂₀)-alkylene-COO-(C₁-C₂₀)-alkyl, (C₀-C₂₀)-alkylene-N(R¹⁰)(R¹¹), SO₃R¹⁷, (C₀-C₂₀)-alkylene-(C₆-C₁₈)-aryl, and (C₀-C₂₀)-alkylene-(5-18-membered)-heteroaryl,

55

or

R⁴ and R⁵ of the same group (CR⁴R⁵) or R⁴ and R⁵ of different groups (CR⁴R⁵) may form together a carbocyclic or heterocyclic ring having from 3 to 6 atoms, additionally, one or more non adjacent groups (CR⁴R⁵) may be replaced by O, CO, OCO, COO, CON(R¹⁹), N(R²⁰)CO, or NR²¹,

R⁶ is independently H, (C₁-C₂₀)-alkyl, (C₂-C₂₀)-alkenyl, (C₂-C₂₀)-alkynyl, OH, O-(C₁-C₈)-alkyl, O-(C₀-C₈)-alkylene-(C₆-C₁₄)-aryl, CO-O-(C₁-C₈)-alkyl, CO-N(R¹²)(R¹³), N(R¹⁴)CO-(C₁-C₈)-alkyl, N(R¹⁵)(R¹⁶), SO₃R¹⁸, (C₀-C₂₀)-alkylene-(5-18-membered)-heteroaryl, or (C₀-C₂₀)-alkylene-(C₆-C₁₈)-aryl, R⁷, R¹⁴, R¹⁷, R¹⁸, R¹⁹, R²⁰, R²¹ are independently of one another H, or (C₁-C₂₀)-alkyl, R⁸, R⁹, R¹⁰, R¹¹, R¹², R¹³, R¹⁵, R¹⁶ are independently of one another H, or (C₁-C₂₀)-alkyl, R²² and R²³ are independently selected from the group consisting of H and (C₁-C₁₅)-alkyl, and x is 1 to 14, wherein the alkyl, alkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, heterocycloalkenyl, alkoxy, aryl, heteroaryl, alkenylene and alkylene groups may be unsubstituted or further substituted.

[0011] In embodiments the use further comprises the step of detecting a resultant inhibition of macular degeneration.

[0012] In other aspects the invention provides a pharmaceutical composition or formulation comprising:

- a) an ophthalmic composition comprising entacapone, an entacapone derivative of claim 2 or a stereoisomer, or pharmaceutically-acceptable salt thereof, wherein the composition does not include dopamine or a dopamine derivative, such as levodopa (L-DOPA) or carbidopa;
- b) a topical ophthalmic composition comprising entacapone, an entacapone derivative of claim 2 or a stereoisomer or pharmaceutically-acceptable salt thereof; or
- c) a composition comprising entacapone, an entacapone derivative of claim 2 or a stereoisomer, or pharmaceutically-acceptable salt thereof, copackaged or coformulated with a second, different medicament for treating or inhibiting macular degeneration or age-related macular degeneration, provided that the composition or formulation does not include another anti-Parkinsons medicament, neuroactive agent, anti-obesity, and/or anti-diabetes.

[0013] In embodiments:

- the composition or formulation does not include another anti-Parkinsons medicament, neuroactive agent, anti-obesity, anti-diabetes, and/or another active pharmaceutical ingredient (API), such as wherein anti-Parkinson's medicaments include L-DOPA, deprenyl, tyrosine hydroxylase, apomorphine, anticholinergic drugs such as benzhexol and orphenadrine, and mGluR4 potentiators such as N-phenyl-7-(hydroxylimino)cyclopropa[b]chromen-1a-carboxamide (PHCCC);
- the composition or formulation is in topical form of eye drops, ointments, gels, or emulsions;
- the composition or formulation is in form of an intravitreal injection formulation or an intraocular implant formulation;
- the composition or formulation wherein the entacapone, entacapone derivative or pharmaceutically-acceptable salt thereof, is in unit dosage form; and/or
- the composition or formulation wherein the second medicament is an antiangiogenic drug (e.g. VEGF inhibitor) selected from revacizumab, ranibizumab, pegaptanib, and aflibercept.

[0014] In embodiments, the entacapone, an entacapone derivative or a stereoisomer, or pharmaceutically-acceptable salt thereof is administered to the eye in topical form of eye drops, ointments, gels, or emulsions, or into the vitreous or sclera of the eye, e.g., administered by an intravitreal injection or an implant, e.g., surgical administration of drug-loaded solid implants within the scleral tissue (i.e. intrascleral delivery); see, e.g. Falavarjani et al., Eye (2013) 27, 787-794; Adverse events and complications associated with intravitreal injection of anti-VEGF agents: a review of literature; Marra, et al. AAPS PharmSciTech. 2011 Mar; 12(1): 362-371. Solution Formulation Development of a VEGF Inhibitor for Intravitreal Injection, etc.

[0015] The invention encompasses all combination of the particular embodiments recited herein, as if each had been separately, laboriously recited.

Brief Description of the Drawings

[0016]

Fig. 1. The eye blood vessel diameter. Values are presented as mean $\pm$ SE (n = 10) . ***p < 0.001, compared with the model group.

Fig. 2. The blood vessel diameter recovery rate. Values are presented as mean $\pm$ SE (n = 10). ***p < 0.001, compared with the model group.

Description of Particular Embodiments of the Invention

[0017] The following descriptions of particular embodiments and examples are provided by way of illustration and not by way of limitation. Those skilled in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

[0018] Unless contraindicated or noted otherwise, in these descriptions and throughout this specification, the terms "a" and "an" mean one or more, the term "or" means and/or and polynucleotide sequences are understood to encompass opposite strands as well as alternative backbones described herein. Furthermore, genres are recited as shorthand for a recitation of all members of the genus; for example, the recitation of (C1-C3) alkyl is shorthand for a recitation of all C1-C3 alkyls: methyl, ethyl and propyl, including isomers thereof.

[0019] A hydrocarbyl group is a substituted or unsubstituted, straight-chain, branched or cyclic alkyl, alkenyl, alkynyl, acyl, aryl, arylalkyl, arylalkenyl, arylalkynyl, alkylaryl, alkenylaryl or alkynylaryl group which comprises 1-15 carbon atoms and optionally includes one or more heteroatoms in its carbon skeleton.

[0020] The term "heteroatom" as used herein generally means any atom other than carbon or hydrogen. Preferred heteroatoms include oxygen (O), phosphorus (P), sulfur (S), nitrogen (N), and halogens, and preferred heteroatom functional groups are haloformyl, hydroxyl, aldehyde, amine, azo, carboxyl, cyanyl, thiocyanyl, carbonyl, halo, hydroperoxyl, imine, aldimine, isocyanide, isocyanate, nitrate, nitrile, nitrite, nitro, nitroso, phosphate, phosphono, sulfide, sulfonyl, sulfo, and sulphydryl.

[0021] The term "alkyl," by itself or as part of another substituent, means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which is fully saturated, having the number of carbon atoms designated (i.e. C1-C8 means one to eight carbons). Examples of alkyl groups include methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, homologs and isomers of, for example, n-pentyl, n-hexyl, n-heptyl, n-octyl and the like.

[0022] The term "alkenyl," by itself or as part of another substituent, means a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which may be mono- or polyunsaturated, having the number of carbon atoms designated (i.e. C2-C8 means two to eight carbons) and one or more double bonds. Examples of alkenyl groups include vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl) and higher homologs and isomers thereof.

[0023] The term "alkynyl," by itself or as part of another substituent, means a straight or branched chain hydrocarbon radical, or combination thereof, which may be mono- or polyunsaturated, having the number of carbon atoms designated (i.e. C2-C8 means two to eight carbons) and one or more triple bonds. Examples of alkynyl groups include ethynyl, 1- and 3-propynyl, 3-butylnyl and higher homologs and isomers thereof.

[0024] The term "alkylene" by itself or as part of another substituent means a divalent radical derived from alkyl, as exemplified by $-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-$. Typically, an alkyl (or alkylene) group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms being preferred in the invention. A "lower alkyl" or "lower alkylene" is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms.

[0025] The terms "alkoxy," "alkylamino" and "alkylthio" (or thioalkoxy) are used in their conventional sense, and refer to those alkyl groups attached to the remainder of the molecule via an oxygen atom, an amino group, or a sulfur atom, respectively.

[0026] The term "heteroalkyl," by itself or in combination with another term, means, unless otherwise stated, a stable straight or branched chain, or cyclic hydrocarbon radical, or combinations thereof, consisting of the stated number of carbon atoms and from one to three heteroatoms selected from the group consisting of O, N, P, Si and S, wherein the nitrogen, sulfur, and phosphorous atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N, P and S may be placed at any interior position of the heteroalkyl group. The heteroatom Si may be placed at any position of the heteroalkyl group, including the position at which the alkyl group is attached to the remainder of the molecule. Examples include $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_3$, $-\text{CH}_2-\text{CH}_2-\text{NH}-\text{CH}_3$, $-\text{CH}_2-\text{CH}_2-\text{N}(\text{CH}_3)-\text{CH}_3$, $-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_3$, $-\text{CH}_2-\text{CH}_2-\text{S}(\text{O})-\text{CH}_3$, $-\text{CH}_2-\text{CH}_2-\text{S}(\text{O})_2-\text{CH}_3$, $-\text{CH}=\text{CH}-\text{O}-\text{CH}_3$, $-\text{Si}(\text{CH}_3)_3$, $-\text{CH}_2-\text{CH}=\text{N}-\text{O}-\text{CH}_3$, and $-\text{CH}=\text{CH}-\text{N}(\text{CH}_3)-\text{CH}_3$. Up to two heteroatoms may be consecutive, such as, for example, $-\text{CH}_2-\text{NH}-\text{O}-\text{CH}_3$ and $-\text{CH}_2-\text{O}-\text{Si}(\text{CH}_3)_3$.

[0027] Similarly, the term "heteroalkylene," by itself or as part of another substituent means a divalent radical derived from heteroalkyl, as exemplified by $-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-$ and $-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-\text{NH}-\text{CH}_2-$. For heteroalkylene groups, heteroatoms can also occupy either or both of the chain termini (e.g., alkyleneoxy, alkyleneedioxy, alkyleneamino, alkylene-diamino, and the like). Still further, for alkylene and heteroalkylene linking groups, no orientation of the linking group is implied.

[0028] The terms "cycloalkyl" and "heterocycloalkyl", by themselves or in combination with other terms, represent,

unless otherwise stated, cyclic versions of "alkyl" and "heteroalkyl", respectively. Accordingly, a cycloalkyl group has the number of carbon atoms designated (i.e., C3-C8 means three to eight carbons) and may also have one or two double bonds. A heterocycloalkyl group consists of the number of carbon atoms designated and from one to three heteroatoms selected from the group consisting of O, N, Si and S, and wherein the nitrogen and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl include cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of heterocycloalkyl include 1-(1,2,5,6-tetrahydropyrid-yl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1-piperazinyl, 2-piperazinyl, and the like.

[0029] The terms "halo" and "halogen," by themselves or as part of another substituent, mean, unless otherwise stated, a fluorine, chlorine, bromine, or iodine atom. Additionally, terms such as "haloalkyl," are meant to include alkyl substituted with halogen atoms, which can be the same or different, in a number ranging from one to $(2m'+1)$, where m' is the total number of carbon atoms in the alkyl group. For example, the term "halo(C1-C4)alkyl" is meant to include trifluoromethyl, 2,2,2-trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, and the like. Thus, the term "haloalkyl" includes monohaloalkyl (alkyl substituted with one halogen atom) and polyhaloalkyl (alkyl substituted with halogen atoms in a number ranging from two to $(2m'+1)$ halogen atoms, where m' is the total number of carbon atoms in the alkyl group). The term "perhaloalkyl" means, unless otherwise stated, alkyl substituted with $(2m'+1)$ halogen atoms, where m' is the total number of carbon atoms in the alkyl group. For example the term "perhalo(C1-C4)alkyl" is meant to include trifluoromethyl, pentachloroethyl, 1,1,1-trifluoro-2-bromo-2-chloroethyl and the like.

[0030] The term "acyl" refers to those groups derived from an organic acid by removal of the hydroxy portion of the acid. Accordingly, acyl is meant to include, for example, acetyl, propionyl, butyryl, decanoyl, pivaloyl, benzoyl and the like.

[0031] The term "aryl" means, unless otherwise stated, a polyunsaturated, typically aromatic, hydrocarbon substituent which can be a single ring or multiple rings (up to three rings) which are fused together or linked covalently. Non-limiting examples of aryl groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl and 1,2,3,4-tetrahydronaphthalene.

[0032] The term "heteroaryl" refers to aryl groups (or rings) that contain from zero to four heteroatoms selected from N, O, and S, wherein the nitrogen and sulfur atoms are optionally oxidized and the nitrogen heteroatom are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of heteroaryl groups include 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalyl, 5-quinoxalyl, 3-quinolyl and 6-quinolyl.

[0033] For brevity, the term "aryl" when used in combination with other terms (e.g., aryloxy, arylthioxy, arylalkyl) includes both aryl and heteroaryl rings as defined above. Thus, the term "arylalkyl" is meant to include those radicals in which an aryl group is attached to an alkyl group (e.g., benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (e.g., a methylene group) has been replaced by, for example, an oxygen atom (e.g., phenoxymethyl, 2-pyridyloxymethyl, 3-(1-naphthyloxy)propyl, and the like).

[0034] Each of the above terms (e.g., "alkyl," "heteroalkyl," "aryl" and "heteroaryl") is meant to include both substituted and unsubstituted forms of the indicated radical. Preferred substituents for each type of radical are provided below.

[0035] Substituents for the alkyl and heteroalkyl radicals (as well as those groups referred to as alkenyl, alkenyl, heteroalkenyl, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl and heterocycloalkenyl) can be a variety of groups selected from: $-OR'$, $=O$, $=NR'$, $=N-OR'$, $-NR'R''$, $-SR'$, halogen, $-SiR'R''R'''$, $-OC(O)R'$, $-C(O)R'$, $-CO_2R'$, $-CONR'R''$, $-OC(O)NR'R''$, $-NR''C(O)R'$, $-NR''C(O)NR''R'''$, $-NR''SO_2NR''$, $-NR''CO_2R'$, $-NH-C(NH_2)=NH$, $-NR''C(NH_2)=NH$, $-NH-C(NH_2)=NR'$, $-S(O)R'$, $-SO_2R'$, $-SO_2NR'R''$, $-NR''SO_2R'$, $-CN$ and $-NO_2$, in a number ranging from zero to three, with those groups having zero, one or two substituents being particularly preferred. R' , R'' and R''' each independently refer to hydrogen, unsubstituted (C1-C8)alkyl and heteroalkyl, unsubstituted aryl, aryl substituted with one to three halogens, unsubstituted alkyl, alkoxy or thioalkoxy groups, or aryl-(C1-C4)alkyl groups. When R' and R'' are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6- or 7-membered ring. For example, $-NR'R''$ is meant to include 1-pyrrolidinyl and 4-morpholinyl. Typically, an alkyl or heteroalkyl group will have from zero to three substituents, with those groups having two or fewer substituents being preferred in the invention. More preferably, an alkyl or heteroalkyl radical will be unsubstituted or monosubstituted. Most preferably, an alkyl or heteroalkyl radical will be unsubstituted. From the above discussion of substituents, one of skill in the art will understand that the term "alkyl" is meant to include groups such as trihaloalkyl (e.g., $-CF_3$ and $-CH_2CF_3$).

[0036] Preferred substituents for the alkyl and heteroalkyl radicals are selected from: $-OR'$, $=O$, $-NR'R''$, $-SR'$, halogen, $-SiR'R''R'''$, $-OC(O)R'$, $-C(O)R'$, $-CO_2R'$, $-CONR'R''$, $-OC(O)NR'R''$, $-NR''C(O)R'$, $-NR''CO_2R'$, $-NR''SO_2NR''R'''$, $-S(O)R'$, $-SO_2R'$, $-SO_2NR'R''$, $-NR''SO_2R'$, $-CN$ and $-NO_2$, where R' and R'' are as defined above. Further preferred substituents are selected from: $-OR'$, $=O$, $-NR'R''$, halogen, $-OC(O)R'$, $-CO_2R'$, $-CONR'R''$, $-OC(O)NR'R''$, $-NR''C(O)R'$, $-NR''CO_2R'$, $-NR''SO_2NR''R'''$, $-SO_2R'$, $-SO_2NR'R''$, $-NR''SO_2R'$, $-CN$ and $-NO_2$.

[0037] Similarly, substituents for the aryl and heteroaryl groups are varied and selected from: halogen, $-OR'$, $-OC(O)R'$,

-NR'R", -SR', -R', -CN, -NO₂, -CO₂R', -CONR'R", -C(O)R', -OC(O)NR'R", -NR"C(O)R', -NR"CO₂R', -NR'-C(O)NR'R", -NR'-SO₂NR'R", -NH-C(NH₂)=NH, -NR'C(NH₂)=NH, -NH-C(NH₂)=NR', -S(O)R', -SO₂R', -SO₂NR'R", -NR"SO₂R', -N₃, -CH(Ph)₂, perfluoro(C1-C4)alkoxy and perfluoro(C1-C4)alkyl, in a number ranging from zero to the total number of open valences on the aromatic ring system; and where R', R" and R''' are independently selected from hydrogen, (C1-C8)alkyl and heteroalkyl, unsubstituted aryl and heteroaryl, (unsubstituted aryl)-(C1-C4)alkyl and (unsubstituted aryl)oxy-(C1-C4)alkyl. When the aryl group is 1,2,3,4-tetrahydronaphthalene, it may be substituted with a substituted or unsubstituted (C3-C7)spirocycloalkyl group. The (C3-C7)spirocycloalkyl group may be substituted in the same manner as defined herein for "cycloalkyl". Typically, an aryl or heteroaryl group will have from zero to three substituents, with those groups having two or fewer substituents being preferred in the invention. In one embodiment of the invention, an aryl or heteroaryl group will be unsubstituted or monosubstituted. In another embodiment, an aryl or heteroaryl group will be unsubstituted.

[0038] Preferred substituents for aryl and heteroaryl groups are selected from: halogen, -OR¹, -OC(O)R', -NR'R", -SR', -R', -CN, -NO₂, -CO₂R', -CONR'R", -C(O)R', -OC(O)NR'R", -NR"C(O)R', -S(O)R', -SO₂R', -SO₂NR'R", -NR"SO₂R', -N₃, -CH(Ph)₂, perfluoro(C1-C4)alkoxy and perfluoro(C1-C4)alkyl, where R' and R" are as defined above. Further preferred substituents are selected from: halogen, -OR¹, -OC(O)R', -NR'R", -R', -CN, -NO₂, -CO₂R', -CONR'R", -NR"C(O)R', -SO₂R', -SO₂NR'R", -NR"SO₂R', perfluoro(C1-C4)alkoxy and perfluoro(C1-C4)alkyl.

[0039] The substituent -CO₂H, as used herein, includes bioisosteric replacements therefor; see, e.g., The Practice of Medicinal Chemistry; Wermuth, C. G., Ed.; Academic Press: New York, 1996; p. 203.

[0040] Two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula -T-C(O)-(CH₂)_q-U-, wherein T and U are independently -NH-, -O-, -CH₂- or a single bond, and q is an integer of from 0 to 2. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula -A-(CH₂)_r-B-, wherein A and B are independently -CH₂-, -O-, -NH-, -S-, -S(O)-, -S(O)₂- or a single bond, and r is an integer of from 1 to 3. One of the single bonds of the new ring so formed may optionally be replaced with a double bond. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula -(CH₂)_s-X-(CH₂)_t-, where s and t are independently integers of from 0 to 3, and X is -O-, -NR'-, -S-, -S(O)-, -S(O)₂-, or -S(O)₂NR'-. The substituent R' in -NR'- and -S(O)₂NR'- is selected from hydrogen or unsubstituted (C1-C6)alkyl.

[0041] Preferred substituents are disclosed herein and exemplified in the tables, structures, examples, and claims, and may be applied across different compounds of the invention, i.e. substituents of any given compound may be combinatorially used with other compounds.

[0042] In particular embodiments applicable substituents are independently substituted or unsubstituted heteroatom, substituted or unsubstituted, optionally heteroatom C1-C6 alkyl, substituted or unsubstituted, optionally heteroatom C2-C6 alkenyl, substituted or unsubstituted, optionally heteroatom C2-C6 alkynyl, or substituted or unsubstituted, optionally heteroatom C6-C14 aryl, wherein each heteroatom is independently oxygen, phosphorus, sulfur or nitrogen.

[0043] In more particular embodiments, applicable substituents are independently aldehyde, aldimine, alkanoyloxy, alkoxy, alkoxy carbonyl, alkyloxy, alkyl, amine, azo, halogens, carbamoyl, carbonyl, carboxamido, carboxyl, cyanyl, ester, halo, haloformyl, hydroperoxyl, hydroxyl, imine, isocyanide, iscyante, N-tert-butoxycarbonyl, nitrate, nitrile, nitrite, nitro, nitroso, phosphate, phosphono, sulfide, sulfonyl, sulfo, sulphydryl, thiol, thiocyanyl, trifluoromethyl or trifluoromethyl ether (OCF₃).

[0044] The term "pharmaceutically acceptable salts" is meant to include salts of the active compounds which are prepared with relatively nontoxic acids or bases, depending on the particular substituents found on the compounds described herein, and suitable for pharmaceutical use. When compounds of the invention contain relatively acidic functionalities, base addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired base, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable base addition salts include sodium, potassium, calcium, ammonium, organic amino, or magnesium salt, or a similar salt. When compounds of the invention contain relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the salts derived from relatively nontoxic organic acids like acetic, propionic, isobutyric, oxalic, maleic, malonic, benzoic, succinic, suberic, fumaric, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the like. Also included are salts of amino acids such as arginate and the like, and salts of organic acids like glucuronic or galacturonic acids and the like. Certain specific compounds of the invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

[0045] The neutral forms of the compounds may be regenerated by contacting the salt with a base or acid and isolating the parent compound in the conventional manner. The parent form of the compound differs from the various salt forms in certain physical properties, such as solubility in polar solvents, but otherwise the salts are equivalent to the parent

form of the compound for the purposes of the invention.

[0046] In addition to salt forms, the disclosure provides in a non-claimed aspect compounds which are in a prodrug form. Prodrugs of the compounds described herein are those compounds that undergo chemical changes under physiological conditions to provide the compounds of the invention. Additionally, prodrugs can be converted to the compounds of the invention by chemical or biochemical methods in an ex vivo environment. For example, prodrugs can be slowly converted to the compounds of the invention when placed in a transdermal patch reservoir with a suitable enzyme or chemical reagent. Prodrugs are often useful because, in some situations, they may be easier to administer than the parent drug. They may, for instance, be more bioavailable by oral administration than the parent drug. The prodrug may also have improved solubility in pharmacological compositions over the parent drug. A wide variety of prodrug derivatives are known in the art, such as those that rely on hydrolytic cleavage or oxidative activation of the prodrug. An example, without limitation, of a prodrug would be a compound which is administered as an ester (the "prodrug"), but then is metabolically hydrolyzed to the carboxylic acid, the active entity. Additional non-claimed examples include peptidyl derivatives of a compound of the invention.

[0047] Certain compounds of the invention can exist in unsolvated forms as well as solvated forms, including hydrated forms. In general, the solvated forms are equivalent to unsolvated forms and are intended to be encompassed within the scope of the invention. Certain compounds of the invention may exist in multiple crystalline or amorphous forms. In general, all physical forms are equivalent for the uses contemplated by the invention and are intended to be within the scope of the invention.

[0048] Some of the subject compounds possess asymmetric carbon atoms (optical centers) or double bonds; the racemates, diastereomers, geometric isomers and specifically designated or depicted chirality is preferred and in many cases critical for optimal activity; however all such isomers are all intended to be encompassed within the scope of the invention.

[0049] The compounds of the invention may also contain unnatural proportions of atomic isotopes at one or more of the atoms that constitute such compounds. For example, the compounds may be radiolabeled with radioactive isotopes, such as for example tritium (^3H), iodine-125 (^{125}I) or carbon-14 (^{14}C). All isotopic variations of the compounds of the invention, whether radioactive or not, are intended to be encompassed within the scope of the invention.

[0050] The term "therapeutically effective amount" refers to the amount of the subject compound that will elicit, to some significant extent, the biological or medical response of a tissue, system, animal or human that is being sought by the researcher, veterinarian, medical doctor or other clinician, such as when administered, is sufficient to prevent development of, or alleviate to some extent, one or more of the symptoms of the condition or disorder being treated. The therapeutically effective amount will vary depending on the compound, the disease and its severity and the age, weight, etc., of the mammal to be treated.

[0051] The invention also provides pharmaceutical compositions comprising the subject compounds and a pharmaceutically acceptable excipient, particularly such compositions comprising a unit dosage of the subject compounds, particularly such compositions copackaged with instructions describing use of the composition to treat an applicable disease or condition (herein).

[0052] The compositions for administration can take the form of bulk liquid solutions or suspensions, or bulk powders. More commonly, however, the compositions are presented in unit dosage forms to facilitate accurate dosing. The term "unit dosage forms" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient. Typical unit dosage forms include prefilled, premeasured ampules or syringes of the liquid compositions or pills, tablets, capsules, lozenges or the like in the case of solid compositions. In such compositions, the compound is usually a minor component (from about 0.1 to about 50% by weight or preferably from about 1 to about 40% by weight) with the remainder being various vehicles or carriers and processing aids helpful for forming the desired dosing form.

[0053] Suitable excipients or carriers and methods for preparing administrable compositions are known or apparent to those skilled in the art and are described in more detail in such publications as Remington's Pharmaceutical Science, Mack Publishing Co, NJ (1991). In addition, the compounds may be advantageously used in conjunction with other therapeutic agents as described herein or otherwise known in the art, particularly other anti-necrosis agents. Hence the compositions may be administered separately, jointly, or combined in a single dosage unit.

[0054] The amount administered depends on the compound formulation, route of administration, etc. and is generally empirically determined in routine trials, and variations will necessarily occur depending on the target, the host, and the route of administration, etc. Generally, the quantity of active compound in a unit dose of preparation may be varied or adjusted from about 1, 5, 25 or 100 to about 5, 25, 100, 500, 1000 or 2000 mg, according to the particular application. In a particular embodiment, unit dosage forms are packaged in a multipack adapted for sequential use, such as blisterpack, comprising sheets of at least 6, 9 or 12 unit dosage forms. The actual dosage employed may be varied depending upon the requirements of the patient and the severity of the condition being treated. Determination of the proper dosage for a particular situation is within the skill of the art. Generally, treatment is initiated with smaller dosages which are less

than the optimum dose of the compound. Thereafter, the dosage is increased by small amounts until the optimum effect under the circumstances is reached. For convenience, the total daily dosage may be divided and administered in portions during the day if desired.

5 **[0055]** The compounds can be administered by a variety of methods including, but not limited to, parenteral, topical, oral, or local administration, such as by aerosol or transdermally, for prophylactic and/or therapeutic treatment. Also, in accordance with the knowledge of the skilled clinician, the therapeutic protocols (e.g., dosage amounts and times of administration) can be varied in view of the observed effects of the administered therapeutic agents on the patient, and in view of the observed responses of the disease to the administered therapeutic agents.

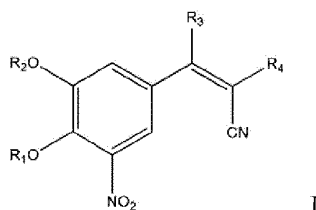
10 **[0056]** The therapeutics of the invention can be administered in a therapeutically effective dosage and amount, in the process of a therapeutically effective protocol for treatment of the patient. For more potent compounds, microgram (ug) amounts per kilogram of patient may be sufficient, for example, in the range of about 1, 10, 100, 1000, 10000, 20000 ug/kg to about 10, 100, 1000, 10000, 20000 or 80000 ug/kg of patient weight though optimal dosages are compound specific, and generally empirically determined for each compound.

15 **[0057]** In general, routine experimentation in clinical trials will determine specific ranges for optimal therapeutic effect, for each therapeutic, each administrative protocol, and administration to specific patients will also be adjusted to within effective and safe ranges depending on the patient condition and responsiveness to initial administrations. However, the ultimate administration protocol will be regulated according to the judgment of the attending clinician considering such factors as age, condition and size of the patient as well as compounds potency, severity of the disease being treated. For example, a dosage regimen of the compounds can be oral administration of from 10 mg to 2000 mg/day, preferably 10 to 1000 mg/day, more preferably 50 to 600 mg/day, in two to four (preferably two) divided doses. Intermittent therapy (e.g., one week out of three weeks or three out of four weeks) may also be used.

20 **[0058]** In particular embodiments the subject entacapone derivative comprises a structure of formula I of WO/2016/206573, a stereoisomer thereof, a hydride thereof, or a pharmaceutically-acceptable salt thereof,:

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wherein :

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(a)

R1 and R2 are independently H or Me;

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R3 is OH or NHR, wherein R is H or an optionally substituted, optionally hetero-, optionally cyclic C1-C18 hydrocarbyl; and

R4 is optionally substituted, optionally hetero-, optionally cyclic C1-C18 hydrocarbyl;

(b)

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R1 and R2 are independently H or Me;

R3 is H, OH or NHR, wherein R is H or C1-C4 alkyl;

R4 is CONHR5; and

R5 is optionally substituted, optionally hetero-, optionally cyclic C1-C18 hydrocarbyl;

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(c)

R1 and R2 are independently H or Me;

R3 is H, OH or NHR, wherein R is H or C1-C4 alkyl;

R4 is COR5; and

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R5 is optionally substituted, heterocyclic C3-C18 hydrocarbyl comprising an n-membered ring wherein n=3-18 including 1 to n-1 heteroatoms independently selected from N, O, S and P; or

(d)

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R1 and R2 are independently H or Me;

R3 is H, OH or NHR, wherein R is H or C1-C4 alkyl; and

R4 is optionally substituted, heterocyclic C3-C18 hydrocarbyl comprising an n-membered ring wherein n=3-18 including 1 to n-1 heteroatoms independently selected from N, O, S and P; particularly wherein excluded from the inhibitor are compounds: CAS IDS: 1364322-41-7, 1150310-12-5, 1150310-15-8, and 143542-72-7.

[0059] In embodiments of the inhibitor or composition the heterocyclic C3-C18 hydrocarbyl comprises:

a 3 membered ring that is an optionally substituted : aziridine, oxirane, oxaziridine;

a 4 membered ring that is an optionally substituted : azetidine, oxetane, oxazetidine;

a 5 membered ring that is an optionally substituted : pyrrole, 1,2-diazole (pyrazole), 1,3 diazole (imidazole), thiazole, isothiazole, oxazole, isoxazole, furan, dioxole, thiophene;

a 6 membered ring that is an optionally substituted: pyridine, diazine, triazine, oxazine, thiazine, dioxine, oxathiine, dithiine;

a 9 membered ring that is an optionally substituted: indole, benzothiazole, benzooxazole, benzofuran, benzodioxole, benzothiophene, benzodithiole; or

a 10 membered ring that is an optionally substituted: quinoline, quinoxaline, quinazoline, chromene, benzodioxine, thiochromene, benzodithiine.

[0060] In embodiments of the inhibitor or composition the optionally substituted, optionally hetero-, optionally cyclic C1-C18 hydrocarbyl in each instance is an optionally substituted C1-C9 alkyl, C2-C9 alkenyl, C2-C9 alkynyl, or C5-C14 aryl hydrocarbon, comprising 1-5 heteroatoms that are N, S, O or P, including 1-5 nitrogen atoms, or a heteroatom substituted with the hydrocarbon.

[0061] In embodiments of the inhibitor or composition:

one or both R1 and R2 is H;

R3 is OH; and/or

R is H or C1-C4 alkyl, esp. Me.

[0062] The invention encompasses all combination of the particular embodiments recited herein, as if each had been separately, laboriously recited. For example, subsection (a) encompasses combinations wherein: R1 and R2 are H; R3 is NH₂; and R4 is a 6 membered ring that is pyridine, and subsection (d) encompasses combinations wherein R1 and R2 are Me; R3 is OH; and R4 is 1,3 diazole.

[0063] In embodiments the inhibitor is of the following Tables.

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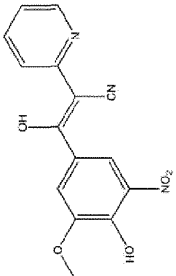
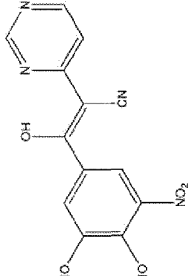
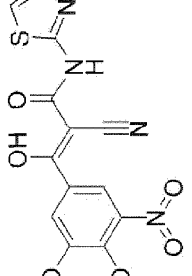
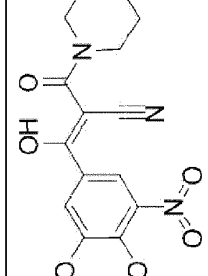
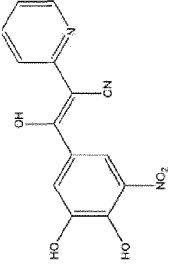
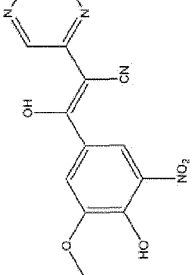
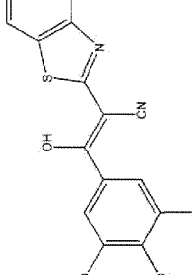
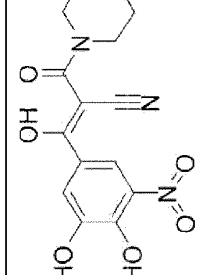
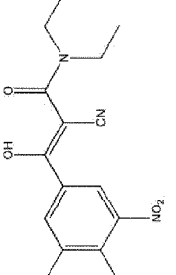
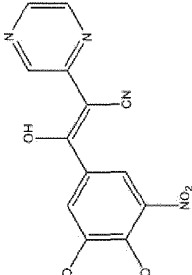
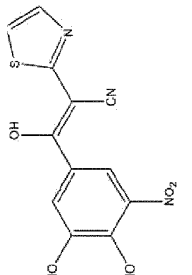
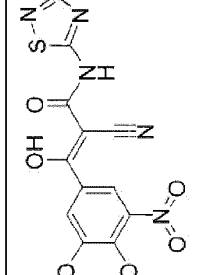
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Table 1. Subsection (a) inhibitors

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 347	 523	 503	 668

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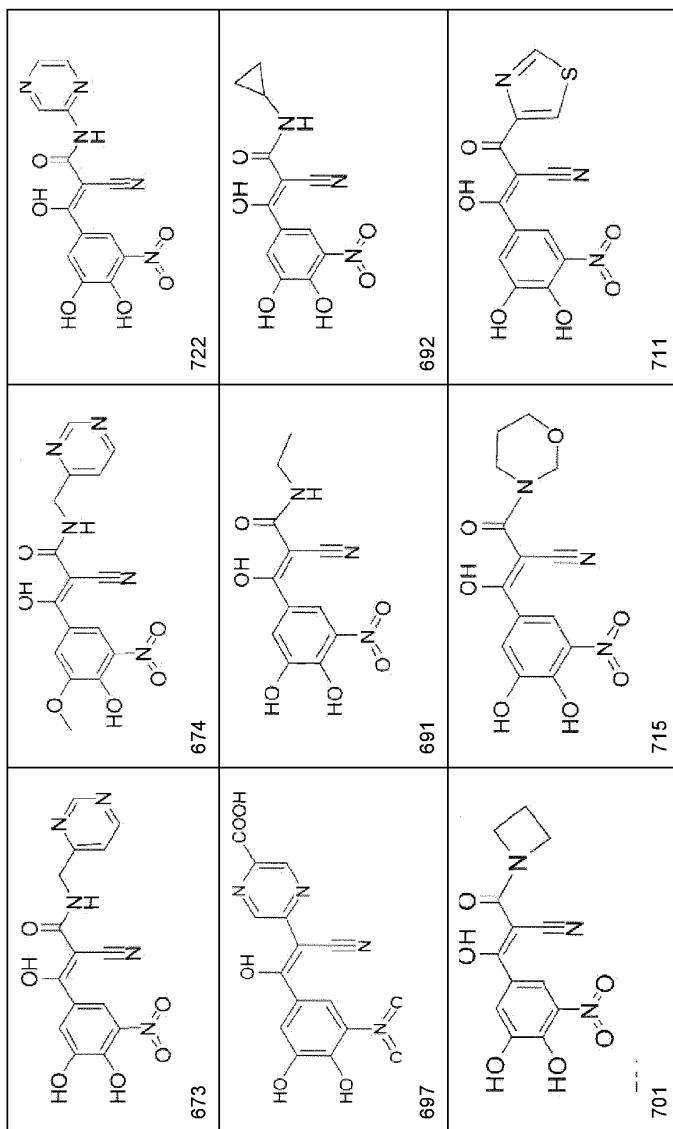
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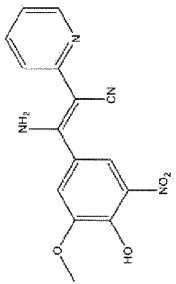
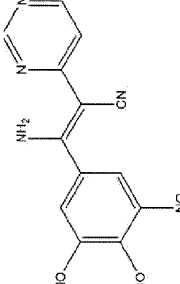
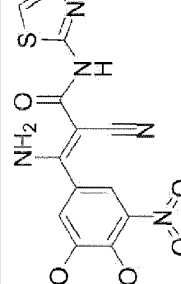
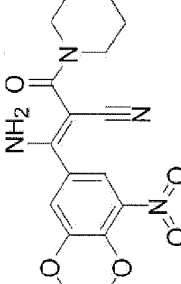
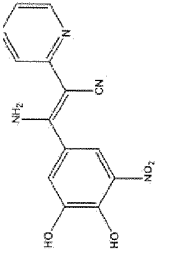
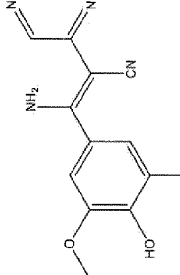
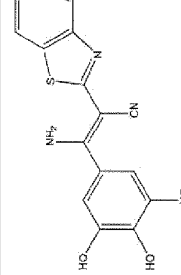
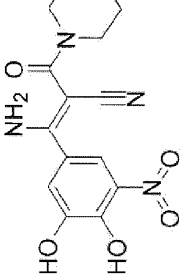
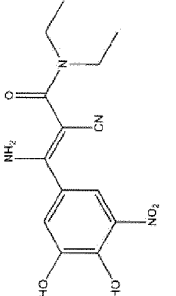
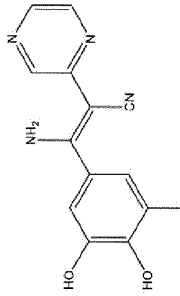
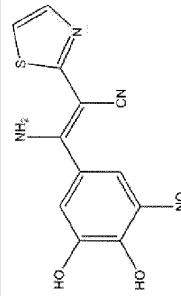
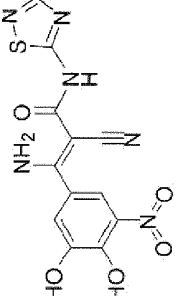
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Table 2. Subsection (a) inhibitors.

 352N	 525N	 374N	 658N
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 347N	 523N	 503N	 668N

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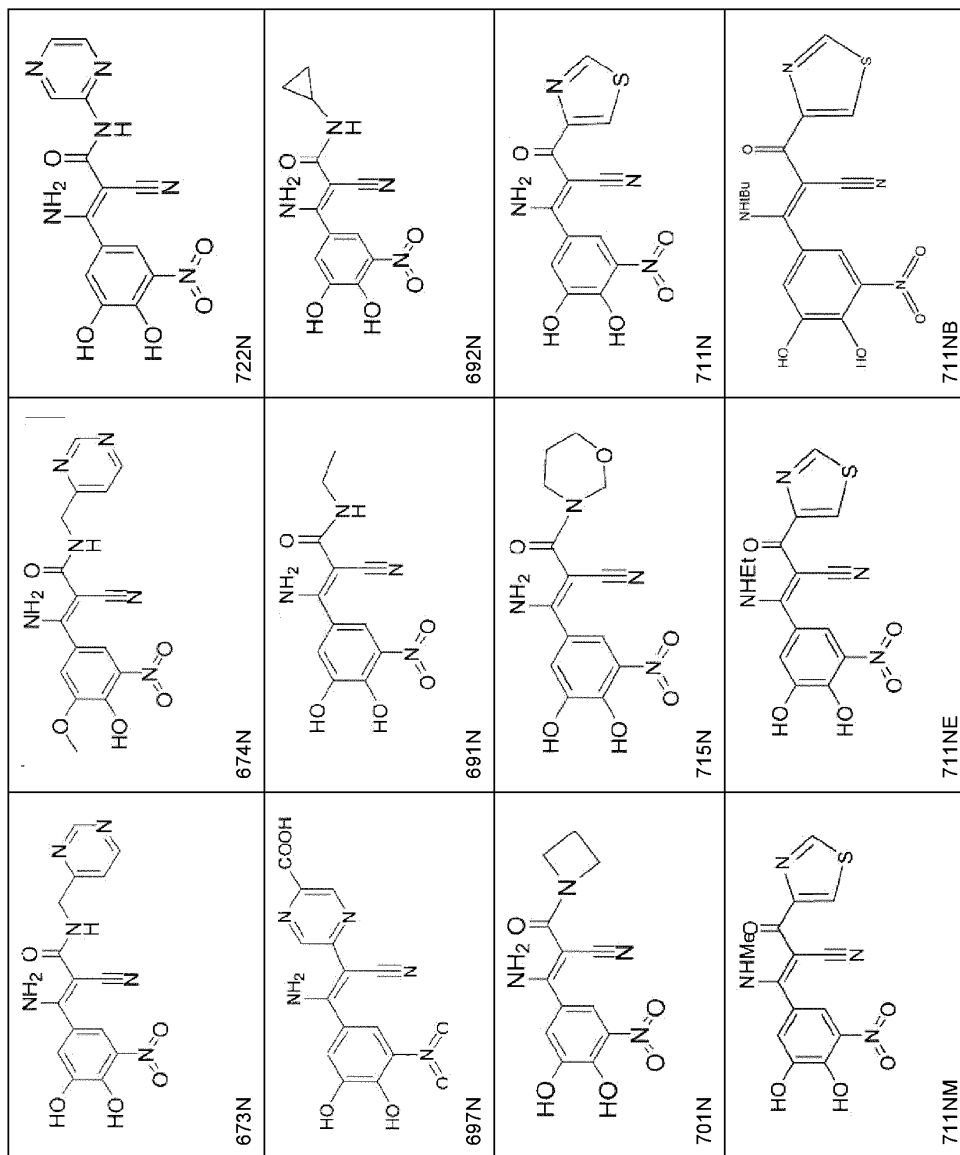
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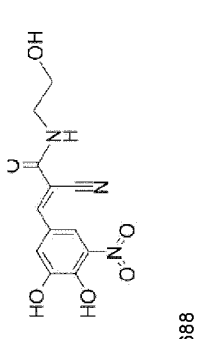
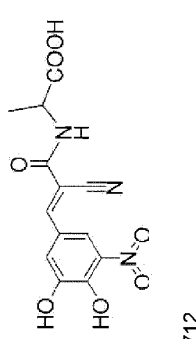
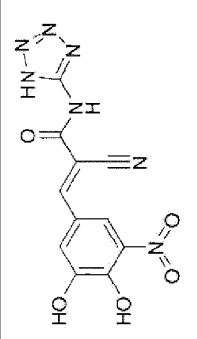
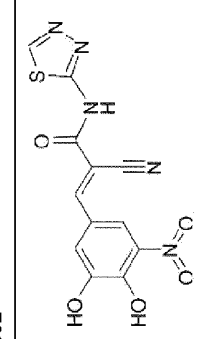
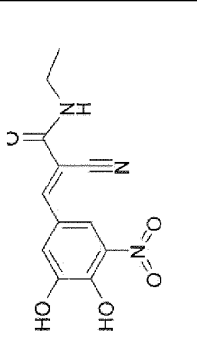
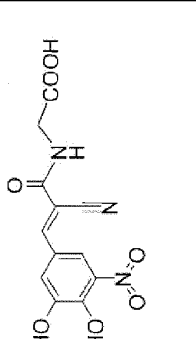
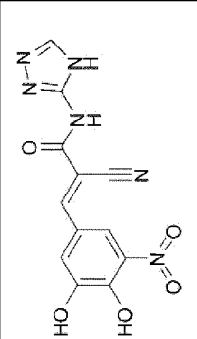
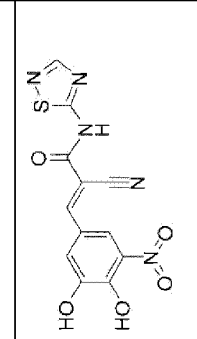
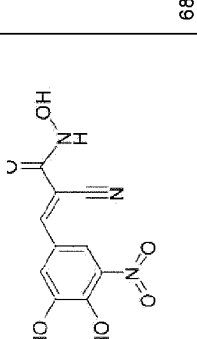
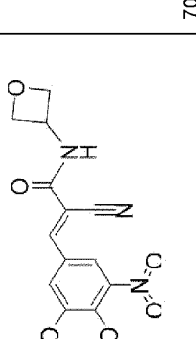
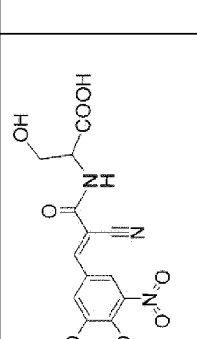
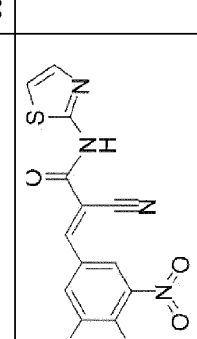
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Table 3. Subsection (b) inhibitors.

 688	 712	 802	 804
 684	 709	 801	 803
 664	 713	 693	 331

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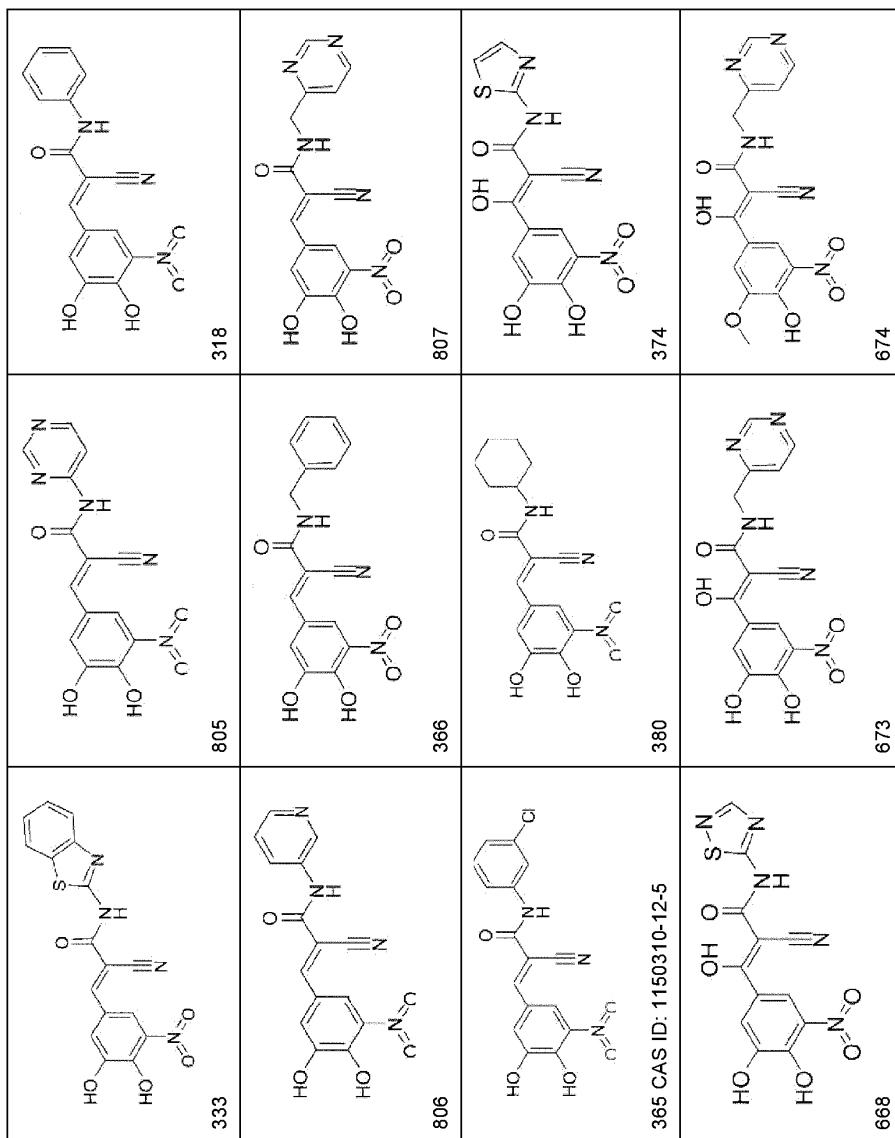
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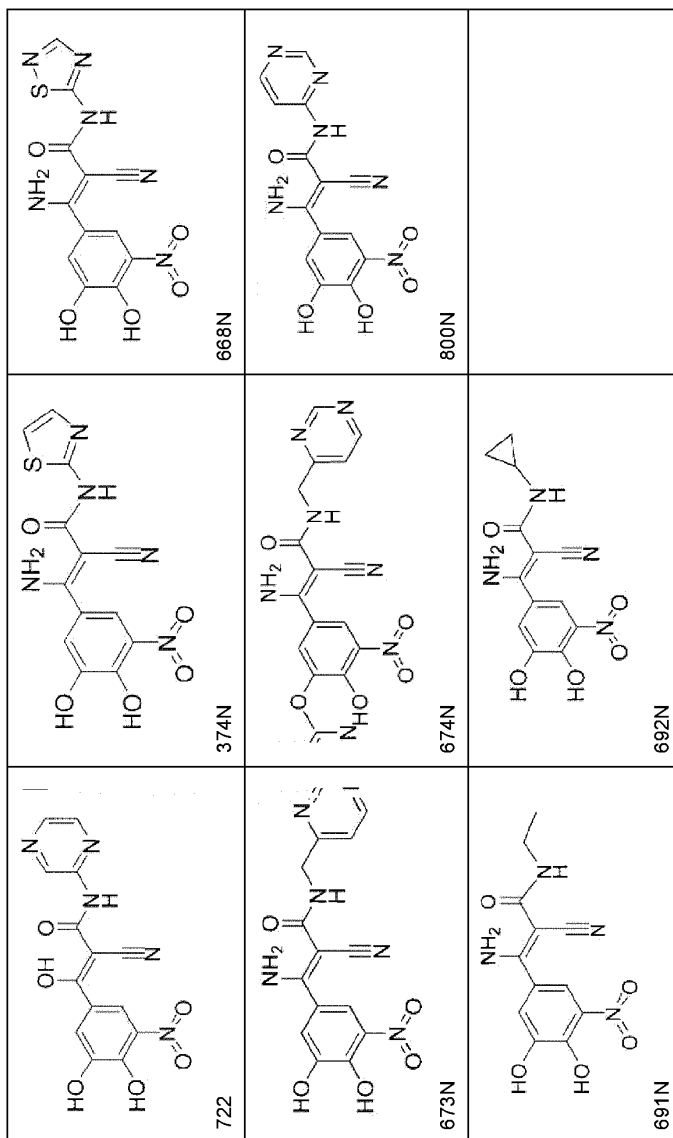
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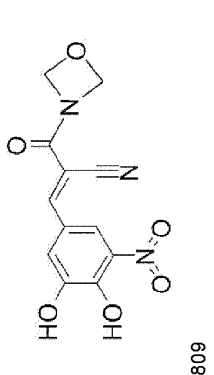
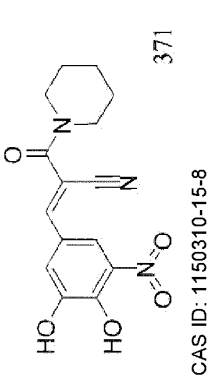
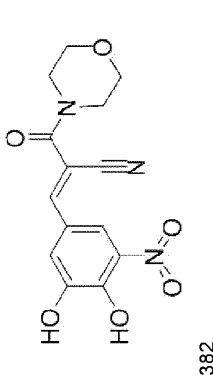
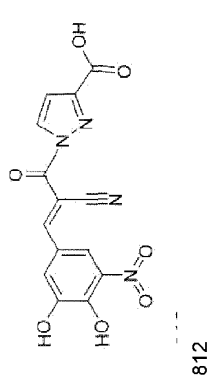
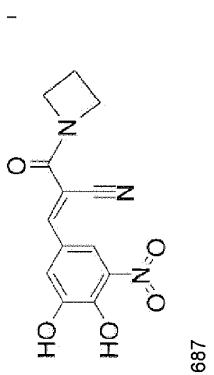
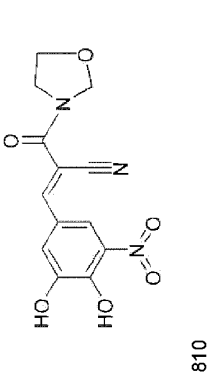
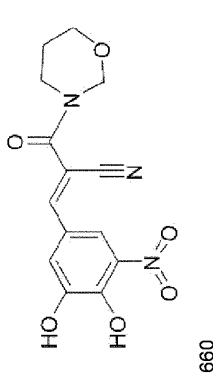
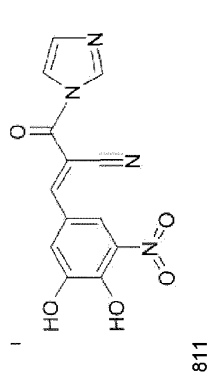
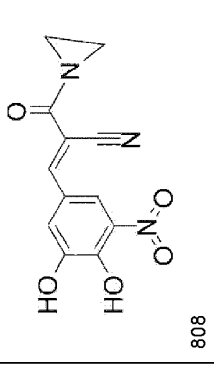
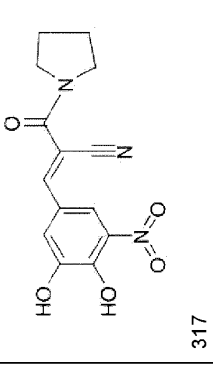
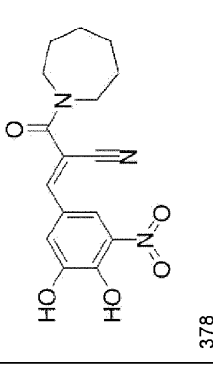
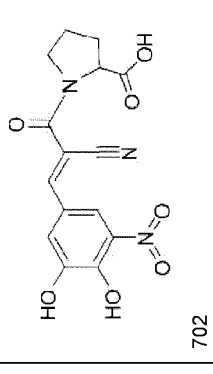
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Table 4. Subsection (c) inhibitors.

 809	 371 CAS ID: 1150310-15-8	 382	 812
 687	 810	 660	 811
 808	 317	 378	 702

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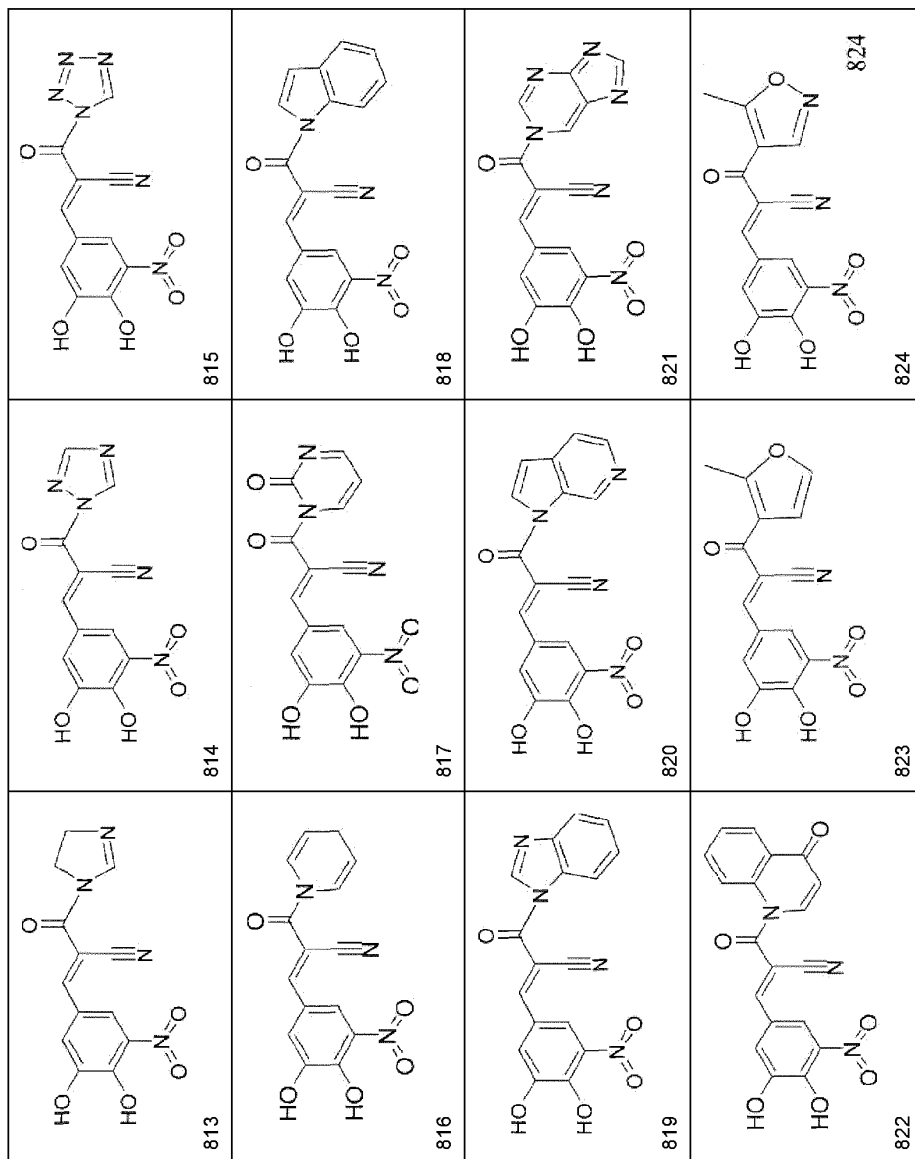
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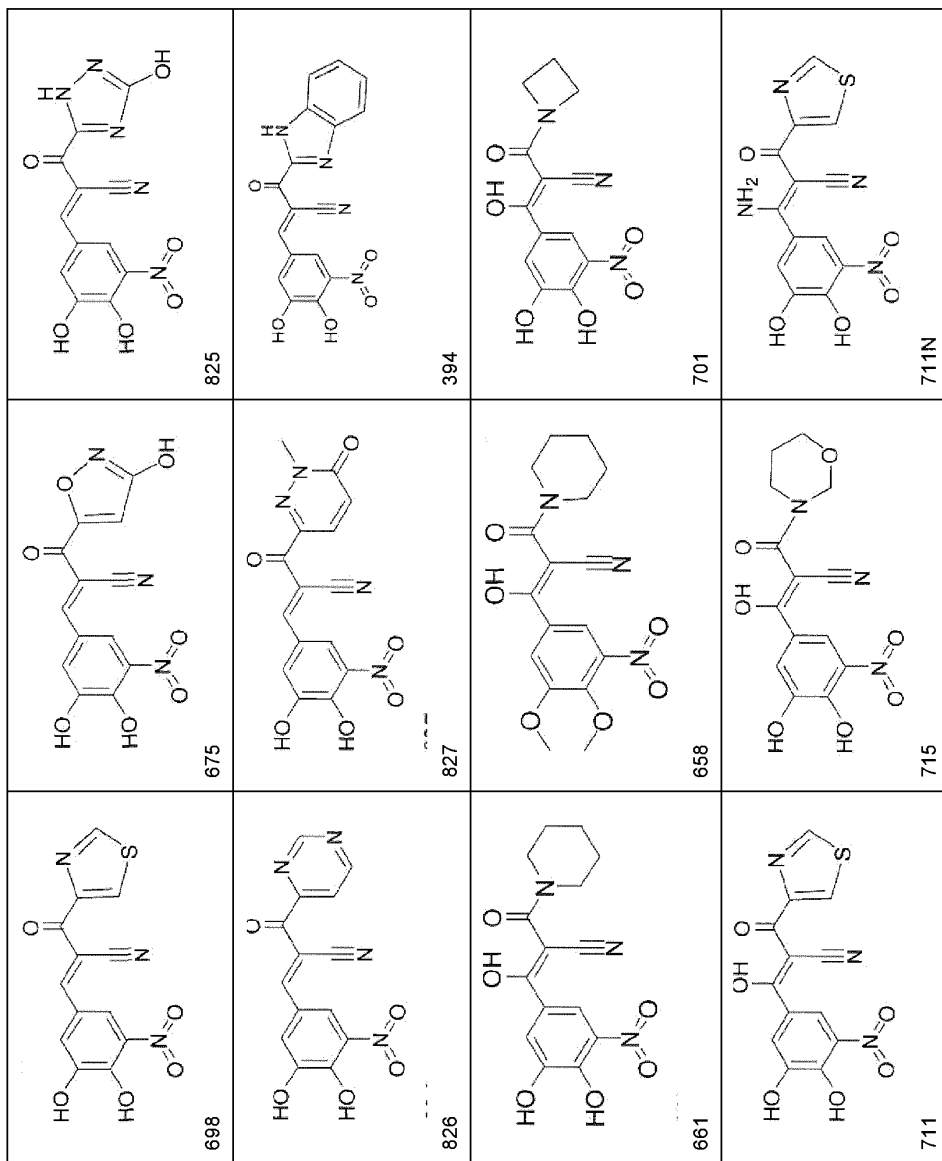
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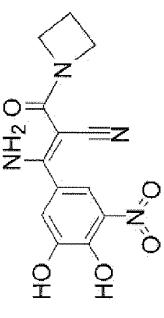
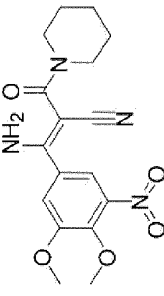
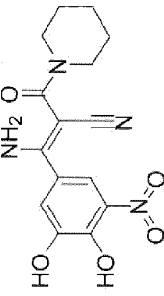
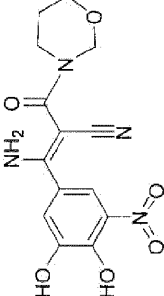
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 658N	
 661N	 715N

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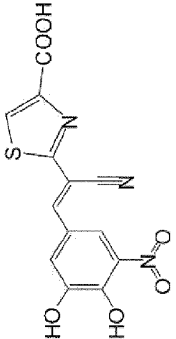
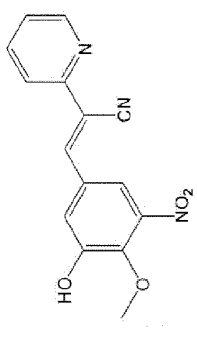
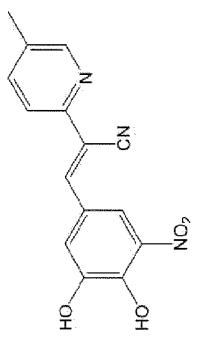
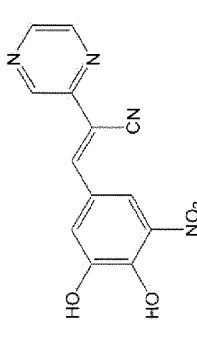
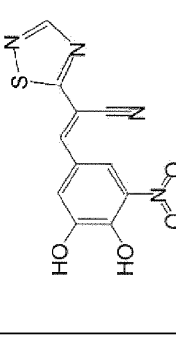
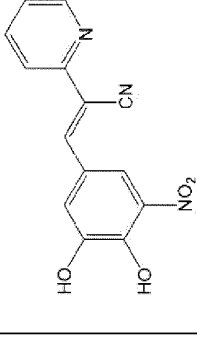
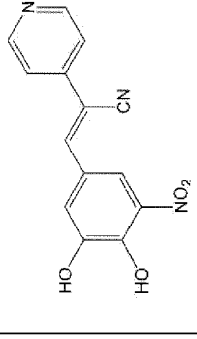
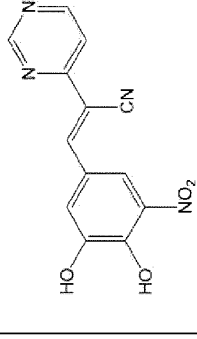
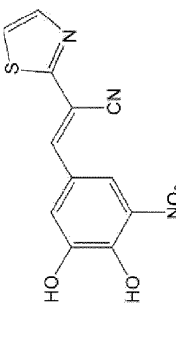
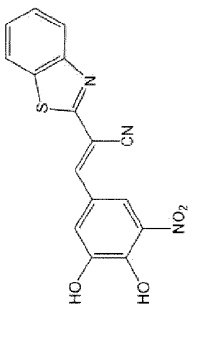
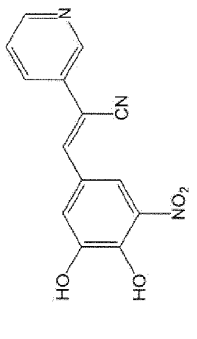
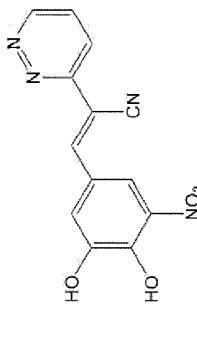
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Table 5. Subsection (d) inhibitors.

 666	 400	 502	 396
 656	 315	 389	 395
 390	 829	 319	 505

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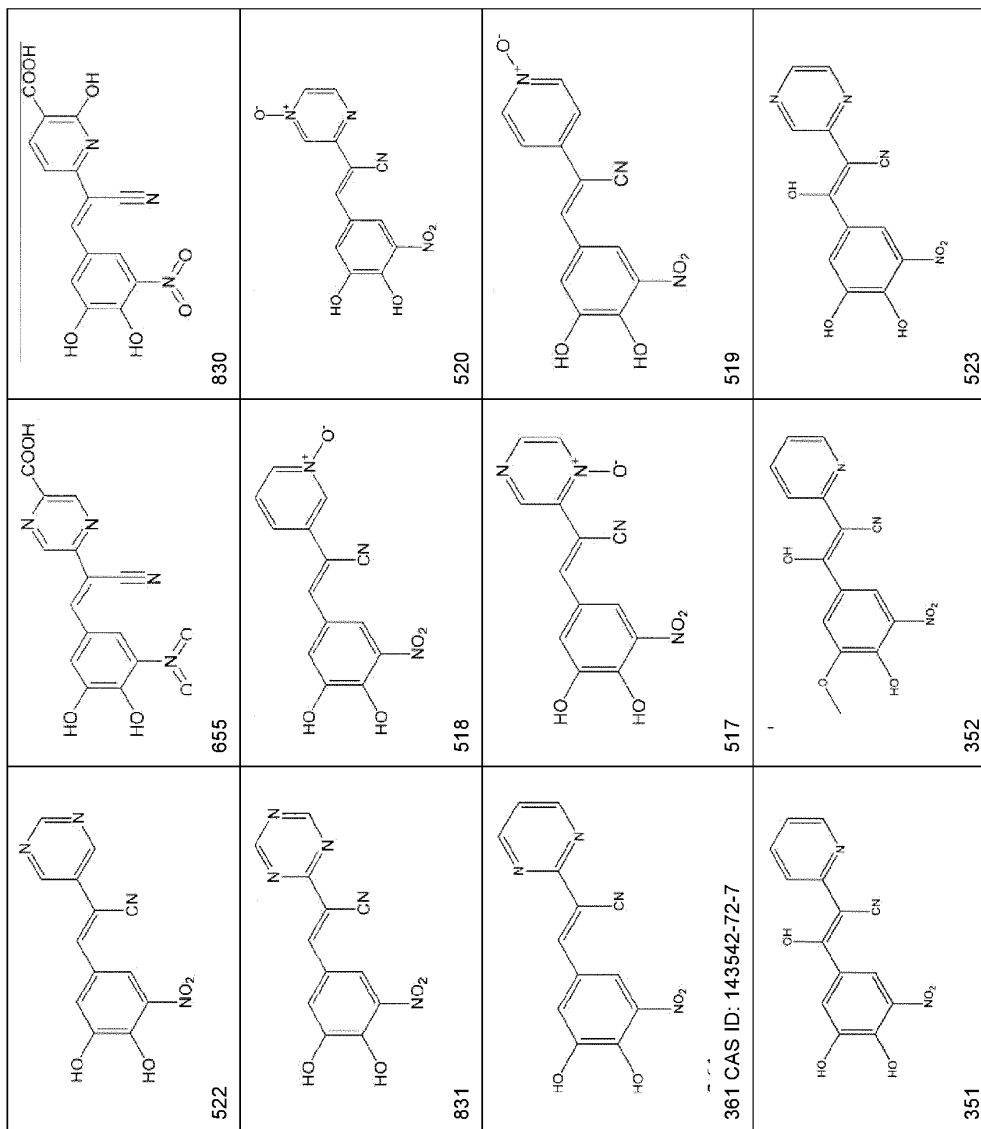
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361 CAS ID: 143542-72-7

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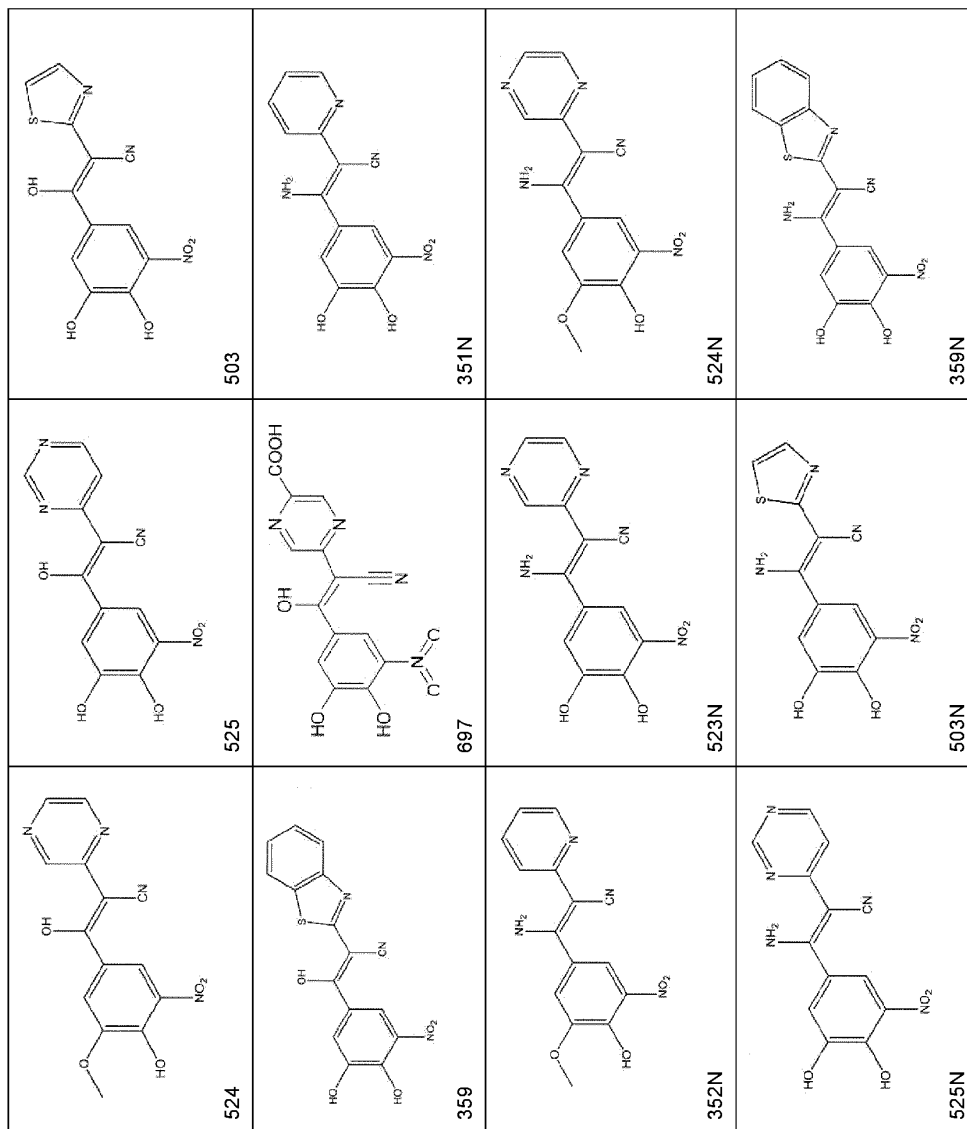
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Compound Preparation.

[0064] EP1978014 discloses processes for preparing entacapone; synthesis of representative derivatives are fully disclosed in WO/2016/206573, and not repeated here.

Therapeutic Activity

[0065] We measured the therapeutic efficacy of entacapone and representative entacapone derivatives in hypoxia-induced retinopathy zebrafish model (Cao et al., Hypoxia-induced retinopathy model in adult zebrafish. Nat Protoc 2010, 5 (12), 1903-10).

[0066] We measured the therapeutic effect of entacapone and several representative entacapone derivatives on hypoxia-induced retinopathy zebrafish model. The transgenic Tg (flila:EGFP) zebrafish were treated with cobalt chloride for 4 days to induce retinopathy. Zebrafish were treated with cobalt chloride and entacapone or derivative on serial concentrations of 5 mM, 15 mM and 50 mM for 4 days. As positive control, zebrafish were treated with cobalt chloride and 100 µL/mL eye-drops augentropfen stulln mono containing 0.3 mg/mL digitalin and 2 mg/mL esculin.

[0067] The eye blood vessel diameter in model group (22.40) was significantly larger than that in untreated group (17.08, $p < 0.001$), the eye ball volume in model group was smaller than that in untreated control group, and the inner plexiform layer and outer plexiform layer in model group was thinner than that in untreated control group. As the positive control, eye-drops augentropfen stulln mono inhibited retinal angiogenesis and repaired the retina structure (Table 1).

[0068] After treatment for 4 days, the retinal blood diameters were 19.33, 17.39 and 17.47 in zebrafish treated with entacapone on concentrations of 5 mM, 10 mM and 50 mM ($p < 0.001$, compared with model group), and the blood vessel diameter recovery rate were 58%, 94% and 93%, respectively (Figure 1). The eye ball volume was larger after entacapone treatment (Figure 2). The inner plexiform layer and outer plexiform layer were thicker in entacapone-treated groups than that in model group. Results with representative entacapone derivatives were consistent, and indicate that entacapone and active derivatives thereof suppress hypoxia-induced retinal neoangiogenesis and improve histopathology of retinopathy.

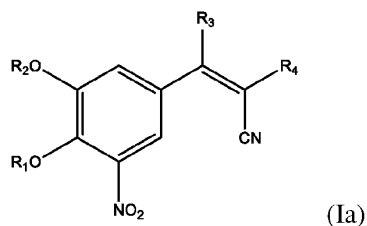
Table 1. Entacapone inhibited retinal angiogenesis in zebrafish (n = 10)

Group	Concentration	Blood vessel diameter (mean $\pm$ SE)	Recovery rate(%)
Untreated control	-	17.08 $\pm$ 0.51	-
Model	-	22.40 $\pm$ 0.44	-
Stulln mono	digitalin 0.3 mg/mL, esculin 2 mg/mL	16.90 $\pm$ 0.72***	103***
	5 mM	19.33 $\pm$ 0.52***	58***
Entacapone	15 mM	17.39 $\pm$ 0.49***	94***
	50 mM	17.47 $\pm$ 0.29***	93***

***p < 0.001, as compared with model group, unit: pixel

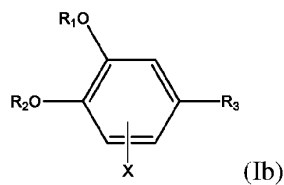
Claims

1. A pharmaceutical composition or formulation for use in treating or inhibiting macular degeneration or age-related macular degeneration, wherein the composition or formulation comprises entacapone, or a pharmaceutically-acceptable salt thereof, provided that the composition or formulation does not include another anti-Parkinsons medicament, neuroactive agent, anti-obesity, and/or anti-diabetes.
2. A pharmaceutical composition or formulation for use in treating or inhibiting macular degeneration or age-related macular degeneration, wherein the composition or formulation comprises an entacapone derivative, or a pharmaceutically-acceptable salt thereof, provided that the composition or formulation does not include another anti-Parkinsons medicament, neuroactive agent, anti-obesity, and/or anti-diabetes, wherein the entacapone derivative is selected from the group consisting of: a structure of formula (Ia), a stereoisomer thereof, or a pharmaceutically-acceptable salt thereof:



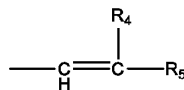
wherein:

R_1 and R_2 are independently H or Me;
 R_3 is H, OH or NHR, wherein R is H, C₁-C₁₈ hydrocarbyl, cyclic C₁-C₁₈ hydrocarbyl or heterocyclic C₁-C₁₈ hydrocarbyl; and
 R_4 is C₁-C₁₈ hydrocarbyl, cyclic C₁-C₁₈ hydrocarbyl or heterocyclic C₁-C₁₈ hydrocarbyl; or
 a structure of formula (Ib), a stereoisomer thereof, or a pharmaceutically-acceptable salt thereof:

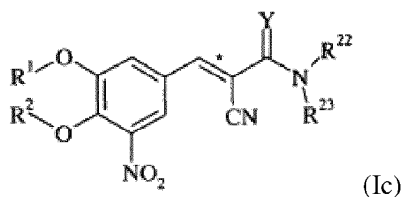


wherein R_1 and R_2 independently represent hydrogen, alkylcarbonyl of 2 to 5 carbon atoms or alkylcarbonyl of 2 to 5 carbon atoms, X represents nitro or cyano and

R_3 represents

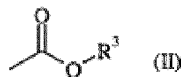


wherein R_4 represents cyano or alkylcarbonyl of 2 to 5 carbon atoms and R_5 represents cyano; alkylcarbonyl of 2 to 5 carbon atoms; or carbamoyl which is unsubstituted or substituted with alkyl of 1 to 8 carbon atoms, or hydroxyalkyl of 1 to 8 carbon atoms; or
 a structure of formula (Ic), a stereoisomer thereof, or a pharmaceutically-acceptable salt thereof:



wherein

Y is sulfur or oxygen,
 R^1 is a group of the following formula II



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or when Y is S, R¹ can be in addition H,

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R² is H or a group of formula II which may be the same as or different from R¹,
 R³ is independently (C₁-C₂₀)-alkyl, (CR⁴R⁵)_x-R⁶, (C₁-C₂₀)-alkylene-(C₁-C₂₀)-alkoxy, (C₂-C₂₀)-alkenyl,
 (C₂-C₂₀)-alkynyl, (C₀-C₂₀)-alkylene- (C₃-C₁₈)-cycloalkyl, (C₀-C₂₀)-alkylene-(3-18-membered)-heterocycloalkyl,
 (C₁-C₂₀)-alkylene-(C₃-C₁₈)-cycloalkenyl, (C₀-C₂₀)-alkylene-(3-18-membered)-heterocycloalkenyl,
 (C₀-C₂₀)-alkylene-(C₆-C₁₈)-aryl, (C₀-C₂₀)-alkylene-(5-18-membered)-heteroaryl, (C₂-C₂₀)-alkenylene-(C₃-C₁₈)-cycloalkyl,
 (C₂-C₂₀)-alkenylene-(3-18-membered)-heterocycloalkyl, (C₂-C₂₀)-alkenylene-(C₃-C₁₈)-cycloalkenyl, (C₃-C₂₀)-alkenylene-(3-18-membered)-heterocycloalkenyl, (C₂-C₂₀)-alkenylene-(C₆-C₁₈)-aryl, or (C₂-C₂₀)-alkenylene-(5-18-membered)-heteroaryl,
 wherein the total number of carbon atoms of R³ is at most 30,
 each R⁴ and R⁵ are independently of one another selected from the group consisting of H, (C₁-C₂₀)-alkyl, (C₁-C₂₀)-alkylene-hydroxy, (C₀-C₂₀)-alkylene- (C₁-C₂₀)-alkoxy, OH, (C₀-C₂₀)-alkylene-N(R⁷)CO-(C₁-C₂₀)-alkyl, (C₀-C₂₀)-alkylene-CON(R⁸)(R⁹), (C₀-C₂₀)-alkylene-COO- (C₁-C₂₀)-alkyl, (C₀-C₂₀)-alkylene- N(R¹⁰)(R¹¹), SO₃R¹⁷, (C₀-C₂₀)-alkylene-(C₆-C₁₈)-aryl, and (C₀-C₂₀)-alkylene-(5-18-membered)-heteroaryl,
 or
 R⁴ and R⁵ of the same group (CR⁴R⁵) or R⁴ and R⁵ of different groups (CR⁴R⁵) may form together a carbocyclic or heterocyclic ring having from 3 to 6 atoms,
 additionally, one or more non adjacent groups (CR⁴R⁵) may be replaced by O, CO, OCO, COO, CON(R¹⁹), N(R²⁰)CO, or NR²¹,
 R⁶ is independently H, (C₁-C₂₀)-alkyl, (C₂-C₂₀)-alkenyl, (C₂-C₂₀)-alkynyl, OH, O-(C₁-C₈)-alkyl, O-(C₀-C₈)-alkylene-(C₆-C₁₄)-aryl, CO-O-(C₁-C₈)-alkyl, CO-N(R¹²)(R¹³), N(R¹⁴)CO-(C₁-C₈)-alkyl, N(R¹⁵)(R¹⁶), SO₃R¹⁸, (C₀-C₂₀)-alkylene-(5-18-membered)-heteroaryl, or (C₀-C₂₀)-alkylene-(C₆-C₁₈)-aryl,
 R⁷, R¹⁴, R¹⁷, R¹⁸, R¹⁹, R²⁰, and R²¹ are independently of one another H, or (C₁-C₂₀)-alkyl,
 R⁸, R⁹, R¹⁰, R¹¹, R¹², R¹³, R¹⁵, and R¹⁶ are independently of one another H, or (C₁-C₂₀)-alkyl,
 R²² and R²³ are independently selected from the group consisting of H and (C₁-C₁₅)-alkyl, and
 x is 1 to 14.

3. The pharmaceutical composition or formulation for use according to claim 1 or 2, wherein the person that does not have Parkinson's disease, obesity, diabetes or diabetic retinopathy.
4. The pharmaceutical composition or formulation for use according to any one of claims 1-3, wherein the pharmaceutical composition or formulation further comprises a second, different medicament for treating or inhibiting macular degeneration or age-related macular degeneration.
5. The composition or formulation for use according to any one of claims 1-4, wherein the composition or formulation is in the topical form of eye drops, ointments, gels, or emulsions.
6. The composition or formulation for use according to any one of claims 1-4, wherein the composition or formulation is in the form of an intravitreal injection formulation or an intraocular implant formulation.
7. The composition or formulation for use according to any one of claims 1-4, wherein the composition or formulation is in a unit dosage form.
8. The composition or formulation for use according to claim 4, wherein the second, different medicament is an antiangiogenic drug selected from revacizumab, ranibizumab, pegaptanib, and aflibercept.

Patentansprüche

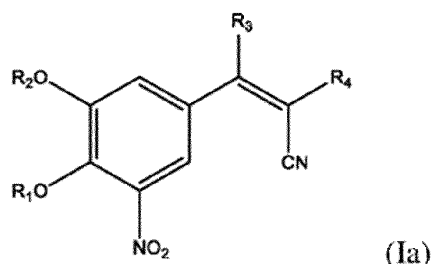
1. Pharmazeutische Zusammensetzung oder Formulierung zur Verwendung beim Behandeln oder Inhibieren von Makuladegeneration oder altersbedingter Makuladegeneration, wobei die Zusammensetzung oder Formulierung

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Entacapon oder ein pharmazeutisch verträgliches Salz davon umfasst, mit der Maßgabe, dass die Zusammensetzung oder Formulierung kein anderes Anti-Parkinson-Medikament, neuroaktives Mittel, Anti-Adipositas und/oder Anti-Diabetes einschließt.

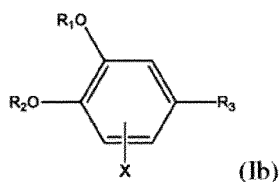
2. Pharmazeutische Zusammensetzung oder Formulierung zur Verwendung beim Behandeln oder Inhibieren von Makuladegeneration oder altersbedingter Makuladegeneration, wobei die Zusammensetzung oder Formulierung ein Entacapon-Derivat oder ein pharmazeutisch verträgliches Salz davon umfasst, mit der Maßgabe, dass die Zusammensetzung oder Formulierung kein anderes Anti-Parkinson-Medikament, neuroaktives Mittel, Anti-Adipositas und/oder Anti-Diabetes einschließt,

wobei das Entacapon-Derivat ausgewählt ist aus der Gruppe bestehend aus:
einer Struktur der Formel (Ia), einem Stereoisomer davon oder einem pharmazeutisch verträglichen Salz davon:

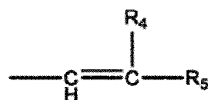


wobei:

R_1 und R_2 unabhängig H oder Me sind;
 R_3 H, OH oder NHR ist, wobei R H, C1-C18-Hydrocarbyl, cyclisches C1-C18-Hydrocarbyl oder heterocyclisches C1-C18-Hydrocarbyl ist; und
 R_4 C1-C18-Hydrocarbyl, cyclisches C1-C18-Hydrocarbyl oder heterocyclisches C1-C18-Hydrocarbyl ist; oder einer Struktur der Formel (Ib), einem Stereoisomer davon oder einem pharmazeutisch verträglichen Salz davon:

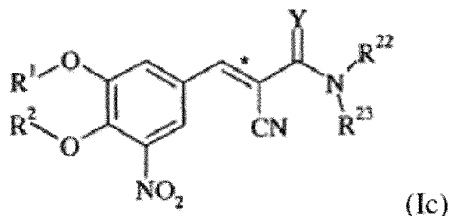


wobei R_1 und R_2 unabhängig Wasserstoff, Alkylcarbonyl von 2 bis 5 Kohlenstoffatomen oder Alkylcarbonyl von 2 bis 5 Kohlenstoffatomen darstellen, X Nitro oder Cyano darstellt und



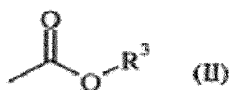
darstellt

wobei R_4 Cyano oder Alkylcarbonyl von 2 bis 5 Kohlenstoffatomen darstellt und R_5 Cyano; Alkylcarbonyl von 2 bis 5 Kohlenstoffatomen; oder Carbamoyl darstellt, das unsubstituiert oder mit Alkyl von 1 bis 8 Kohlenstoffatomen oder Hydroxyalkyl von 1 bis 8 Kohlenstoffatomen substituiert ist; oder einer Struktur der Formel (Ic), einem Stereoisomer davon oder einem pharmazeutisch verträglichen Salz davon:



wobei

Y Schwefel oder Sauerstoff ist,
R¹ eine Gruppe der folgenden Formel II ist



oder, wenn Y S ist, R¹ zusätzlich H sein kann,

R² H oder eine Gruppe der Formel II ist, die gleich oder verschieden von R¹ sein kann,

R³ unabhängig (C₁-C₂₀)-Alkyl, (CR⁴R⁵)_x-R⁶, (C₁-C₂₀)-Alkyl-(C₁-C₂₀)-Alkoxy, (C₂-C₂₀)-Alkenyl, (C₂-C₂₀)-Alkynyl, (C₀-C₂₀)-Alkyl-(C₃-C₁₈)-Cycloalkyl, (C₀-C₂₀)-Alkyl-(3-18-gliedriges)-Heterocycloalkyl, (C₁-C₂₀)-Alkyl-(C₃-C₁₈)-Cycloalkenyl, (C₀-C₂₀)-Alkyl-(3-18-gliedriges)-Heterocycloalkenyl, (C₀-C₂₀)-Alkyl-(C₆-C₁₈)-Aryl, (C₀-C₂₀)-Alkyl-(5-18-gliedriges)-Heteroaryl, (C₂-C₂₀)-Alkenyl-(C₃-C₁₈)-Cycloalkyl, (C₂-C₂₀)-Alkenyl-(3-18-gliedriges)-Heterocycloalkyl, (C₂-C₂₀)-Alkenyl-(C₃-C₁₈)-Cycloalkenyl, (C₂-C₂₀)-Alkenyl-(3-18-gliedriges)-Heterocycloalkenyl, (C₂-C₂₀)-Alkenyl-(C₆-C₁₈)-Aryl oder (C₂-C₂₀)-Alkenyl-(5-18-gliedriges)-Heteroaryl ist,

wobei die Gesamtanzahl an Kohlenstoffatomen von R³ höchstens 30 beträgt,

jeder R⁴ und R⁵ unabhängig voneinander ausgewählt sind aus der Gruppe bestehend aus H, (C₁-C₂₀)-Alkyl, (C₁-C₂₀)-Alkylhydroxy, (C₀-C₂₀)-Alkyl-(C₁-C₂₀)-Alkoxy, OH, (C₀-C₂₀)-Alkyl-N(R⁷)CO-(C₁-C₂₀)-Alkyl, (C₀-C₂₀)-Alkyl-CON(R⁸)(R⁹), (C₀-C₂₀)-Alkyl-COO-(C₁-C₂₀)-Alkyl, (C₀-C₂₀)-Alkyl-N(R¹⁰)(R¹¹), SO₃R¹⁷, (C₀-C₂₀)-Alkyl-(C₆-C₁₈)-Aryl und (C₀-C₂₀)-Alkyl-(5-18-gliedrigem)-Heteroaryl,

oder

R⁴ und R⁵ der gleichen Gruppe (CR⁴R⁵) oder R⁴ und R⁵ von verschiedenen Gruppen (CR⁴R⁵) zusammen einen carbocyclischen oder heterocyclischen Ring mit von 3 bis 6 Atomen bilden können, zusätzlich eine oder mehrere nicht benachbarte Gruppen (CR⁴R⁵) durch O, CO, OCO, COO, CON(R¹⁹), N(R²⁰)CO oder NR²¹ ersetzt sein können,

R⁶ unabhängig H, (C₁-C₂₀)-Alkyl, (C₂-C₂₀)-Alkenyl, (C₂-C₂₀)-Alkynyl, OH, O-(C₁-C₈)-Alkyl, O-(C₀-C₈)-Alkyl-(C₆-C₁₄)-Aryl, CO-O-(C₁-C₈)-Alkyl, CO-N(R¹²)(R¹³), N(R¹⁴)CO-(C₁-C₈)-Alkyl, N(R¹⁵)(R¹⁶), SO₃R¹⁸, (C₀-C₂₀)-Alkyl-(5-18-gliedriges)-Heteroaryl oder (C₀-C₂₀)-Alkyl-(C₆-C₁₈)-Aryl ist,

R⁷, R¹⁴, R¹⁷, R¹⁸, R¹⁹, R²⁰ und R²¹ unabhängig voneinander H oder (C₁-C₂₀)-Alkyl sind,

R⁸, R⁹, R¹⁰, R¹¹, R¹², R¹³, R¹⁵ und R¹⁶ unabhängig voneinander H oder (C₁-C₂₀)-Alkyl sind,

R²² und R²³ unabhängig ausgewählt sind aus der Gruppe bestehend aus H und (C₁-C₁₅)-Alkyl und x 1 bis 14 ist.

3. Pharmazeutische Zusammensetzung oder Formulierung zur Verwendung nach Anspruch 1 oder 2, wobei die Person, die keine Parkinson-Erkrankung, Adipositas, Diabetes oder diabetische Retinopathie aufweist.

4. Pharmazeutische Zusammensetzung oder Formulierung zur Verwendung nach einem der Ansprüche 1-3, wobei die pharmazeutische Zusammensetzung oder Formulierung ferner ein zweites, verschiedenes Medikament zum Behandeln oder Inhibieren von Makuladegeneration oder altersbedingter Makuladegeneration umfasst.

5. Zusammensetzung oder Formulierung zur Verwendung nach einem der Ansprüche 1-4, wobei die Zusammensetzung oder Formulierung in der topischen Form von Augentropfen, Salben, Gelen oder Emulsionen vorliegt.

6. Zusammensetzung oder Formulierung zur Verwendung nach einem der Ansprüche 1-4, wobei die Zusammensetzung

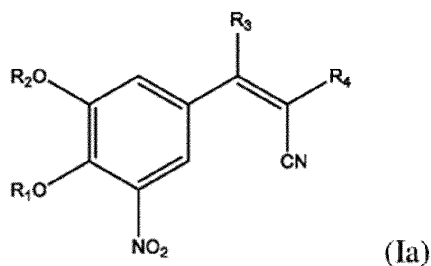
zung oder Formulierung in der Form einer intravitrealen Injektionsformulierung oder einer intraokularen Implantat-
formulierung vorliegt.

7. Zusammensetzung oder Formulierung zur Verwendung nach einem der Ansprüche 1-4, wobei die Zusammensetzung oder Formulierung in einer Einheitsdosierungsform vorliegt.
8. Zusammensetzung oder Formulierung zur Verwendung nach Anspruch 4, wobei das zweite, verschiedene Medikament ein antiangiogenes Arzneimittel ist, ausgewählt aus Revacizumab, Ranibizumab, Pegaptanib und Aflibercept.

Revendications

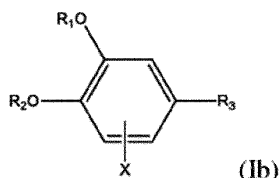
1. Composition ou formulation pharmaceutique pour utilisation dans le traitement ou l'inhibition d'une dégénérescence maculaire ou d'une dégénérescence maculaire liée à l'âge, dans laquelle la composition ou formulation comprend de l'entacapone, ou un sel pharmaceutiquement acceptable de celle-ci, à condition que la composition ou formulation n'inclue pas un autre médicament anti-Parkinsons, un agent neuroactif, un anti-obésité, et/ou un anti-diabète.
2. Composition ou formulation pharmaceutique pour utilisation dans le traitement ou l'inhibition d'une dégénérescence maculaire ou d'une dégénérescence maculaire liée à l'âge, dans laquelle la composition ou formulation comprend un dérivé d'entacapone, ou un sel pharmaceutiquement acceptable de celui-ci, à condition que la composition ou formulation n'inclue pas un autre médicament anti-Parkinson, un agent neuroactif, un anti-obésité, et/ou un anti-diabète,

dans laquelle le dérivé d'entacapone est choisi dans le groupe constitué de :
une structure de formule (Ia), un stéréo-isomère de celle-ci, ou un sel acceptable sur le plan pharmaceutique de celle-ci :



dans laquelle :

R₁ et R₂ sont indépendamment H ou Me ;
R₃ est H, OH ou NHR, dans laquelle R est H, hydrocarbyle en C1 à C18, hydrocarbyle cyclique en C1 à C18 ou hydrocarbyle hétérocyclique en C₁ à C₁₈ ; et
R₄ est hydrocarbyle en C₁ à C₁₈, hydrocarbyle cyclique en C₁ à C₁₈ ou hydrocarbyle hétérocyclique en C₁ à C₁₈ ; ou
une structure de formule (Ib), un stéréo-isomère de celle-ci, ou un sel acceptable sur le plan pharmaceutique de celle-ci :



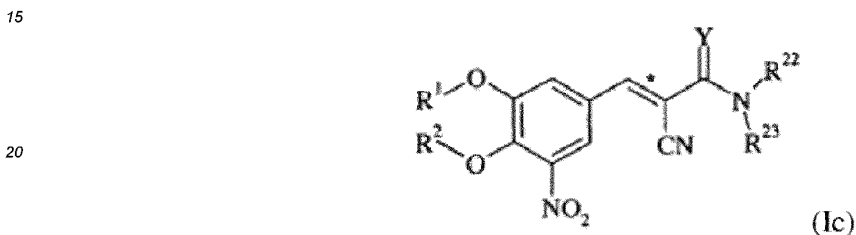
dans laquelle R₁ et R₂ représentent indépendamment hydrogène, alkylcarbamoyle de 2 à 5 atomes de carbone

ou alkylcarbonyle de 2 à 5 atomes de carbone, X représente nitro ou cyano et



R₃ représente

10 dans laquelle R₄ représente cyano ou alkylcarbonyle de 2 à 5 atomes de carbone et R₅ représente cyano ; alkylcarbonyle de 2 à 5 atomes de carbone ; ou carbamoyle qui est non substitué ou substitué avec un alkyle de 1 à 8 atomes de carbone, ou un hydroxyalkyle de 1 à 8 atomes de carbone ; ou une structure de formule (Ic), un stéréo-isomère de celle-ci, ou un sel acceptable sur le plan pharmaceutique de celle-ci :



25 dans laquelle

Y est soufre ou oxygène,
R¹ est un groupe de la formule II suivante



35 ou lorsque Y est S, R¹ peut être en plus H,

R² est H ou un groupe de formule II qui peut être le même que ou différent de R¹,

38 R³ est indépendamment alkyle en (C₁ à C₂₀), (CR⁴R⁵)x-R⁶, alkylène en (C₁ à C₂₀)-alcoxy en (C₁ à C₂₀), alcénylène en (C₂ à C₂₀), alcynyle en (C₂ à C₂₀), alkylène en (C₀ à C₂₀)-cycloalkyle en (C₃ à C₁₈), alkylène en (C₀ à C₂₀)-hétérocycloalkyle de (3 à 18 chaînons), alkylène en (C₁ à C₂₀)-cycloalcénylène en (C₃ à C₁₈), alkylène en (C₀ à C₂₀)-hétérocycloalcénylène de (3 à 18 chaînons), alkylène en (C₀ à C₂₀)-aryle en (C₆ à C₁₈), alkylène en (C₀ à C₂₀)-hétéroaryle de (5 à 18 chaînons), alcénylène en (C₂ à C₂₀)-cycloalkyle en (C₃ à C₁₈), alcénylène en (C₂ à C₂₀)-hétérocycloalkyle de (3 à 18 chaînons), alcénylène en (C₂ à C₂₀)-cycloalcénylène en (C₃ à C₁₈), alcénylène en (C₂ à C₂₀)-hétérocycloalkyle de (3 à 18 chaînons), alcénylène en (C₂ à C₂₀)-aryle en (C₆ à C₁₈), ou alcénylène en (C₂ à C₂₀)-hétéroaryle de (5 à 18 chaînons),
42 dans laquelle le nombre total d'atomes de carbone de R³ est d'au plus 30,

45 chaque R⁴ et R⁵ sont indépendamment l'un de l'autre choisis dans le groupe constitué de H, alkyle en (C₁ à C₂₀), alkylène en (C₁ à C₂₀)-hydroxy, alkylène en (C₀ à C₂₀)-alcoxy en (C₁ à C₂₀), OH, alkylène en (C₀ à C₂₀)-N(R⁷)CO-alkyle en (C₁ à C₂₀), alkylène en (C₀ à C₂₀)-CON(R⁸)(R⁹), alkylène en (C₀ à C₂₀)-COO-alkyle en (C₁ à C₂₀), alkylène en (C₀ à C₂₀)-N(R¹⁰)(R¹¹), SO₃R¹⁷, alkylène en (C₀ à C₂₀)-aryle en (C₆ à C₁₈), et alkylène en (C₀ à C₂₀)-hétéroaryle de (5 à 18 chaînons)-hétéroaryle,
48 ou

50 R⁴ et R⁵ du même groupe (CR⁴R⁵) ou R⁴ et R⁵ de groupes différents (CR⁴R⁵) peuvent former conjointement un noyau carbocyclique ou hétérocyclique ayant de 3 à 6 atomes, en complément, un ou plusieurs groupes non adjacents (CR⁴R⁵) peuvent être remplacés par O, CO, OCO, COO, CON(R¹⁹), N(R²⁰)CO, ou NR²¹,
52

55 R⁶ est indépendamment H, alkyle en (C₁ à C₂₀), alcénylène en (C₂ à C₂₀), alcynyle en (C₂ à C₂₀), OH, O-alkyle en (C₁ à C₈), O-alkylène en (C₀ à C₈)-aryle en (C₆ à C₁₄), CO-O-alkyle en (C₁ à C₈), CO-N(R¹²)(R¹³), N(R¹⁴)CO-alkyle en (C₁ à C₈), N(R¹⁵)(R¹⁶), SO₃R¹⁸, alkylène en (C₀ à C₂₀)-hétéroaryle de (5 à 18 chaînons),

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ou alkylène en (C₀ à C₂₀)-aryle en (C₆ à C₁₈),
R⁷, R¹⁴, R¹⁷, R¹⁸, R¹⁹, R²⁰, et R²¹ sont indépendamment l'un de l'autre H, ou alkyle en (C₁ à C₂₀),
R⁸, R⁹, R¹⁰, R¹¹, R¹², R¹³, R¹⁵, et R¹⁶ sont indépendamment l'un de l'autre H, ou alkyle en (C₁ à C₂₀),
R²² et R²³ sont indépendamment choisis dans le groupe constitué de H et alkyle en (C₁ à C₁₅), et
x va de 1 à 14.

5

3. Composition ou formulation pharmaceutique pour utilisation selon la revendication 1 ou 2, dans laquelle la personne n'a pas de maladie de Parkinson, d'obésité, de diabète ou de rétinopathie diabétique.

10

4. Composition ou formulation pharmaceutique pour utilisation selon l'une quelconque des revendications 1 à 3, dans laquelle la composition ou formulation pharmaceutique comprend en outre un deuxième médicament différent pour traiter ou inhiber une dégénérescence maculaire ou une dégénérescence maculaire liée à l'âge.

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5. Composition ou formulation pour utilisation selon l'une quelconque des revendications 1 à 4, dans laquelle la composition ou formulation est sous la forme topique de collyres, onguents, gels, ou émulsions.

20

6. Composition ou formulation pour utilisation selon l'une quelconque des revendications 1 à 4, dans laquelle la composition ou formulation est sous la forme d'une formulation pour injection intravitréenne ou d'une formulation d'implant intraoculaire.

7. Composition ou formulation pour utilisation selon l'une quelconque des revendications 1 à 4, dans laquelle la composition ou formulation est sous une forme pharmaceutique unitaire.

25

8. Composition ou formulation pour utilisation selon la revendication 4, dans laquelle le deuxième médicament différent est un médicament anti-angiogénique choisi parmi révacizumab, ranibizumab, pégaptanib, et aflibercept.

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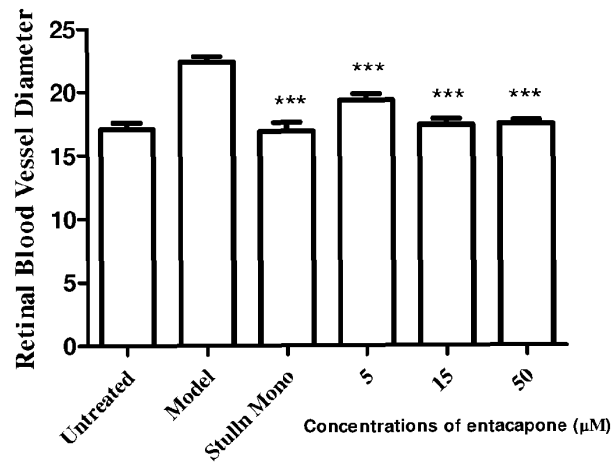


Fig 1

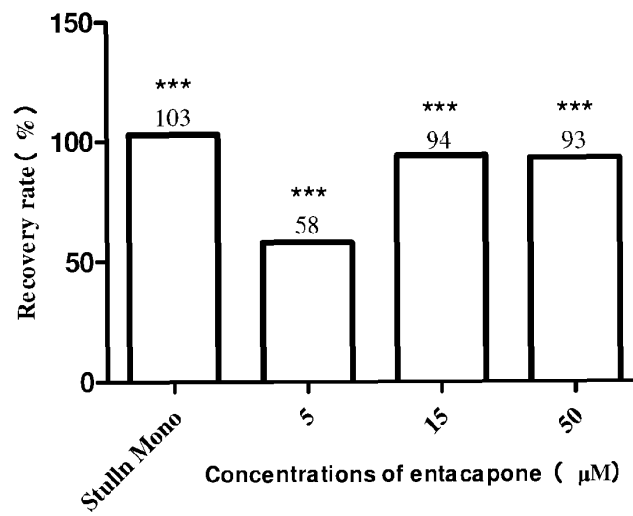


Fig. 2

REFERENCES CITED IN THE DESCRIPTION

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(11) **EP 3 716 992 B1**

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A61K 38/17 ^(2006.01) **A61P 27/02** ^(2006.01)
- (52) Cooperative Patent Classification (CPC):
A61K 38/179; A61P 27/02
- (86) International application number:
PCT/US2018/063025
- (87) International publication number:
WO 2019/108770 (06.06.2019 Gazette 2019/23)

(54) **USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS**

VERWENDUNG EINES VEGF-ANTAGONISTEN ZUR BEHANDLUNG ANGIOGENER AUGENERKRANKUNGEN

UTILISATION D'UN ANTAGONISTE DE VEGF POUR TRAITER DES TROUBLES OCULAIRES ANGIOGÉNIQUES

- | | |
|---|---|
| <p>(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR
Designated Extension States:
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22.10.2018 US 201862748782 P</p> <p>(43) Date of publication of application:
07.10.2020 Bulletin 2020/41</p> <p>(60) Divisional application:
22181390.0</p> <p>(73) Proprietor: Regeneron Pharmaceuticals, Inc.
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London EC1M 5QU (GB)</p> <p>(56) References cited:
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EP 3 716 992 B1

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Remarks:

The file contains technical information submitted after the application was filed and not included in this specification

Description

[0001] This application claims the benefit of U.S. provisional patent application nos. 62/593,033, filed November 30, 2017; 62/644,425, filed March 17, 2018; and 62/748,782, filed October 22, 2018.

FIELD OF THE INVENTION

[0002] The present invention relates to the field of therapeutic treatments of eye disorders. More specifically, the invention relates to a VEGF antagonist for treating or preventing diabetic retinopathy in a patient that does not suffer from center involved diabetic macular edema.

BACKGROUND

[0003] Several eye disorders are associated with pathological angiogenesis. For example, the development of age-related macular degeneration (AMD) is associated with a process called choroidal neovascularization (CNV). Leakage from the CNV causes macular edema and collection of fluid beneath the macula resulting in vision loss. Diabetic macular edema (DME) is another eye disorder with an angiogenic component. DME is the most prevalent cause of moderate vision loss in patients with diabetes and is a common complication of diabetic retinopathy, a disease affecting the blood vessels of the retina. Clinically significant DME occurs when fluid leaks into the center of the macula, the light-sensitive part of the retina responsible for sharp, direct vision. Fluid in the macula can cause severe vision loss or blindness. Yet another eye disorder associated with abnormal angiogenesis is central retinal vein occlusion (CRVO). CRVO is caused by obstruction of the central retinal vein that leads to a back-up of blood and fluid in the retina. The retina can also become ischemic, resulting in the growth of new, inappropriate blood vessels that can cause further vision loss and more serious complications. Release of vascular endothelial growth factor (VEGF) contributes to increased vascular permeability in the eye and inappropriate new vessel growth. Thus, inhibiting the angiogenic-promoting properties of VEGF appears to be an effective strategy for treating angiogenic eye disorders.

[0004] FDA-approved treatments of angiogenic eye disorders such as AMD and CRVO include the administration of an anti-VEGF antibody called ranibizumab (Lucentis®, Genentech, Inc.) on a monthly basis by intravitreal injection.

[0005] Methods for treating eye disorders using VEGF antagonists are mentioned in, e.g., US 7,303,746; US 7,306,799; US 7,300,563; US 7,303,748; and US 2007/0190058. Nonetheless, there remains a need in the art for new administration regimens for angiogenic eye disorders, especially those which allow for less frequent dosing while maintaining a high level of efficacy.

BRIEF SUMMARY OF THE INVENTION

[0006] The present invention is defined in the claims. The present invention is concerned with a VEGF antagonist for use in methods for treating or preventing diabetic retinopathy, e.g., nonproliferative diabetic retinopathy, wherein the patient does not suffer from center involved diabetic macular edema. The VEGF antagonist of the present invention is for use in methods comprising sequentially administering multiple doses of a VEGF antagonist to a patient over time. In particular, the methods comprise sequentially administering to the eye of the patient five monthly doses of a VEGF antagonist, followed by one or more secondary doses of the VEGF antagonist every eight weeks, which may be followed by one or more tertiary doses of the VEGF antagonists. The present inventors have surprisingly discovered that beneficial therapeutic effects can be achieved in patients suffering from angiogenic eye disorders by administering a VEGF antagonist to a patient at a frequency of once every 8 or more weeks, when such doses are preceded by five monthly doses. One advantage of such a dosing regimen is that, for most of the course of treatment it allows for less frequent dosing (once every 8 weeks) compared to prior administration regimens for angiogenic eye disorders which require monthly administrations throughout the entire course of treatment. (See, e.g., prescribing information for Lucentis® [ranibizumab], Genentech, Inc.).

[0007] Hence, the present invention provides a VEGF antagonist for use in a method for treating or preventing diabetic retinopathy in a patient in need of such treatment or prevention, wherein said patient does not suffer from center involved diabetic macular edema, said method comprising administering, to an eye of the patient, 5 monthly doses followed by one or more secondary doses every 8 weeks of about 2 mg of VEGF antagonist that is a VEGF receptor-based chimeric molecule comprising (1) an immunoglobulin-like (Ig) domain 2 of a VEGFR1 and (2) Ig domain 3 of VEGFR2, and (3) a multimerizing component.

[0008] The VEGF antagonist of the present invention is for use in a method where it is administered to an eye of the patient (e.g., by intravitreal injection). The VEGF antagonist comprises one or more VEGF receptor-based chimeric molecule(s), (also referred to herein as a "VEGF-Trap" or "VEGFT"). In particular, the VEGF antagonist is a VEGF receptor-based chimeric molecule comprising (1) an immunoglobulin-like (Ig) domain 2 of a VEGFR1 and (2) Ig domain

3 of VEGFR2, and (3) a multimerizing component. An exemplary VEGF antagonist that can be used in the context of the present invention is a multimeric VEGF-binding protein comprising two or more VEGF receptor-based chimeric molecules referred to herein as "VEGFR1R2-FcΔC1(a)" or "aflibercept."

5 [0009] Various administration routes are contemplated for use in the methods of the present invention, provided that the VEGF antagonist is administered to an eye of the patient, including, *e.g.*, topical administration or intraocular administration (*e.g.*, intravitreal administration).

[0010] Aflibercept (EYLEA™, Regeneron Pharmaceuticals, Inc) was approved by the FDA in November 2011, for the treatment of patients with neovascular (wet) age-related macular degeneration, with a recommended dose of 2 mg administered by intravitreal injection every 4 weeks for the first three months, followed by 2 mg administered by intravitreal injection once every 8 weeks.

10 [0011] The VEGF antagonist of the present invention may be for use in a method for treating or preventing diabetic retinopathy of any severity level, for example, nonproliferative diabetic retinopathy (NPDR) (*e.g.*, moderately severe to severe NPDR, for example, characterized by a Diabetic Retinopathy Severity Scale level of about 47-53, *e.g.*, 47 or 53) in a patient (*e.g.*, a human, for example, 18 years of age or older, *e.g.*, having type 1 or 2 diabetes) in need of such treatment, provided that the patient does not suffer from center involved diabetic macular edema, said method comprising administering (*e.g.*, by intravitreal injection), to an eye of the patient,

(i) 5 monthly doses followed by one or more secondary doses every 8 weeks, wherein the secondary doses initiate 8 weeks after the final of the 5 monthly doses and continues with a dose given every 8 weeks thereafter;

20 of about 2 mg of VEGF antagonist which is a VEGF receptor-based chimeric molecule, for example, that comprises (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2; (2) a VEGFR2 component comprising amino acids 130-231 of SEQ ID NO:2; and (3) a multimerization component comprising amino acids 232-457 of SEQ ID NO:2. For example, in an embodiment of the invention, the VEGF antagonist is Aflibercept. In an embodiment of the invention, 25 the VEGF antagonist comprises VEGFR1R2-FcΔC1(a) encoded by the nucleic acid sequence of SEQ ID NO:1. In an embodiment of the invention, the patient is characterized as having a baseline best-corrected visual acuity (BCVA) ETDRS letter score of 69 or greater; having vision characterized by a Snellen visual acuity of 20/40 or better; does not suffer from retinal neovascularization; does not suffer from anterior segment neovascularization (ASNV); does not suffer from vitreous hemorrhage; and/or does not suffer from tractional retinal detachment. In an embodiment of the invention, "treating" NPDR in a patient, as discussed herein, refers to bringing about at least a 2-step improvement in DRSS (Diabetic Retinopathy Severity Scale) from baseline (before the first VEGF antagonist administration), for example, by week 24 or 48 or 52 (relative to commencement of first VEGF antagonist administration). The patient has diabetic retinopathy and does not suffer from center involved diabetic macular edema.

30 [0012] The present invention provides a VEGF antagonist for use in a method for treating or preventing proliferative diabetic retinopathy (PDR) in a patient (*e.g.*, a human) in need of such treatment or prevention, wherein said patient does not suffer from center involved diabetic macular edema (CI-DME), wherein the patient is initially treated for non-proliferative diabetic retinopathy (*e.g.*, wherein the patient has type 1 or 2 diabetes; has a hemoglobin A1c of about 8.5; has an ETDRS BCVA score of about 82; has a central retinal thickness of about 247 μm; has a diabetic retinopathy severity score of 47 or 53; and/or is about 56 years of age), said method comprising administering, to an eye of the patient (*e.g.*, by intravitreal injection),

5 monthly doses followed by one or more secondary doses every 8 weeks;

45 of about 2 mg of VEGF antagonist, *e.g.*, that comprises (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2; (2) a VEGFR2 component comprising amino acids 130-231 of SEQ ID NO:2; and (3) a multimerization component comprising amino acids 232-457 of SEQ ID NO:2 (*e.g.*, aflibercept). The VEGF antagonist is for use in a method where the patient is treated for at least about 24 weeks, for example 52 weeks or 100 weeks, *e.g.*, wherein the patient receives 6 injections over the 24 week period. In an embodiment of the invention, the VEGF antagonist is for use in a method where the patient is treated and achieves one or more of the following benefits:

- 50 (i) at least a 2 step improvement from baseline in diabetic retinopathy severity scale (DRSS) score;
 (ii) at least a 3 step improvement from baseline in diabetic retinopathy severity scale (DRSS) score;
 (iii) an improvement in best corrected visual acuity of at least about 1.9 letters;
 (iv) does not experience a reduction in best corrected visual acuity of any more than 4 letters;
 (v) does not develop diabetic macular edema;
 55 (vi) does not develop center involved diabetic macular edema;
 (vii) does not experience a vision threatening complication;
 (viii) does not develop proliferative diabetic retinopathy;
 (ix) does not develop anterior segment neovascularization;

and/or (x) experiences a reduction in central retinal thickness of about 19 μ m. Thus, the present invention also includes the VEGF antagonist of the invention for use in methods for causing a patient with non-proliferative diabetic retinopathy to achieve one or more of the following benefits

- 5 (i) at least a 2 step improvement from baseline in diabetic retinopathy severity scale (DRSS) score;
- (ii) at least a 3 step improvement from baseline in diabetic retinopathy severity scale (DRSS) score;
- (iii) an improvement in best corrected visual acuity of at least about 1.9 letters;
- (iv) does not experience a reduction in best corrected visual acuity of any more than 4 letters;
- (v) does not develop diabetic macular edema;
- 10 (vi) does not develop center involved diabetic macular edema;
- (vii) does not experience a vision threatening complication;
- (viii) does not develop proliferative diabetic retinopathy;
- (ix) does not develop anterior segment neovascularization;
- and/or
- 15 (x) experiences a reduction in central retinal thickness of about 19 μ m; by administering a VEGF antagonist according to the dosing regimen set forth above.

[0013] For example, the VEGF antagonist of the present invention may be for use in a method that causes a reduction or preventing an increase in the Diabetic Retinopathy Severity Scale (DRSS) level (see *e.g.*, Figure 18) (*e.g.*, by at least 2 or 3 levels) of nonproliferative diabetic retinopathy in a patient, wherein said patient does not suffer from center involved diabetic macular edema, comprising administering, to an eye of the patient (*e.g.*, by intravitreal injection),

5 monthly doses followed by one or more secondary doses every 8 weeks; of about 2 mg of VEGF antagonist, *e.g.*, that comprises (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2; (2) a VEGFR2 component comprising amino acids 130-231 of SEQ ID NO:2; and (3) a multimerization component comprising amino acids 232-457 of SEQ ID NO:2 (*e.g.*, aflibercept).

[0014] The VEGF antagonist of the present invention may be for use in method for treating or preventing the occurrence or re-occurrence of a vision threatening complication or blindness in the eye of a subject (*e.g.*, a human) whose eye has nonproliferative diabetic retinopathy, wherein said patient does not suffer from center involved diabetic macular edema, the method comprising administering (*e.g.*, by intravitreal injection), to an eye of the subject (*e.g.*, by intravitreal injection), (5 monthly doses followed by one or more secondary doses every 8 weeks; of about 2 mg of VEGF antagonist that is a VEGF receptor-based chimeric molecule comprising (1) an immunoglobulin-like (Ig) domain 2 of a VEGFR1 and (2) Ig domain 3 of VEGFR2, and (3) a multimerizing component. Optionally, the other eye of the subject is also administered the VEGF antagonist even if not so afflicted, afflicted with DRSS of a lower level or afflicted with another angiogenic eye disorder. For example, in an embodiment of the invention, the VEGF antagonist is a VEGF receptor-based chimeric molecule which (i) comprises (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2; (2) a VEGFR2 component comprising amino acids 130-231 of SEQ ID NO:2; and (3) a multimerization component comprising amino acids 232-457 of SEQ ID NO:2; (ii) comprises (1) an immunoglobulin-like (Ig) domain 2 of a first VEGF receptor and (2) Ig domain 3 of a second VEGF receptor, and (3) a multimerizing component; (iii) is aflibercept; or (iv) is conbercept.

[0015] Other embodiments of the present invention will become apparent from a review of the ensuing detailed description.

BRIEF DESCRIPTION OF THE FIGURES

[0016]

Figure 1 shows a dosing regimen. In this regimen, a single "initial dose" of VEGF antagonist ("VEGFT") is administered at the beginning of the treatment regimen (*i.e.* at "week 0"), two "secondary doses" are administered at weeks 4 and 8, respectively, and at least six "tertiary doses" are administered once every 8 weeks thereafter, *i.e.*, at weeks 16, 24, 32, 40, 48, 56, *etc.*

Figure 2 shows a further dosing regimen. In this regimen, three monthly doses ("Q4wk doses") of a VEGF antagonist are administered at the beginning of the treatment regimen (*i.e.*, at weeks 0, 4 and 8), a single dose is then administered 8 weeks after the last Q4wk dose ("Q8wk dose") (*i.e.*, at week 16), followed by three or more additional doses administered 16 weeks after the Q8wk dose and once every 16 weeks thereafter ("Q16wk doses") (*i.e.*, at weeks 32, 48, 64, *etc.*).

Figure 3 summarizes the phase 3, double-masked, randomized, controlled PANORAMA study design. Patients with moderately severe to severe NPDR received (i) a sham injection ("sham"), (ii) 3 monthly doses followed by one or

more secondary doses every 16 weeks, wherein the first secondary dose initiates 8 weeks after the third monthly dose (week 16) ("Group 1"), or (iii) 5 monthly doses followed by one or more secondary doses every 8 weeks ("Group 2").

Figure 4 summarizes the PANORAMA dosing schedule of the sham, Group 1 and Group 2 groups. X=Active injections; O=sham injections.

Figure 5 describes the baseline disposition and demographics of the PANORAMA study population in each dosing group including: sham, Group 1, Group 2 and the combination of Group 1 and Group 2 (All IAI). IAI=Intravitreal aflibercept injection.

Figure 6 describes the baseline disease characteristics of the PANORAMA study population in each dosing group including: sham, Group 1, Group 2 and the combination of Group 1 and Group 2 (All IAI).

Figure 7 sets forth the number of injections received by PANORAMA subjects in each dosing group (sham, Group 1, Group 2 and the combination of Group 1 and Group 2: "All IAI").

Figure 8 shows the proportion (%) of PANORAMA patients in each dosing group (sham, Group 1, Group 2 and All IAI (combined Group 1 and Group 2)) achieving ≥ 2 -step improvement from baseline on the diabetic retinopathy severity scale (DRSS).

Figure 9 shows the proportion (%) of PANORAMA patients in each dosing group (sham, Group 1, Group 2 and All IAI (combined Group 1 and Group 2)) achieving ≥ 3 -step improvement from baseline on the diabetic retinopathy severity scale (DRSS).

Figure 10 shows the proportion (%) of PANORAMA patients in each dosing group (sham, Group 1, Group 2 and All IAI (combined Group 1 and Group 2)) experiencing vision threatening complications (VTC; proliferative diabetic retinopathy (PDR)/anterior segment neovascularization (ASNV)) and/or center involved diabetic macular edema (CI-DME) through week 24.

Figure 11 summarizes the mean change in best corrected visual acuity (BCVA) score (early treatment diabetic retinopathy study (ETDRS) letters) of each PANORAMA dosing group (sham, Group 1, Group 2 and the combination of Group 1 and Group 2: "All IAI") up to 24 weeks.

Figure 12 summarizes the mean change in central retinal thickness (CRT, μm) of each PANORAMA dosing group (sham, Group 1, Group 2 and All IAI (combined Group 1 and Group 2)) through Week 24.

Figure 13 summarizes the study eye ocular treatment emergent adverse events (TEAEs) in the sham and All IAI (combined Group 1 and Group 2) PANORAMA groups through week 24. FAS/SAF=full analysis set/safety analysis set

Figure 14 summarizes the study eye ocular serious treatment emergent adverse events (TEAEs) in the sham and All IAI (combined Group 1 and Group 2) PANORAMA groups through week 24. FAS/SAF=full analysis set/safety analysis set.

Figure 15 summarizes the study eye intra-ocular inflammation experienced by the sham and All IAI (combined Group 1 and Group 2) PANORAMA groups through week 24.

Figure 16 summarizes the anti-platelet trialists' collaboration (APTC) events experienced by sham and All IAI (combined Group 1 and Group 2) PANORAMA groups through week 24.

Figure 17 summarizes the deaths of PANORAMA subjects through week 24.

Figure 18 is an International Clinical Diabetic Retinopathy Disease Severity Scale (DRSS) Detailed Table.

Figure 19 is a summary of the results of the PANORAMA trial after 52 weeks-percentage of patients in each treatment group experiencing at least a 2 step improvement in DRSS and the percentage of subjects developing a vision threatening complication (VTC) and/or center-involved diabetic macular edema (CI-DME).

Figure 20 summarizes the number of active injections given to subjects in treatment groups sham, 2q16 (out of 6) and 2q8 (out of 9) through week 52.

Figure 21 summarizes the proportion of patients, in the sham, 2q16 and 2q8 groups, with at least a 2 step improvement, from baseline in DRSS at week 52. LOCF=last observation carried forward through week 52.

Figure 22 summarizes the percentage of subjects in each treatment group (sham, 2q16 and 2q8) with at least a 2 step improvement, from baseline in DRSS at weeks 8, 12, 24, 40 and 52.

Figure 23 summarizes the percentage of patients in each treatment group (sham, 2q16 and 2q8) developing a vision threatening complication (VTC) and/or center-involved diabetic macular edema (CI-DME) through week 52.

Figure 24 is a Kaplan-Meier plot of the probability of developing a vision threatening complication over time for subjects in each treatment group (sham, 2q16 and 2q8). VTE=VEGF Trap eye. VTC=vision threatening complication.

Figure 25 summarizes the time for subjects in each treatment group (sham, 2q16 and 2q8) to develop CI-DME.

Figure 26 summarizes the mean change in best corrected visual acuity (ETDRS letters) for subjects in each treatment group (sham, 2q16 and 2q8) over 52 weeks.

Figure 27 summarizes the central retinal thickness (μm) for subjects in each treatment group (sham, 2q16 and 2q8) over 52 weeks.

Figure 28 summarizes the incidence of ocular treatment-emergent adverse events (TEAEs) experienced by subjects,

in the treatment eye, in each treatment group (sham, 2q16 and 2q8) after 52 weeks.

Figure 29 summarizes the incidence of serious ocular treatment-emergent adverse events (TEAEs) experienced by subjects, in the treatment eye, in each treatment group (sham, 2q16 and 2q8) after 52 weeks.

Figure 30 summarizes the incidence of ocular inflammation experienced by subjects, in the treatment eye, in each treatment group (sham, 2q16 and 2q8) after 52 weeks.

Figure 31 summarizes the incidence of anti-platelet trialists' collaboration (APTC) events experienced by subjects, in the treatment eye, in each treatment group (sham, 2q16 and 2q8) after 52 weeks.

Figure 32 summarizes the proportion of patients with a three step improvement or more in each treatment group (sham, 2q16 and 2q8) after 52 weeks.

DETAILED DESCRIPTION

[0017] The references to methods of treatment in the subsequent paragraphs of the description are to interpreted as reference to the products of the present invention, in particular the VEGF antagonist of the present invention, for use in a method of treatment of the human or animal body by therapy or diagnosis.

[0018] The present invention includes a VEGF antagonist for use in an exceptionally effective method for preventing the progression of non-proliferative diabetic retinopathy without diabetic macular edema in a patient to more advanced and vision threatening disorders such as proliferative diabetic retinopathy, diabetic macular edema and/or anterior segment neovascularization of the eye. Indeed, moderately severe to severe non-proliferative diabetic retinopathy patients on the aflibercept dosing regimens experienced a reversal of disease progression achieving a two or more step improvement in DRSS level. The eyes of subjects on the dosing regimens also experienced a reduction in the occurrence of vision threatening complications relative to untreated eyes. This prevention can be achieved by the present invention. In particular, the present invention provides a VEGF antagonist for use in a method for treating or preventing diabetic retinopathy, in a patient in need of such treatment or prevention, wherein said patient does not suffer from center involved diabetic macular edema, said method comprising administering, to an eye of the patient, monthly doses followed by one or more secondary doses every 8 weeks of about 2 mg of VEGF antagonist that is a VEGF receptor-based chimeric molecule comprising (1) an immunoglobulin-like (Ig) domain 2 of a VEGFR1 and (2) Ig domain 3 of VEGFR2, and (3) a multimerizing component. The VEGF antagonist may be aflibercept.

[0019] Before the present invention is described, it is to be understood that this invention is not limited to particular methods and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0020] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. As used herein, the term "about," when used in reference to a particular recited numerical value, means that the value may vary from the recited value by no more than 1%. For example, as used herein, the expression "about 100" includes 99 and 101 and all values in between (e.g., 99.1, 99.2, 99.3, 99.4, etc.).

[0021] A subject or a patient can be a mammal, for example a human (e.g., a human 50, 55, 60, 65 or 70 years of age or older), rabbit, mouse, non-human primate, monkey or rat. In an embodiment of the invention, the subject or patient previously received a different treatment for DR, e.g., PDR (e.g., panretinal photocoagulation (laser) therapy). In an embodiment of the invention, the previous treatment failed to sufficiently treat the DR. The patient does not suffer from center involved diabetic macular edema. In an embodiment of the invention, the patient or subject does not suffer from DME. In an embodiment of the invention, the subject or patient has diabetes (e.g., type 1 or type 2).

DOSING REGIMENS

[0022] The present invention provides a VEGF antagonist for use in a method for treating or preventing diabetic retinopathy in a patient in need of such treatment or prevention, wherein said patient does not suffer from center involved diabetic macular edema. The diabetic retinopathy may be of any severity level, for example, proliferative or nonproliferative diabetic retinopathy (NPDR), e.g., moderately severe NPDR or severe NPDR, provided that the patient does not suffer from center involved diabetic macular edema, said method comprising administering, to an eye of the patient, 5 monthly doses followed by one or more secondary doses every 8 weeks of about 2 mg of VEGF antagonist that is a VEGF receptor-based chimeric molecule comprising (1) an immunoglobulin-like (Ig) domain 2 of a VEGFR1 and (2) Ig domain 3 of VEGFR2, and (3) a multimerizing component. The VEGF antagonist of the present invention is for use in methods that comprise sequentially administering to the eye of a patient (e.g., a human such as a human 18 years of age or older) multiple doses of a VEGF antagonist that is a VEGF receptor-based chimeric molecule comprising (1) an immunoglobulin-like (Ig) domain 2 of a VEGFR1 and (2) Ig domain 3 of VEGFR2, and (3) a multimerizing component (e.g., aflibercept). In an embodiment of the invention, the patient has diabetes (e.g., type 1 or type 2). As used herein, "se-

quentially administering" means that each dose of VEGF antagonist is administered to the eye of a patient at a different point in time, e.g., on different days separated by a predetermined interval (e.g., hours, days, weeks or months).

[0023] Also disclosed are methods for treating or preventing an angiogenic eye disorder (e.g., NPDR); preventing progression of DR (e.g., NPDR) to a more severe form or complication thereof, e.g., to PDR, ASNV, DME and/or CI-DME; causing a reduction in DRSS of NPDR; treating or preventing the occurrence or re-occurrence of a VTC or blindness, in a subject, comprising administering, to the eye of the subject, five initial monthly doses of VEGF antagonist (e.g., aflibercept) followed by one or more secondary doses every eight weeks. In one instance, the eye suffering from the disorder is administered the antagonist and, optionally, the other eye is also treated with the same or a different dosing regimen even if the disorder has not manifested in that eye or if a less severe form of the disorder has manifested or if another angiogenic eye disorder afflicts the other eye. The VEGF antagonist, thus, may be for use in a method including administering a 0.5 or 2 mg dose of VEGF antagonist (e.g., by intravitreal injection) to an eye of a subject (e.g., a human) as 3 or 4 or 5 monthly doses followed by a dose every 8 weeks counted from the last of the initial 3 or 4 or 5 monthly doses. In one instance, the eye suffering from the disorder is administered the antagonist and, optionally, the other eye is also treated with the same or a different dosing regimen even if the disorder has not manifested in that eye or if a less severe form of the disorder has manifested or if another angiogenic eye disorder afflicts the other eye. In one instance, a single initial dose of a VEGF antagonist (e.g., 2 mg) is administered to a patient's eye (e.g., by intravitreal injection) on the first day of the treatment regimen (*i.e.*, at week 0), followed by two secondary doses, each administered four weeks after the immediately preceding dose (*i.e.*, at week 4 and at week 8), followed by at least 5 tertiary doses, each administered eight weeks after the immediately preceding dose (*i.e.*, at weeks 16, 24, 32, 40 and 48). The tertiary doses may continue (at intervals of 8 or more weeks) indefinitely during the course of the treatment regimen. This administration regimen is depicted graphically in Figure 1.

[0024] Also disclosed are methods for treating or preventing an angiogenic eye disorder (e.g., NPDR); preventing progression of DR (e.g., NPDR) to a more severe form or complication thereof, e.g., to PDR, ASNV, DME and/or CI-DME; causing a reduction in DRSS of NPDR; treating or preventing the occurrence or re-occurrence of a VTC or blindness, in a subject, the methods comprising administering (e.g., by intravitreal injection) to an eye of the subject:

(A) three initial monthly doses (e.g., once every 4 weeks or "Q4wk") of a VEGF antagonist (e.g., 2 mgs); followed by
(B) administering to the eye of the subject a single dose (e.g., 2 mg) of the VEGF antagonist 8 weeks after the immediately preceding dose ("Q8wk"); followed by
(C) administering to the eye of the subject one or more further (maintenance) doses (e.g., 2 mg) of the VEGF antagonist (e.g., aflibercept) 16 weeks after the immediately preceding dose and once every 16 weeks ("Q16wk") thereafter (at weeks 32, 48 and 64, *etc.*, counted from the first of the initial monthly doses). See, for example, Figure 2. Also disclosed is a method that comprises administering a VEGF antagonist as (i) 3 monthly doses followed by one or more secondary doses every 16 weeks, wherein the first secondary dose initiates 8 weeks after the third monthly dose (week 16). In one instance, the eye suffering from the disorder is administered the antagonist and, optionally, the other eye is also treated with the same or a different dosing regimen even if the disorder has not manifested in that eye or if a less severe form of the disorder has manifested or if another angiogenic eye disorder afflicts the other eye.

[0025] In one exemplary embodiment of the present invention, each tertiary dose is administered at least 8 (e.g., 8, 8½, 9, 9½, 10, 10½, 11, 11½, 12, 12½, 13, 13½, 14, 14½, or more) weeks after the immediately preceding dose.

[0026] Also disclosed are methods for treating or preventing an angiogenic eye disorder (e.g., NPDR); preventing progression of DR (e.g., NPDR) to a more severe form or complication thereof, e.g., to PDR, ASNV, DME and/or CI-DME; causing a reduction in DRSS of NPDR; treating or preventing the occurrence or re-occurrence of a VTC or blindness, in a subject, the methods comprising administering (e.g., by intravitreal injection) to an eye of the subject:

(A) three initial monthly doses (e.g., once every 4 weeks or "Q4wk") of a VEGF antagonist (e.g., aflibercept or conbercept, e.g., 0.5 mg or 2.0 mg); followed by
(B)
• administering the VEGF antagonist to the eye of the subject one or more doses of the VEGF antagonist 3 months after the immediately preceding dose (or every 12 weeks or quarterly); or
• administering the VEGF antagonist to the eye of the subject on an as needed/*pro re nata* (PRN) basis, based on visual and/or anatomical outcomes as assessed by a physician or other qualified medical professional.

[0027] The VEGF antagonist of the present invention is for use in a method comprising administering, to an eye of the patient, 5 monthly doses followed by one or more secondary doses every 8 weeks. The "tertiary doses" are the

doses which are administered after the secondary doses. The 5 monthly doses followed by one or more secondary doses every 8 weeks and tertiary doses may all contain the same amount of VEGF antagonist, but will generally differ from one another in terms of frequency of administration. In certain embodiments, however, the amount of VEGF antagonist contained in the initial, secondary and/or tertiary doses will vary from one another (e.g., adjusted up or down as appropriate) during the course of treatment.

[0028] The phrase "the immediately preceding dose," as used herein, means, in a sequence of multiple administrations, the dose of VEGF antagonist which is administered to the eye of a patient prior to the administration of the very next dose in the sequence with no intervening doses.

[0029] In an embodiment of the invention, the VEGF antagonist dosing regimen is conducted over the course of 24, 48, 52, 96 or 100 weeks (or more).

[0030] In an embodiment of the invention, patients (e.g., suffering from NPDR) receiving such a dosing regimen exhibit at least a 2-step improvement in DRSS (Diabetic Retinopathy Severity Scale) from baseline (before treatment commences) at week 24 or 48 or 52 in the eye. In an embodiment of the invention, a 3-step improvement is experienced in the eye.

[0031] The VEGF antagonist of the invention may for use in methods that comprise administering to the eye of a patient any number of secondary and/or tertiary doses of a VEGF antagonist. For example, in certain embodiments, only a single secondary dose is administered to the patient's eye. In other embodiments, two or more (e.g., 2, 3, 4, 5, 6, 7, 8, or more) secondary doses are administered to the patient's eye. Likewise, in certain embodiments, only a single tertiary dose is administered to the patient's eye. In other embodiments, two or more (e.g., 2, 3, 4, 5, 6, 7, 8, or more) tertiary doses are administered to the patient's eye.

[0032] In embodiments involving multiple secondary doses, each secondary dose is administered every 8 weeks after the immediately preceding dose. Similarly, in embodiments involving multiple tertiary doses, each tertiary dose may be administered at the same frequency as the other tertiary doses. For example, each tertiary dose may be administered to the patient's eye 8 weeks after the immediately preceding dose. Alternatively, the frequency at which the tertiary doses are administered to a patient's eye can vary over the course of the treatment regimen. For example, the present invention includes the VEGF antagonist for use in methods which comprise administering to the patient's eye 5 monthly doses followed by one or more secondary doses every 8 weeks, followed by at least 5 tertiary doses of the VEGF antagonist, wherein the first four tertiary doses are administered 8 weeks after the immediately preceding dose, and wherein each subsequent tertiary dose is administered from 8 to 12 (e.g., 8, 8½, 9, 9½, 10, 10½, 11, 11½, 12) weeks after the immediately preceding dose. The frequency of administration may also be adjusted during the course of treatment by a physician depending on the needs of the individual patient following clinical examination.

[0033] In an embodiment of the invention, a VEGF antagonist is for use in a method where it is administered to a subject according to a dosing regimen of the present invention in association with a further therapeutic agent (e.g., a vitamin or dietary supplement) or therapeutic procedure (e.g. laser therapy or surgery). For example, in an embodiment of the invention, the subject receives laser therapy, such as pan-retinal photocoagulation (laser) therapy, in association with the VEGF antagonist. In an embodiment of the invention, the subject received the laser therapy previously, for example to treat DR (e.g., PDR), but switched to a VEGF antagonist dosing regimen according to the present invention.

[0034] The term "in association with" indicates that the components, a VEGF antagonist along with a further therapeutic agent can be formulated into a single composition, e.g., for simultaneous delivery, or formulated separately into two or more compositions (e.g., a kit). Each component can be administered to a subject at a different time than when the other component is administered; for example, each administration may be given non-simultaneously (e.g., separately or sequentially) at intervals over a given period of time. Moreover, the separate components may be administered to a subject by the same or by a different route.

VEGF ANTAGONISTS

[0035] The VEGF antagonist of the present invention is for use in methods that comprise administering to a patient's eye a VEGF antagonist according to specified dosing regimens set forth herein. As used herein, the expression "VEGF antagonist" means any molecule that blocks, reduces or interferes with the normal biological activity of VEGF.

[0036] VEGF antagonists include molecules which interfere with the interaction between VEGF and a natural VEGF receptor, e.g., molecules which bind to VEGF or a VEGF receptor and prevent or otherwise hinder the interaction between VEGF and a VEGF receptor. Specific exemplary VEGF antagonists include anti-VEGF antibodies and antigen-binding fragments thereof (e.g., Fab or F(ab)₂), anti-VEGF receptor antibodies and antigen-binding fragments thereof, anti-VEGF and anti-VEGF receptor single chain antibodies, anti-VEGF and anti-VEGF receptor bispecific antibodies and antigen-binding fragments thereof, anti-VEGF and anti-VEGF receptor DARPins (designed ankyrin repeat proteins) and VEGF receptor-based chimeric molecules (also referred to herein as "VEGF-Traps"). The VEGF antagonist employed in the present invention is a VEGF antagonist that is a VEGF receptor-based chimeric molecule comprising (1) an immunoglobulin-like (Ig) domain 2 of a VEGFR1 and (2) Ig domain 3 of VEGFR2, and (3) a multimerizing component.

[0037] VEGF receptor-based chimeric molecules include chimeric polypeptides which comprise two or more immunoglobulin (Ig)-like domains of a VEGF receptor such as VEGFR1 (also referred to as Flt1) and/or VEGFR2 (also referred to as Flk1 or KDR), and may also contain a multimerizing domain (e.g., an Fc domain which facilitates the multimerization [e.g., dimerization] of two or more chimeric polypeptides). An exemplary VEGF receptor-based chimeric molecule is a molecule referred to as VEGFR1R2-FcΔC1(a) which is encoded by the nucleic acid sequence of SEQ ID NO:1. VEGFR1R2-FcΔC1(a) comprises three components: (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2; (2) a VEGFR2 component comprising amino acids 130 to 231 of SEQ ID NO:2; and (3) a multimerization component ("FcΔC1(a)") comprising amino acids 232 to 457 of SEQ ID NO:2 (the C-terminal amino acid of SEQ ID NO:2 [i.e., K458] which may or may not be included in the VEGF antagonist used in the methods of the invention; see e.g., US Patent 7,396,664). Amino acids 1-26 of SEQ ID NO: 2 are the signal sequence.

[0038] The VEGF antagonist used in the Examples set forth herein below is a molecule comprising the VEGFR1R2-FcΔC1(a) molecule (e.g., a homodimer thereof) and is referred to herein as "VEGFT." Additional VEGF receptor-based chimeric molecules which can be used in the context of the present invention are disclosed in US 7,396,664, 7,303,746 and WO 00/75319.

[0039] The VEGF antagonist employed in the invention is a VEGF receptor based chimeric molecule that is a polypeptide (or a homodimer thereof) that comprises:

- (1) an immunoglobulin-like (Ig) domain 2 of a VEGFR1;
- (2) Ig domain 3 of VEGFR2; and
- (3) a multimerizing component (e.g., an Fc or variant thereof).

[0040] In an embodiment of the invention, the VEGF antagonist is a VEGF receptor based chimeric molecule that is a polypeptide (or homodimer thereof) that comprises:

- (1) an immunoglobulin-like (Ig) domain 2 of VEGFR1;
- (2) Ig domain 3 of VEGFR2;
- (3) an immunoglobulin-like (Ig) domain 4 of the second VEGF receptor (e.g., VEGFR2); and
- (4) a multimerizing component (e.g., an Fc or variant thereof).

[0041] Exemplary VEGF antagonists include, e.g., VEGF mini-Trap (see e.g., US7087411), aflibercept, an anti-VEGF DARPin such as the Abicipar Pegol DARPin), a single chain (e.g., VL-VH) anti-VEGF antibody such as brolicizumab (RTH258), a monospecific, multispecific or bispecific anti-VEGF antibody or antigen-binding fragment thereof, e.g., which also binds to ANG2, such as RG7716, ranibizumab (LUCENTIS), or bevacizumab (AVASTIN), and conbercept. The VEGF antagonist employed in the present invention is a VEGF antagonist that is a VEGF receptor-based chimeric molecule comprising (1) an immunoglobulin-like (Ig) domain 2 of a VEGFR1 and (2) Ig domain 3 of VEGFR2, and (3) a multimerizing component.

ANGIOGENIC EYE DISORDERS

[0042] The VEGF antagonist of the present invention is for use in a method for treating or preventing diabetic retinopathy, wherein the patient does not suffer from center involved diabetic macular edema.

[0043] The expression "angiogenic eye disorder," as used herein, means any disease of the eye which is caused by or associated with the growth or proliferation of blood vessels or by blood vessel leakage. Non-limiting examples of angiogenic eye disorders that are treatable include age-related macular degeneration (e.g., wet AMD, exudative AMD, etc.), retinal vein occlusion (RVO), central retinal vein occlusion (CRVO; e.g., macular edema following CRVO), branch retinal vein occlusion (BRVO), diabetic macular edema (DME), choroidal neovascularization (CNV; e.g., myopic CNV), iris neovascularization, neovascular glaucoma, post-surgical fibrosis in glaucoma, proliferative vitreoretinopathy (PVR), optic disc neovascularization, corneal neovascularization, retinal neovascularization, vitreal neovascularization, pannus, pterygium, vascular retinopathy and diabetic retinopathies such as nonproliferative diabetic retinopathy and proliferative diabetic retinopathy.

[0044] Diabetic retinopathy is a progressive condition that can occur in people who have diabetes. Typically, it causes progressive damage to the retina, the light-sensitive lining at the back of the eye. Diabetic retinopathy is a serious sight-threatening complication of diabetes. Over time, diabetes damages the blood vessels in the retina and diabetic retinopathy occurs when these tiny blood vessels leak blood and other fluids. The condition typically affects both eyes. The longer a person has diabetes, the more likely they will develop diabetic retinopathy. If left untreated, diabetic retinopathy can cause blindness.

[0045] Symptoms of diabetic retinopathy may include:

- Seeing spots or floaters
- Blurred vision
- Having a dark or empty spot in the center of your vision
- Difficulty seeing well at night

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[0046] Thus, in order to preserve vision, it is critical to halt or impede progression of diabetic retinopathy to its more sight-threatening later stages, *e.g.*, proliferative diabetic retinopathy, diabetic macular edema and/or anterior segment neovascularization (ASNV). Klein et al., Changes in Retinal Vessel Diameter and Incidence and Progression of Diabetic Retinopathy, Arch. Ophthalmol. 130(6): 749-755 (2012).

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[0047] Anterior segment neovascularization is neovascularization of the iris, and/or definitive neovascularization of the iridocorneal angle.

[0048] As diabetic retinopathy progresses, it reaches more advanced stages including:

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1. Mild nonproliferative retinopathy. This stage is characterized by small areas of balloon-like swelling in the retina's tiny blood vessels, called microaneurysms. These microaneurysms may leak fluid into the retina; then

2. Moderate nonproliferative retinopathy. As the disease progresses, blood vessels that nourish the retina may swell and distort. They may also lose their ability to transport blood. Both conditions cause characteristic changes to the appearance of the retina and may contribute to diabetic macular edema; then

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3. Severe nonproliferative retinopathy. In this stage, many more blood vessels are blocked, depriving blood supply to areas of the retina. These areas secrete growth factors that signal the retina to grow new blood vessels; and then

4. Proliferative diabetic retinopathy (PDR). See below.

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[0049] Nonproliferative diabetic retinopathy (NPDR) is an early retinopathy in diabetic patients which is not characterized by neovascularization and whose stage may also be graded according to the Diabetic Retinopathy Severity Scale (DRSS). Grades of NPDR include, for example, early, moderate, moderately severe and severe. In an embodiment of the invention, moderately severe to severe NPDR is accorded a severity level of 47 to 53 (*e.g.*, 47 or 53). In an embodiment of the invention, a moderately severe to severe NPDR patient (*e.g.*, a human, for example 18 years of age or older with type 1 or type 2 diabetes) is characterized with one or more of the following:

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- without macular edema (*e.g.*, threatening the center of the macula);
- can safely defer panretinal photocoagulation for at least 6 months;
- has a baseline best-corrected visual acuity (BCVA) ETDRS letter score of 69 or greater (*e.g.*, Snellen visual acuity of 20/40 or better);
- without retinal neovascularization;
- without anterior segment neovascularization (ASNV);
- without vitreous hemorrhage; and/or
- without tractional retinal detachment.

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[0050] Proliferative diabetic retinopathy (PDR) is the more advanced form of diabetic retinopathy. At this stage, new fragile blood vessels can begin to grow in the retina and into the vitreous, the gel-like fluid that fills the back of the eye. The new blood vessel may leak blood into the vitreous, clouding vision.

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[0051] Thus, the present invention includes a VEGF antagonist for use in methods for treating or preventing diabetic retinopathy in a patient's eye, wherein the patient does not suffer from center involved diabetic macular edema, whether the subject's eye has moderately severe NPDR (nonproliferative diabetic retinopathy) or severe NPDR (*e.g.*, moderately severe to severe NPDR) or PDR (proliferative diabetic retinopathy), *e.g.*, where the DRSS for the subject's diabetic retinopathy is 47 or 53, by administering a VEGF antagonist (*e.g.*, 2 mg of aflibercept) to the subject's eye under a dosing regimen set forth herein. The subject does not suffer from center-involved diabetic macular edema (CI-DME). In a patient with a nonproliferative diabetic retinopathy, the dosing regimens set forth herein may be used to prevent the progression of the patient to a more severe form of NPDR or to a proliferative diabetic retinopathy, ASNV, DME and/or CI-DME. Indeed, the dosing regimens may be used to reverse an increase in (or prevent an increase in) DRSS in a patient suffering from NPDR (*e.g.*, who does not suffer from DME and/or CI-DME) by as much as, for example, 2 levels or more, *e.g.*, 3 levels.

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[0052] The International Clinical Diabetic Retinopathy Disease Severity Scale (DRSS), including levels thereof, is detailed in a table set forth in Figure 18.

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[0053] Subjects suffering from NPDR are at risk of suffering from various vision threatening complications or events (VTCs) and blindness (*e.g.*, blindness secondary to such a VTC). Vision threatening complications are defined as composite outcome of PDR (inclusive of patients who have vitreous hemorrhage or tractional retinal detachment believed to be due to PDR) and ASNV. ASNV is defined as neovascularization of the iris (at least 2 cumulative clock hours),

and/or definitive neovascularization of the iridocorneal angle. The dosing regimens reduce incidence of such VTCs and/or blindness in subjects suffering from NPDR. Thus, the present invention provides the VEGF antagonist for use in methods for preventing the occurrence or re-occurrence (following one or more initial occurrences) of a VTC and/or blindness in a subject who, for example, suffers from NPDR or PDR by administering, to the subject's eye, a VEGF antagonist (aflibercept, *e.g.*, 2.0 mg) under a dosing regimen set forth herein. Such methods may include not only the step of treating one eye suffering a VTC, but also treating the other eye even if no VTC and/or blindness has occurred in the other eye so as to prevent a VTC and/or blindness.

PHARMACEUTICAL FORMULATIONS

[0054] The present invention includes the VEGF antagonist for use in methods in which the VEGF antagonist that is administered to the patient's eye is contained within a pharmaceutical formulation. The pharmaceutical formulation may comprise the VEGF antagonist along with at least one inactive ingredient such as, *e.g.*, a pharmaceutically acceptable carrier. Other agents may be incorporated into the pharmaceutical composition to provide improved transfer, delivery, tolerance, and the like. The term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly, in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the antibody is administered. A multitude of appropriate formulations can be found in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences (15th ed, Mack Publishing Company, Easton, Pa., 1975), particularly Chapter 87 by Blaug, Seymour, therein. These formulations include, for example, powders, pastes, ointments, jellies, waxes, oils, lipids, lipid (cationic or anionic) containing vesicles (such as LIPOFECTIN™), DNA conjugates, anhydrous absorption pastes, oil-in-water and water-in-oil emulsions, emulsions carbowax (polyethylene glycols of various molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax. Any of the foregoing mixtures may be appropriate in the context of the present invention, provided that the VEGF antagonist is not inactivated by the formulation and the formulation is physiologically compatible and tolerable with the route of administration. See also Powell et al. PDA (1998) J Pharm Sci Technol. 52:238-311 and the citations therein for additional information related to excipients and carriers well known to pharmaceutical chemists.

[0055] Pharmaceutical formulations useful for administration by injection in the context of the present invention may be prepared by dissolving, suspending or emulsifying a VEGF antagonist in a sterile aqueous medium or an oily medium conventionally used for injections. As the aqueous medium for injections, there are, for example, physiological saline, an isotonic solution containing glucose and other auxiliary agents, *etc.*, which may be used in combination with an appropriate solubilizing agent such as an alcohol (*e.g.*, ethanol), a polyalcohol (*e.g.*, propylene glycol, polyethylene glycol), a nonionic surfactant [*e.g.*, polysorbate 80, HCO-50 (polyoxyethylene (50 mol) adduct of hydrogenated castor oil)], *etc.* As the oily medium, there may be employed, *e.g.*, sesame oil, soybean oil, *etc.*, which may be used in combination with a solubilizing agent such as benzyl benzoate, benzyl alcohol, *etc.* The injection thus prepared can be filled in an appropriate ampoule if desired.

[0056] In an embodiment of the invention, the pharmaceutical formulation administered to a subject comprises aflibercept, *e.g.*, about 40 mg/mL aflibercept, in 10 mM sodium phosphate, 40 mM sodium chloride, 0.03% polysorbate 20, and 5% sucrose, pH 6.2.

[0057] In an embodiment of the invention, the pharmaceutical formulation administered to a subject comprises a VEGF antagonist (*e.g.*, aflibercept or conbercept, for example, 40 mg/ml thereof) and:

- (a) pyrophosphate (*e.g.*, 5 mM - 250 mM). In an embodiment of the invention, the formulation further includes NaCl, sodium citrate, citric acid, mannitol and polysorbate 80, *e.g.*, pH 5.2;
- (b) a buffer such as a histidine salt such as histidine-HCl or histidine-acetate (*e.g.*, at 10 mM to 50 mM), *e.g.*, pH 5.7 to 6.2; a sugar such as sucrose, trehalose, mannitol, or glucose (*e.g.*, more than 6%, but not more than 10%, for example, 2.5% to 10%); and a surfactant such as polysorbate 20 and polysorbate 80 (*e.g.*, 0% or 0.01% to 0.03%);
- (c) a histidine containing buffer such as L-histidine/histidine hydrochloride; a non-ionic surfactant such as polysorbate 20 (*e.g.*, 0.03%), an inorganic salt such as NaCl (*e.g.*, 40 mM), and a carbohydrate such as sucrose (*e.g.*, 5%), *e.g.* pH 6.0-6.5 (*e.g.*, 6.2 or 6.5);
- (d) a buffer consisting of a histidine salt (*e.g.*, wherein the histidine salt is histidine-HCl or histidine-acetate, for example, 10 mM to 50 mM) and having pH ranging from 5.7 to 6.2; a sugar selected from the group consisting of sucrose, trehalose, mannitol, and glucose (*e.g.*, more than 6%, but not more than 10%); a surfactant selected from the group consisting of polysorbate 20 and polysorbate 80 (*e.g.*, 0% to 0.1%);
- (e) citric acid (*e.g.*, 5 mM, 10 mM, 15 mM, 20 mM, 25 mM or 30 mM), sucrose (*e.g.*, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% or 10%), arginine (*e.g.*, 5 mM, 10 mM, 15 mM, 20 mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM or 50 mM or 100 mM), and polysorbate 20 (*e.g.*, 0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, 0.08%, 0.09% or 0.10%);
- (f) a buffer such as phosphate, histidine, acetate, succinate, citrate, glutamate, and/or lactate (*e.g.*, at 5-20 or 5-50

mM); a non-ionic surfactant such as a polysorbate (e.g., PS20 or PS80), a polyethylene glycol dodecyl ether, a poloxamer, 4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol, an alkylsaccharide or an alkylglycoside, a tonicifying agent such as a polyol or an amino acid, for example, sucrose, trehalose, sorbitol, mannitol, glycerol, proline, arginine, methionine, glycine, or lysine, wherein the formulation has a final osmolality of about 300 mOsm/kg, and wherein the concentration of chloride anion is less than about 10 mM; pH 5.0-6.5;

(g) sodium acetate (e.g., 10-15 mM); sucrose (e.g., 7%) or trehalose (e.g., 8%); and polysorbate 20 (e.g., 0.03%), pH 5.5; or

(h) any of: 10 mM sodium phosphate, 40 mM sodium chloride, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, pH 6.2; 10 mM sodium phosphate, 9% (w/v) sucrose, 0.03% (w/v) polysorbate 20, pH 6.2; 10 mM sodium phosphate, 40 mM sodium chloride, 2% (w/v) proline, 0.03% (w/v) polysorbate 20, pH 6.2; 10 mM sodium phosphate, 3% (w/v) proline, 0.03% (w/v) polysorbate 20, pH 6.2; 10 mM sodium phosphate, 9% (w/v) Trehalose, 0.03% (w/v) polysorbate 20, pH 6.2; 10 mM histidine, 3% (w/v) proline, 0.03% (w/v) polysorbate 20, pH 6.2; 10 mM histidine, 9% (w/v) sucrose, 0.03% (w/v) polysorbate 20, pH 6.2; or 10 mM acetate, 9% (w/v) sucrose, 0.03% (w/v) polysorbate 20, pH 5.2.

15 MODES OF ADMINISTRATION

[0058] The VEGF antagonist (or pharmaceutical formulation comprising the VEGF antagonist) may be administered to any eye of the patient by any known delivery system and/or administration method. In certain embodiments, the VEGF antagonist is for use in a method where it is administered to the patient by ocular, intraocular, intravitreal or subconjunctival injection. In other embodiments, the VEGF antagonist can be administered to the patient by topical administration, e.g., via eye drops or other liquid, gel, ointment or fluid which contains the VEGF antagonist and can be applied directly to the eye. Administration to a patient's or subject's eye refers to any acceptable method for delivering a VEGF antagonist (e.g., aflibercept) to the tissues of the eye of the patient or subject (e.g., intravitreal injection). In an embodiment of the invention, administration to the subject's or patient's eye refers to delivering a VEGF antagonist to an eye suffering an angiogenic eye disorder (e.g., as discussed herein), such as NPDR, and, optionally, to delivery to the other eye even if not so afflicted. In an embodiment of the invention, the VEGF antagonist is for use in a method where administration is by intravitreal injection using a syringe with a 30-gauge, ½-inch injection needle. For example, in an embodiment of the invention, about 50 µl is intravitreally injected to deliver about 2 mg of VEGF antagonist (e.g., aflibercept; for example, in a pharmaceutical formulation including aflibercept, e.g., about 40 mg/mL aflibercept, in 10 mM sodium phosphate, 40 mM sodium chloride, 0.03% polysorbate 20, and 5% sucrose, pH 6.2). In an embodiment of the invention, 0.5 or 2.0 mg of conbercept in a pharmaceutical formulation comprising conbercept, citric acid, sucrose, arginine and polysorbate 20 is injected into the eye.

35 AMOUNT OF VEGF ANTAGONIST ADMINISTERED

[0059] The VEGF antagonist of the present invention is for use in a method comprising administering, to an eye of the patient, 5 monthly doses followed by one or more secondary doses every 8 weeks of about 2 mg of VEGF antagonist. Each dose of VEGF antagonist (e.g. 2 mg of aflibercept) administered to the patient's eye over the course of the treatment regimen may contain the same, or substantially the same, amount of VEGF antagonist. Alternatively, the quantity of VEGF antagonist contained within the individual doses may vary over the course of the treatment regimen, provided that the method comprises administering, to an eye of the patient, 5 monthly doses followed by one or more secondary doses every 8 weeks of about 2 mg of VEGF antagonist. The present invention contemplates dosing schemes in which the quantity of VEGF antagonist contained within the individual doses increases over time (e.g., each subsequent dose contains more VEGF antagonist than the last), decreases over time (e.g., each subsequent dose contains less VEGF antagonist than the last), initially increases then decreases, initially decreases then increases, or remains the same throughout the course of the administration regimen, provided that the method comprises administering, to an eye of the patient, 5 monthly doses followed by one or more secondary doses every 8 weeks of about 2 mg of VEGF antagonist.

[0060] The amount of VEGF antagonist administered to the patient's eye in each dose (e.g., 0.5 mg or 2 mg, for example of aflibercept or conbercept) is, in most cases, a therapeutically effective amount. As used herein, the phrase "therapeutically effective amount" means a dose of VEGF antagonist that results in a detectable improvement in one or more symptoms or indicia of an angiogenic eye disorder, or a dose of VEGF antagonist that inhibits, prevents, lessens, or delays the progression of an angiogenic eye disorder. In the case of an anti-VEGF antibody or a VEGF receptor-based chimeric molecule such as VEGFR1R2-FcΔC1(a), a therapeutically effective amount can be from about 0.05 mg to about 5 mg, e.g., about 0.05 mg, about 0.1 mg, about 0.15 mg, about 0.2 mg, about 0.25 mg, about 0.3 mg, about 0.35 mg, about 0.4 mg, about 0.45 mg, about 0.5 mg, about 0.55 mg, about 0.6 mg, about 0.65 mg, about 0.7 mg, about 0.75 mg, about 0.8 mg, about 0.85 mg, about 0.9 mg, about 1.0 mg, about 1.05 mg, about 1.1 mg, about 1.15 mg, about 1.2 mg, about 1.25 mg, about 1.3 mg, about 1.35 mg, about 1.4 mg, about 1.45 mg, about 1.5 mg, about 1.55 mg, about 1.6 mg, about 1.65 mg, about 1.7 mg, about 1.75 mg, about 1.8 mg, about 1.85 mg, about 1.9 mg, about 2.0 mg, about

2.05 mg, about 2.1 mg, about 2.15 mg, about 2.2 mg, about 2.25 mg, about 2.3 mg, about 2.35 mg, about 2.4 mg, about 2.45 mg, about 2.5 mg, about 2.55 mg, about 2.6 mg, about 2.65 mg, about 2.7 mg, about 2.75 mg, about 2.8 mg, about 2.85 mg, about 2.9 mg, about 3.0 mg, about 3.5 mg, about 4.0 mg, about 4.5 mg, or about 5.0 mg of the antibody or receptor-based chimeric molecule. The VEGF antagonist of the present invention is for use in a method comprises administering, to an eye of the patient, 5 monthly doses followed by one or more secondary doses every 8 weeks of about 2 mg of VEGF antagonist

[0061] The amount of VEGF antagonist contained within the individual doses may be expressed in terms of milligrams of antibody per kilogram of patient body weight (*i.e.*, mg/kg). For example, the VEGF antagonist may be administered to a patient at a dose of about 0.0001 to about 10 mg/kg of patient body weight.

TREATMENT POPULATION AND EFFICACY

[0062] The VEGF antagonist of the present invention is for use in methods that are useful for treating angiogenic eye disorders in patients that have been diagnosed with or are at risk of being afflicted with an angiogenic eye disorder, where the method treats or prevents diabetic retinopathy, wherein the patient does not suffer from center involved macular edema. Generally, the methods demonstrate efficacy within 104 weeks of the initiation of the treatment regimen (with the initial dose administered at "week 0"), *e.g.*, by the end of week 16, by the end of week 24, by the end of week 32, by the end of week 40, by the end of week 48, by the end of week 52, by the end of week 56, *etc.*

[0063] In an embodiment of the invention, in the context of methods for treating angiogenic eye disorders such as DR, PDR, NPDR, AMD, CRVO, and DME, "efficacy" means that, from the initiation of treatment, the patient exhibits:

- a loss of 15 or fewer (*e.g.*, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1) letters on the Early Treatment Diabetic Retinopathy Study (ETDRS) visual acuity chart;
- a gain of one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or more) letters on the ETDRS chart;
- maintenance of or an improvement in DRSS score, *e.g.*, reduction in DRSS by 2 or 3 steps;
- a reduction in the incidence or the prevention of vision threatening complications (VTC) and/or blindness and/or center involved diabetic macular edema; and/or
- a reduction in or maintenance of central retinal thickness;

for example, wherein one or more of such goals are achieved within about 24 or 52 weeks of treatment initiation.

PACKAGES

[0064] Also disclosed is a package, comprising:

a drug container; and
instructions for using the drug in a method for treating or preventing diabetic retinopathy in a patient in need of such treatment, wherein said patient does not suffer from center involved diabetic macular edema, wherein the drug is a VEGF antagonist and said method comprises administering, to an eye of the patient, 5 monthly doses followed by one or more secondary doses every 8 weeks of about 2 mg of VEGF antagonist that is a VEGF receptor-based chimeric molecule comprising (1) an immunoglobulin-like (Ig) domain 2 of a VEGFR1 and (2) Ig domain 3 of VEGFR2, and (3) a multimerizing component.

[0065] Also disclosed is a package wherein the VEGF antagonist

(i) comprises (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2; (2) a VEGFR2 component comprising amino acids 130-231 of SEQ ID NO:2; and (3) a multimerization component comprising amino acids 232-457 of SEQ ID NO:2;

(ii) comprises (1) an immunoglobulin-like (Ig) domain 2 of a first VEGF receptor and (2) Ig domain 3 of a second VEGF receptor, and (3) a multimerizing component;

(iii) is aflibercept; or

(iv) is conbercept.

[0066] Also disclosed is a package wherein the instructions indicate the drug is administered by intravitreal injection.

[0067] Also disclosed is a package wherein the instructions indicate the drug is administered 3 or 5 monthly doses followed by one or more doses every 8 weeks.

EXAMPLES

[0068] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the methods and compositions of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

[0069] The exemplary VEGF antagonist used in all Examples set forth below is (unless otherwise indicated) a dimeric molecule having two functional VEGF binding units. Each functional binding unit is comprised of Ig domain 2 from VEGFR1 fused to Ig domain 3 from VEGFR2, which in turn is fused to the hinge region of a human IgG1 Fc domain (VEGFR1R2-FcΔC1(a); encoded by SEQ ID NO:1). This VEGF antagonist is referred to in the examples below as "VEGFT". For purposes of the following Examples, "monthly" dosing is equivalent to dosing once every four weeks.

Example 1: Phase I Clinical Trial of Intravitreally Administered VEGF Receptor-Based Chimeric Molecule (VEGFT) in Subjects with Neovascular AMD

[0070] In this Phase I study, 21 subjects with neovascular AMD received a single intravitreal (IVT) dose of VEGFT. Five groups of three subjects each received either 0.05, 0.15, 0.5, 2 or 4 mg of VEGFT, and a sixth group of six subjects received 1 mg. No serious adverse events related to the study drug, and no identifiable intraocular inflammation was reported. Preliminary results showed that, following injection of VEGFT, a rapid decrease in foveal thickness and macular volume was observed that was maintained through 6 weeks. At Day 43 across all dose groups, mean excess retinal thickness [excess retinal thickness = (retinal thickness - 179μm)] on optical coherence tomography (OCT) was reduced from 119μm to 27μm as assessed by Fast Macular Scan and from 194μm to 60μm as assessed using a single Posterior Pole scan. The mean increase in best corrected visual acuity (BCVA) was 4.75 letters, and BCVA was stable or improved in 95% of subjects. In the 2 highest dose groups (2 and 4 mg), the mean increase in BCVA was 13.5 letters, with 3 of 6 subjects demonstrating improvement of ≥ 3 lines.

Example 2: Phase II Clinical Trial of Repeated Doses of Intravitreally Administered VEGF Receptor-Based Chimeric Molecule (VEGFT) in Subjects with Neovascular AMD

[0071] This study was a double-masked, randomized study of 3 doses (0.5, 2, and 4 mg) of VEGFT tested at 4-week and/or 12-week dosing intervals. There were 5 treatment arms in this study, as follows: 1) 0.5 mg every 4 weeks, 2) 0.5 mg every 12 weeks, 3) 2 mg every 4 weeks, 4) 2 mg every 12 weeks and 5) 4 mg every 12 weeks. Subjects were dosed at a fixed interval for the first 12 weeks, after which they were evaluated every 4 weeks for 9 months, during which additional doses were administered based on pre-specified criteria. All subjects were then followed for one year after their last dose of VEGFT. Preliminary data from a pre-planned interim analysis indicated that VEGFT met its primary endpoint of a statistically significant reduction in retinal thickness after 12 weeks compared with baseline (all groups combined, decrease of 135μm, $p < 0.0001$). Mean change from baseline in visual acuity, a key secondary endpoint of the study, also demonstrated statistically significant improvement (all groups combined, increase of 5.9 letters, $p < 0.0001$). Moreover, patients in the dose groups that received only a single dose, on average, demonstrated a decrease in excess retinal thickness ($p < 0.0001$) and an increase in visual acuity ($p = 0.012$) at 12 weeks. There were no drug-related serious adverse events, and treatment with the VEGF antagonists was generally well-tolerated. The most common adverse events were those typically associated with intravitreal injections.

Example 3: Phase I Clinical Trial of Systemically Administered VEGF Receptor-Based Chimeric Molecule (VEGFT) in Subjects with Neovascular AMD

[0072] This study was a placebo-controlled, sequential-group, dose-escalating safety, tolerability and bioeffect study of VEGFT by IV infusion in subjects with neovascular AMD. Groups of 8 subjects meeting eligibility criteria for subfoveal choroidal neovascularization (CNV) related to AMD were assigned to receive 4 IV injections of VEGFT or placebo at dose levels of 0.3, 1, or 3 mg/kg over an 8-week period.

[0073] Most adverse events that were attributed to VEGFT were mild to moderate in severity, but 2 of 5 subjects treated with 3 mg/kg experienced dose-limiting toxicity (DLT) (one with Grade 4 hypertension and one with Grade 2 proteinuria); therefore, all subjects in the 3 mg/kg dose group did not enter the study. The mean percent changes in excess retinal thickness were: -12%, -10%, -66%, and -60% for the placebo, 0.3, 1, and 3 mg/kg dose groups at day 15 (ANOVA $p < 0.02$), and -5.6%, +47.1%, and -63.3% for the placebo, 0.3, and 1 mg/kg dose groups at day 71 (ANOVA $p < 0.02$). There was a numerical improvement in BCVA in the subjects treated with VEGFT. As would be expected in

such a small study, the results were not statistically significant.

Example 4: Phase III Clinical Trials of the Efficacy, Safety, and Tolerability of Repeated Doses of Intravitreal VEGFT in Subjects with Neovascular Age-Related Macular Degeneration

A. Objectives, Hypotheses and Endpoints

[0074] Two parallel Phase III clinical trials were carried out to investigate the use of VEGFT to treat patients with the neovascular form of age-related macular degeneration (Study 1 and Study 2). The primary objective of these studies was to assess the efficacy of IVT administered VEGFT compared to ranibizumab (Lucentis®, Genentech, Inc.), in a non-inferiority paradigm, in preventing moderate vision loss in subjects with all subtypes of neovascular AMD.

[0075] The secondary objectives were (a) to assess the safety and tolerability of repeated IVT administration of VEGFT in subjects with all sub-types of neovascular AMD for periods up to 2 years; and (b) to assess the effect of repeated IVT administration of VEGFT on Vision-Related Quality of Life (QOL) in subjects with all sub-types of neovascular AMD.

[0076] The primary hypothesis of these studies was that the proportion of subjects treated with VEGFT with stable or improved BCVA (<15 letters lost) is similar to the proportion treated with ranibizumab who have stable or improved BCVA, thereby demonstrating non-inferiority.

[0077] The primary endpoint for these studies was the prevention of vision loss of greater than or equal to 15 letters on the ETDRS chart, compared to baseline, at 52 weeks. Secondary endpoints were as follows: (a) change from baseline to Week 52 in letter score on the ETDRS chart; (b) gain from baseline to Week 52 of 15 letters or more on the ETDRS chart; (c) change from baseline to Week 52 in total NEI VFQ-25 score; and (d) change from baseline to Week 52 in CNV area.

B. Study Design

[0078] For each study, subjects were randomly assigned in a 1:1:1:1 ratio to 1 of 4 dosing regimens: (1) 2 mg VEGFT administered every 4 weeks (2Q4); (2) 0.5 mg VEGFT administered every 4 weeks (0.5Q4); (3) 2 mg VEGFT administered every 4 weeks to week 8 and then every 8 weeks (with sham injection at the interim 4-week visits when study drug was not administered (2Q8); and (4) 0.5 mg ranibizumab administered every 4 weeks (RQ4). Subjects assigned to (2Q8) received the 2 mg injection every 4 weeks to week 8 and then a sham injection at interim 4-week visits (when study drug is not to be administered) during the first 52 weeks of the studies. (No sham injection were given at Week 52).

[0079] The study duration for each subject was scheduled to be 96 weeks plus the recruitment period. For the first 52 weeks (Year 1), subjects received an IVT or sham injection in the study eye every 4 weeks. (No sham injections were given at Week 52). During the second year of the study, subjects will be evaluated every 4 weeks and will receive IVT injection of study drug at intervals determined by specific dosing criteria, but at least every 12 weeks. (During the second year of the study, sham injections will not be given.) During this period, injections may be given as frequently as every 4 weeks, but no less frequently than every 12 weeks, according to the following criteria: (i) increase in central retinal thickness of ≥ 100 μ m compared to the lowest previous value as measured by optical coherence tomography (OCT); or (ii) a loss from the best previous letter score of at least 5 ETDRS letters in conjunction with recurrent fluid as indicated by OCT; or (iii) new or persistent fluid as indicated by OCT; or (iv) new onset classic neovascularization, or new or persistent leak on fluorescein angiography (FA); or (v) new macular hemorrhage; or (vi) 12 weeks have elapsed since the previous injection. According to the present protocol, subjects must receive an injection at least every 12 weeks.

[0080] Subjects were evaluated at 4 weeks intervals for safety and best corrected visual acuity (BCVA) using the 4 meter ETDRS protocol. Quality of Life (QOL) was evaluated using the NEI VFQ-25 questionnaire. OCT and FA examinations were conducted periodically.

[0081] Approximately 1200 subjects were enrolled, with a target enrollment of 300 subjects per treatment arm.

[0082] To be eligible for this study, subjects were required to have subfoveal choroidal neovascularization (CNV) secondary to AMD. "Subfoveal" CNV was defined as the presence of subfoveal neovascularization, documented by FA, or presence of a lesion that is juxtafoveal in location angiographically but affects the fovea. Subject eligibility was confirmed based on angiographic criteria prior to randomization.

[0083] Only one eye was designated as the study eye. For subjects who met eligibility criteria in both eyes, the eye with the worse VA was selected as the study eye. If both eyes had equal VA, the eye with the clearest lens and ocular media and least amount of subfoveal scar or geographic atrophy was selected. If there was no objective basis for selecting the study eye, factors such as ocular dominance, other ocular pathology and subject preference were considered in making the selection.

[0084] Inclusion criteria for both studies were as follows: (i) signed Informed consent; (ii) at least 50 years of age; (iii) active primary subfoveal CNV lesions secondary to AMD, including juxtafoveal lesions that affect the fovea as evidenced by FA in the study eye; (iv) CNV at least 50% of total lesion size; (v) early treatment diabetic retinopathy study (ETDRS)

best-corrected visual acuity of: 20/40 to 20/320 (letter score of 73 to 25) in the study eye; (vi) willing, committed, and able to return for all clinic visits and complete all study-related procedures; and (vii) able to read, understand and willing to sign the informed consent form (or, if unable to read due to visual impairment, be read to verbatim by the person administering the informed consent or a family member).

- 5 **[0085]** Exclusion criteria for both studies were as follows: 1. Any prior ocular (in the study eye) or systemic treatment or surgery for neovascular AMD except dietary supplements or vitamins. 2. Any prior or concomitant therapy with another investigational agent to treat neovascular AMD in the study eye, except dietary supplements or vitamins. 3. Prior treatment with anti-VEGF agents as follows: (a) Prior treatment with anti-VEGF therapy in the study eye was not allowed; (b) Prior treatment with anti-VEGF therapy in the fellow eye with an investigational agent (not FDA approved, e.g. bevacizumab) was allowed up to 3 months prior to first dose in the study, and such treatments were not allowed during the study. Prior treatment with an approved anti-VEGF therapy in the fellow eye was allowed; (c) Prior systemic anti-VEGF therapy, investigational or FDA/Health Canada approved, was only allowed up to 3 months prior to first dose, and was not allowed during the study. 4. Total lesion size > 12 disc areas (30.5 mm², including blood, scars and neovascularization) as assessed by FA in the study eye. 5. Subretinal hemorrhage that is either 50% or more of the total lesion area, or if the blood is under the fovea and is 1 or more disc areas in size in the study eye. (If the blood is under the fovea, then the fovea must be surrounded 270 degrees by visible CNV.) 6. Scar or fibrosis, making up > 50% of total lesion in the study eye. 7. Scar, fibrosis, or atrophy involving the center of the fovea. 8. Presence of retinal pigment epithelial tears or rips involving the macula in the study eye. 9. History of any vitreous hemorrhage within 4 weeks prior to Visit 1 in the study eye. 10. Presence of other causes of CNV, including pathologic myopia (spherical equivalent of -8 diopters or more negative, or axial length of 25 mm or more), ocular histoplasmosis syndrome, angioid streaks, choroidal rupture, or multifocal choroiditis in the study eye. 11. History or clinical evidence of diabetic retinopathy, diabetic macular edema or any other vascular disease affecting the retina, other than AMD, in either eye. 12. Prior vitrectomy in the study eye. 13. History of retinal detachment or treatment or surgery for retinal detachment in the study eye. 14. Any history of macular hole of stage 2 and above in the study eye. 15. Any intraocular or periocular surgery within 3 months of Day 1 on the study eye, except lid surgery, which may not have taken place within 1 month of day 1, as long as it was unlikely to interfere with the injection. 16. Prior trabeculectomy or other filtration surgery in the study eye. 17. Uncontrolled glaucoma (defined as intraocular pressure greater than or equal to 25 mm Hg despite treatment with anti-glaucoma medication) in the study eye. 18. Active intraocular inflammation in either eye. 19. Active ocular or periocular infection in either eye. 20. Any ocular or periocular infection within the last 2 weeks prior to Screening in either eye. 21. Any history of uveitis in either eye. 22. Active scleritis or episcleritis in either eye. 23. Presence or history of scleromalacia in either eye. 24. Aphakia or pseudophakia with absence of posterior capsule (unless it occurred as a result of a yttrium aluminum garnet [YAG] posterior capsulotomy) in the study eye. 25. Previous therapeutic radiation in the region of the study eye. 26. History of corneal transplant or corneal dystrophy in the study eye. 27. Significant media opacities, including cataract, in the study eye which might interfere with visual acuity, assessment of safety, or fundus photography. 28. Any concurrent intraocular condition in the study eye (e.g. cataract) that, in the opinion of the investigator, could require either medical or surgical intervention during the 96 week study period. 29. Any concurrent ocular condition in the study eye which, in the opinion of the investigator, could either increase the risk to the subject beyond what is to be expected from standard procedures of intraocular injection, or which otherwise may interfere with the injection procedure or with evaluation of efficacy or safety. 30. History of other disease, metabolic dysfunction, physical examination finding, or clinical laboratory finding giving reasonable suspicion of a disease or condition that contraindicates the use of an investigational drug or that might affect interpretation of the results of the study or render the subject at high risk for treatment complications. 31. Participation as a subject in any clinical study within the 12 weeks prior to Day 1. 32. Any systemic or ocular treatment with an investigational agent in the past 3 months prior to Day 1. 33. The use of long acting steroids, either systemically or intraocularly, in the 6 months prior to day 1. 34. Any history of allergy to povidone iodine. 35. Known serious allergy to the fluorescein sodium for injection in angiography. 36. Presence of any contraindications indicated in the FDA Approved label for ranibizumab (Lucentis®). 37. Females who were pregnant, breastfeeding, or of childbearing potential, unwilling to practice adequate contraception throughout the study. Adequate contraceptive measures include oral contraceptives (stable use for 2 or more cycles prior to screening); IUD; Depo-Provera®; Norplant® System implants; bilateral tubal ligation; vasectomy; condom or diaphragm plus either contraceptive sponge, foam or jelly.
- 50 **[0086]** Subjects were not allowed to receive any standard or investigational agents for treatment of their AMD in the study eye other than their assigned study treatment with VEGFT or ranibizumab as specified in the protocol until they completed the Completion/Early Termination visit assessments. This includes medications administered locally (e.g., IVT, topical, juxtascleral or periorbital routes), as well as those administered systemically with the intent of treating the study and/or fellow eye.

- 55 **[0087]** The study procedures are summarized as follows:

Best Corrected Visual Acuity: Visual function of the study eye and the fellow eye were assessed using the ETDRS protocol (The Early Treatment Diabetic Retinopathy Study Group) at 4 meters. Visual Acuity examiners were certified to ensure consistent measurement of BCVA. The VA examiners were required to remain masked to treatment assignment.

[0088] Optical Coherence Tomography: Retinal and lesion characteristics were evaluated using OCT on the study eye. At the Screen Visit (Visit 1) images were captured and transmitted for both eyes. All OCT images were captured using the Zeiss Stratus OCT™ with software Version 3 or greater. OCT images were sent to an independent reading center where images were read by masked readers at visits where OCTs were required. All OCTs were electronically archived at the site as part of the source documentation. A subset of OCT images were read. OCT technicians were required to be certified by the reading center to ensure consistency and quality in image acquisition. Adequate efforts were made to ensure that OCT technicians at the site remained masked to treatment assignment.

[0089] Fundus Photography and Fluorescein Angiography (FA): The anatomical state of the retinal vasculature of the study eye was evaluated by funduscopy examination, fundus photography and FA. At the Screen Visit (Visit 1) funduscopy examination, fundus photography and FA were captured and transmitted for both eyes. Fundus and angiographic images were sent to an independent reading center where images were read by masked readers. The reading center confirmed subject eligibility based on angiographic criteria prior to randomization. All FAs and fundus photographs were archived at the site as part of the source documentation. Photographers were required to be certified by the reading center to ensure consistency and quality in image acquisition. Adequate efforts were made to ensure that all photographers at the site remain masked to treatment assignment.

[0090] Vision-Related Quality of Life: Vision-related QOL was assessed using the National Eye Institute 25-Item Visual Function Questionnaire (NEI VFQ-25) in the interviewer-administered format. NEI VFQ-25 was administered by certified personnel at a contracted call center. At the screening visit, the sites assisted the subject and initiated the first call to the call center to collect all of the subject's contact information and to complete the first NEI VFQ-25 on the phone prior to randomization and IVT injection. For all subsequent visits, the call center called the subject on the phone, prior to IVT injection, to complete the questionnaire.

[0091] Intraocular Pressure: Intraocular pressure (IOP) of the study eye was measured using applanation tonometry or Tonopen. The same method of IOP measurement was used in each subject throughout the study.

C. Results Summary (52 Week Data)

[0092] The primary endpoint (prevention of moderate or severe vision loss as defined above) was met for all three VEGFT groups (2Q4, 0.5Q4 and 2Q8) in this study. The results from both studies are summarized in Table 1.

Table 1

	Ranibizumab 0.5 mg monthly (RQ4)	VEGFT 0.5 mg monthly (0.5Q4)	VEGFT 2 mg monthly (2Q4)	VEGFT 2 mg every 8 weeks ^[a] (2Q8)
Maintenance of vision* (% patients losing <15 letters) at week 52 versus baseline				
Study 1	94.4%	95.9%**	95.1%**	95.1%**
Study 2	94.4%	96.3%**	95.6%**	95.6%**
Mean improvement in vision* (letters) at 52 weeks versus baseline (p-value vs RQ4)***				
Study 1	8.1	6.9 (NS)	10.9 (p<0.01)	7.9 (NS)
Study 2	9.4	9.7 (NS)	7.6 (NS)	8.9 (NS)
^[a] Following three initial monthly doses * Visual acuity was measured as the total number of letters read correctly on the Early Treatment Diabetic Retinopathy Study (ETDRS) eye chart. ** Statistically non-inferior based on a non-inferiority margin of 10%, using confidence interval approach (95.1 % and 95% for Study 1 and Study 2, respectively) *** Test for superiority NS = non-significant				

[0093] In Study 1, patients receiving VEGFT 2mg monthly (2Q4) achieved a statistically significant greater mean improvement in visual acuity at week 52 versus baseline (secondary endpoint), compared to ranibizumab 0.5mg monthly (RQ4); patients receiving VEGFT 2mg monthly on average gained 10.9 letters, compared to a mean 8.1 letter gain with ranibizumab 0.5mg dosed every month (p<0.01). All other dose groups of VEGFT in Study 1 and all dose groups in Study 2 were not statistically different from ranibizumab in this secondary endpoint.

[0094] A generally favorable safety profile was observed for both VEGFT and ranibizumab. The incidence of ocular treatment emergent adverse events was balanced across all four treatment groups in both studies, with the most frequent events associated with the injection procedure, the underlying disease, and/or the aging process. The most frequent

ocular adverse events were conjunctival hemorrhage, macular degeneration, eye pain, retinal hemorrhage, and vitreous floaters. The most frequent serious non-ocular adverse events were typical of those reported in this elderly population who receive intravitreal treatment for wet AMD; the most frequently reported events were falls, pneumonia, myocardial infarction, atrial fibrillation, breast cancer, and acute coronary syndrome. There were no notable differences among the study arms.

Example 5: Phase II Clinical Trial of VEGFT in Subjects with Diabetic Macular Edema (DME)

[0095] In this study, 221 patients with clinically significant DME with central macular involvement were randomized, and 219 patients were treated with balanced distribution over five groups. The control group received macular laser therapy at baseline, and patients were eligible for repeat laser treatments, but no more frequently than at 16 week intervals. The remaining four groups received VEGFT by intravitreal injection as follows: Two groups received 0.5 or 2 mg of VEGFT once every four weeks throughout the 12-month dosing period (0.5Q4 and 2Q4, respectively). Two groups received three initial doses of 2 mg VEGFT once every four weeks (*i.e.*, at baseline, and weeks 4 and 8), followed through week 52 by either once every 8 weeks dosing (2Q8) or as needed dosing with very strict repeat dosing criteria (PRN). Mean gains in visual acuity versus baseline were as shown in Table 2:

Table 2

	n	Mean change in visual acuity at week 24 versus baseline (letters)	Mean change in visual acuity at week 52 versus baseline (letters)
Laser	44	2.5	-1.3
VEGFT 0.5 mg monthly (0.5Q4)	44	8.6**	11.0**
VEGFT 2 mg monthly (2Q4)	44	11.4**	13.1**
VEGFT 2 mg every 8 weeks ^[a] (2Q8)	42	8.5**	9.7**
VEGFT 2 mg as needed ^[a] (PRN)	45	10.3**	12.0**
^[a] Following three initial monthly doses			
** p < 0.01 versus laser			

[0096] In this study, the visual acuity gains achieved with VEGFT administration at week 24 were maintained or numerically improved up to completion of the study at week 52 in all VEGFT study groups, including 2 mg dosed every other month

[0097] As demonstrated in the foregoing Examples, the administration of VEGFT to patients suffering from angiogenic eye disorders (*e.g.*, AMD and DME) at a frequency of once every 8 weeks, following a single initial dose and two secondary doses administered four weeks apart, resulted in significant prevention of moderate or severe vision loss or improvements in visual acuity.

Example 6: A Randomized, Multicenter, Double-Masked Trial in Treatment Naive Patients with Macular Edema Secondary to CRVO

[0098] In this randomized, double-masked, Phase 3 study, patients received 6 monthly injections of either 2 mg intravitreal VEGFT (114 patients) or sham injections (73 patients). From Week 24 to Week 52, all patients received 2 mg VEGFT as-needed (PRN) according to retreatment criteria. Thus, "sham-treated patients" means patients who received sham injections once every four weeks from Week 0 through Week 20, followed by intravitreal VEGFT as needed from Week 24 through Week 52. "VEGFT-treated patients" means patients who received VEGFT intravitreal injections once every four weeks from Week 0 through Week 20, followed by intravitreal VEGFT as needed from Week 24 through Week 52. The primary endpoint was the proportion of patients who gained ≥ 15 ETDRS letters from baseline at Week 24. Secondary visual, anatomic, and Quality of Life NEI VFQ-25 outcomes at Weeks 24 and 52 were also evaluated.

[0099] At Week 24, 56.1% of VEGFT-treated patients gained ≥ 15 ETDRS letters from baseline vs 12.3% of sham-treated patients ($P < 0.0001$). Similarly, at Week 52, 55.3% of VEGFT-treated patients gained ≥ 15 letters vs 30.1% of

sham-treated patients ($P < 0.01$). At Week 52, VEGFT-treated patients gained a mean of 16.2 letters vs 3.8 letters for sham-treated patients ($P < 0.001$). Mean number of injections was 2.7 for VEGFT-treated patients vs 3.9 for sham-treated patients. Mean change in central retinal thickness was -413.0 μm for VEGFT-treated patients vs -381.8 μm for sham-treated patients. The proportion of patients with ocular neovascularization at Week 24 were 0% for VEGFT-treated patients and 6.8% for sham-treated patients, respectively; at Week 52 after receiving VEGFT PRN, proportions were 0% and 6.8% for VEGFT-treated and sham-treated. At Week 24, the mean change from baseline in the VFQ-25 total score was 7.2 vs 0.7 for the VEGFT-treated and sham-treated groups; at Week 52, the scores were 7.5 vs 5.1 for the VEGFT-treated and sham-treated groups.

[0100] This Example confirms that dosing monthly with 2 mg intravitreal VEGFT injection resulted in a statistically significant improvement in visual acuity at Week 24 that was maintained through Week 52 with PRN dosing compared with sham PRN treatment. VEGFT was generally well tolerated and had a generally favorable safety profile.

Example 7: Dosing Regimens

[0101] Specific, non-limiting examples of dosing regimens are as follows:

VEGFT 2 mg (0.05 mL) administered by intravitreal injection once every 4 weeks (monthly).

[0102] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 8 weeks, followed by 2 mg (0.05 mL) via intravitreal injection once every 8 weeks.

[0103] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 8 weeks, followed by 2 mg (0.05 mL) via intravitreal injection on a less frequent basis based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0104] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 8 weeks, followed by 2 mg (0.05 mL) via intravitreal injection administered *pro re nata* (PRN) based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0105] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 12 weeks, followed by 2 mg (0.05 mL) via intravitreal injection once every 8 weeks.

[0106] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 12 weeks, followed by 2 mg (0.05 mL) via intravitreal injection on a less frequent basis based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0107] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 12 weeks, followed by 2 mg (0.05 mL) via intravitreal injection administered *pro re nata* (PRN) based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0108] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 16 weeks, followed by 2 mg (0.05 mL) via intravitreal injection once every 8 weeks.

[0109] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 16 weeks, followed by 2 mg (0.05 mL) via intravitreal injection on a less frequent basis based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0110] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 16 weeks, followed by 2 mg (0.05 mL) via intravitreal injection administered *pro re nata* (PRN) based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0111] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 20 weeks, followed by 2 mg (0.05 mL) via intravitreal injection once every 8 weeks.

[0112] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 20 weeks, followed by 2 mg (0.05 mL) via intravitreal injection on a less frequent basis based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0113] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 20 weeks, followed by 2 mg (0.05 mL) via intravitreal injection administered *pro re nata* (PRN) based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0114] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 24 weeks, followed by 2 mg (0.05 mL) via intravitreal injection once every 8 weeks.

[0115] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 24 weeks, followed by 2 mg (0.05 mL) via intravitreal injection on a less frequent basis based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0116] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 24 weeks, followed by 2 mg (0.05 mL) via intravitreal injection administered *pro re nata* (PRN) based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0117] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 28 weeks, followed by 2 mg (0.05 mL) via intravitreal injection once every 8 weeks.

[0118] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 28 weeks, followed by 2 mg (0.05 mL) via intravitreal injection on a less frequent basis based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

5 [0119] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 28 weeks, followed by 2 mg (0.05 mL) via intravitreal injection administered *pro re nata* (PRN) based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0120] VEGFT 2 mg (0.05 mL) administered by intravitreal injection as a single initial dose, followed by additional doses administered *pro re nata* (PRN) based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

10 [0121] Variations on the above-described dosing regimens would be appreciated by persons of ordinary skill in the art. For example, the amount of VEGFT and/or volume of formulation administered to a patient may be varied based on patient characteristics, severity of disease, and other diagnostic assessments by a physician or other qualified medical professional.

15 [0122] Any of the foregoing administration regimens may be used for the treatment of, *e.g.*, age-related macular degeneration (*e.g.*, wet AMD, exudative AMD, *etc.*), retinal vein occlusion (RVO), central retinal vein occlusion (CRVO; *e.g.*, macular edema following CRVO), branch retinal vein occlusion (BRVO), diabetic macular edema (DME), choroidal neovascularization (CNV; *e.g.*, myopic CNV), iris neovascularization, neovascular glaucoma, post-surgical fibrosis in glaucoma, proliferative vitreoretinopathy (PVR), optic disc neovascularization, corneal neovascularization, retinal neovascularization, vitreal neovascularization, pannus, pterygium, vascular retinopathy, *etc.*

20 **Example 8: Phase 3, Double-Masked, Randomized Study Of The Efficacy And Safety Of Intravitreal IAI In Patients With Moderately Severe To Severe NPDR (PANORAMA)-Week 24 and 52 results**

25 [0123] This was a phase 3, double-masked, randomized study of the efficacy and safety of IVT (intravitreal injection) aflibercept (IAI) for the improvement of moderately severe to severe non-proliferative diabetic retinopathy (NPDR). These data relate to results achieved after 24 weeks and 52 weeks.

[0124] Eligible patients were enrolled into 1 of 3 treatment groups in a 1:1:1 randomization scheme, and are stratified based on their Diabetic Retinopathy Severity Scale (DRSS) score (level 47 vs. level 53) (see Figure 5 and Figure 6). Only 1 eye was selected as the study eye.

30 **Study design**

[0125] The primary outcome measure of the study is the proportion of patients who improved by ≥ 2 steps from baseline on the DRSS in the combined 2Q8 and 2Q16 groups at week 24, and in each group separately at week 52.

35 [0126] Patients are evaluated for efficacy (best corrected visual acuity [BCVA] using the 4-meter Early Treatment Diabetic Retinopathy Study [ETDRS] protocol, spectral domain optical coherence tomography [SD OCT], and fluorescein angiography [FA]/fundus photography [FP]) and for ocular and systemic safety (including ophthalmic exams, visual field testing, and laboratory assessments) through week 100.

40 [0127] The secondary outcome measures are also tested at week 52 and are as follows:

(1) Proportion of patients developing a vision threatening complication (VTC) due to diabetic retinopathy through week 52. Vision threatening complications are defined as composite outcome of PDR (inclusive of patients who have vitreous hemorrhage or tractional retinal detachment believed to be due to PDR) and ASNV. ASNV is defined as neovascularization of the iris (at least 2 cumulative clock hours), and/or definitive neovascularization of the iridocorneal angle

(2) Proportion of patients who develop CI DME through week 52

(3) Time to development of a vision threatening complication through week 52

(4) Time to development of CI DME (center-involved DME) through week 52

45 (5) Proportion of patients who receive PRP (panretinal photocoagulation) through week 52, inclusive of patients undergoing vitrectomy with endolaser

50 (6) Area under the curve (AUC) for change in BCVA from baseline at week 52.

Study timeline

55 [0128]

Day -21 to -1: Screening visit (visit 1)

Day 1: Baseline visit (visit 2)

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Week 24: Primary Outcome Measure (2Q8 & 2Q16 combined) (visit 7)

Week 52: Primary Outcome Measure (2Q8 & 2Q16 separately) and all secondary outcome measures (visit 11)

Week 100: End of Study (visit 18)

5 **[0129] Exclusion criteria:** Patients who met any of the following criteria at either the screening visit or at day 1 were excluded from the study:

- (1) Presence of DME threatening the center of the macula (within 1,000 microns of the foveal center) in the study eye;
- (2) Evidence of retinal neovascularization on clinical examination or FA (fluorescein angiography);
- 10 (3) Any prior focal or grid laser photocoagulation (within 1,000 microns of the foveal center) or any prior PRP in the study eye;
- (4) Any prior systemic anti-VEGF treatment or IVT anti-VEGF treatment in the study eye;
- (5) Any prior intraocular steroid in the study eye; periocular steroid in the study eye within 120 days of day 1;
- (6) History of vitreoretinal surgery in the study eye;
- 15 (7) Intraocular pressure (IOP) ≥ 25 mm Hg in the study eye;
- (8) Evidence of active infectious blepharitis, keratitis, scleritis, or conjunctivitis in either eye;
- (9) Any intraocular inflammation or infection in either eye within 3 months of the screening visit;
- (10) Current ASNV, vitreous hemorrhage, or tractional retinal detachment visible at the screening assessments in the study eye;
- 20 (11) Ocular media of insufficient quality to obtain fundus and optical coherence tomography (OCT) images in the study eye; allergy to fluorescein precluding ability to perform fluorescein angiography;
- (12) Hemoglobin A1c (HbA1c) $>12\%$, or if HbA1c is $\leq 12\%$, diabetes mellitus is uncontrolled in the opinion of the investigator;
- (13) Uncontrolled blood pressure (defined as systolic >160 mm Hg or diastolic >95 mm Hg while patient is sitting);
- 25 (14) History of cerebrovascular accident or myocardial infarction within 180 days of day 1;
- (15) Renal failure, dialysis, or history of renal transplant;
- (16) Women who are breastfeeding or who have a positive serum hCG/urine pregnancy test at the screening or baseline visit;
- 30 (17) Any concurrent ocular condition in the study eye which, in the opinion of the investigator, could either increase the risk to the patient beyond what is to be expected from standard procedures of IVT injections, or which otherwise may interfere with the injection procedure or with evaluation of efficacy or safety;
- (18) History of other disease, metabolic dysfunction, physical examination finding, or clinical laboratory finding giving reasonable suspicion of a disease or condition that contraindicates the use of an investigational drug or that might affect interpretation of the results of the study or render the patient at high risk for treatment complications;
- 35 (19) Participation as a patient in any interventional clinical study within the 12 weeks prior to day 1 of the study;
- (20) Sexually active men* or women of childbearing potential** who are unwilling to practice adequate contraception prior to the initial dose/start of the first treatment, during the study, and for at least 3 months after the last dose. Adequate contraceptive measures include stable use of oral contraceptives or other prescription pharmaceutical contraceptives for 2 or more menstrual cycles prior to screening; intrauterine device; bilateral tubal ligation; vasectomy; condom plus contraceptive sponge, foam, or jelly, or diaphragm plus contraceptive sponge, foam, or jelly.
- 40

* Contraception is not required for men with documented vasectomy.

45 ** Postmenopausal women must be amenorrheic for at least 12 months in order not to be considered of child-bearing potential. Pregnancy testing and contraception are not required for women with documented hysterectomy or tubal ligation.

(21) Patients who are on systemic anti-VEGF treatment (i.e., bevacizumab, ziv-aflibercept) for oncology treatment (if a patient requires systemic anti-VEGF treatment during the study, the patient will be withdrawn)

50 **[0130] Treatment regimen:** The 3 treatment groups have the following dosing regimens scheduled from day 1 to week 48:

2Q8: aflibercept 2 mg Q8 to week 48 (after 5 initial monthly doses), followed by a flexible treatment regimen with aflibercept 2 mg after week 52;

55 2Q16: aflibercept 2 mg Q16 to week 96 (after 3 initial monthly doses and 1 Q8 interval);

Sham: sham injections every 4 weeks (Q4) to week 16, followed by sham injections Q8 to week 96.

[0131] Data herein may refer to the 2Q16 dosing group as "Group 1"; the 2Q8 dosing group as "Group 2" and the

sham group as "Sham".

[0132] See Figure 3 and Figure 4.

[0133] The number of injections received by each dose group after 24 weeks is shown in Figure 7 and the number of injections after 52 weeks is shown in Figure 20.

5 **[0134]** To preserve the masking, sham injections are performed for the 2Q8 and 2Q16 groups at treatment visits in which patients will not receive an active injection through week 96, and at all treatment visits for the sham group from baseline to week 96. Masking is maintained to the end of the study (week 100).

[0135] Rescue treatment in the study eye: Patients who develop PDR, ASNV, or center-involved DME (CI DME) in the study eye are treated, if deemed appropriate by the masked physician. For any of these complications, an FP (fundus photography) is performed before rescue treatment is given.

10 **[0136]** Patients who develop CI DME receive IVT aflibercept or laser photocoagulation, and no longer receive their randomized treatment. Rescue treatment is given by the masked or unmasked physician.

[0137] Patients who develop PDR and/or ASNV receive PRP or vitrectomy with endolaser, if necessary, but remain on their randomized treatment schedule. Panretinal photocoagulation or surgical intervention is performed by either the masked or unmasked physician. In addition, 1 injection of aflibercept is given, which must be administered by the unmasked physician.

15 **[0138]** If treatment for DME, ASNV, or PDR is given, patient data is censored from the time of treatment for the primary analysis.

20 **Study population**

[0139] 402 patients were enrolled. The patient population included men or women with type 1 or 2 diabetes mellitus who had moderately severe to severe NPDR (without DME threatening the center of the macula), in whom PRP can be safely deferred for at least 6 months. See Figure 5 and Figure 6. About 75% of patients had a DRSS of 47 and about 25% had a DRSS of 53.

25 **[0140]** For patients who meet eligibility criteria in both eyes, the eye with the most severe DRSS score is selected as the study eye. If both eyes have equivalent scores, factors such as ocular dominance and patient preference are considered in making the selection.

30 **Ocular Procedures (Efficacy and Safety)**

[0141] Best Corrected Visual Acuity (BCVA): Visual function of the study eye and the fellow eye is assessed using the ETDRS protocol (The Early Treatment Diabetic Retinopathy Study Group 1985) at 4 meters at each study visit. Visual acuity examiners are certified to ensure consistent measurement of BCVA. The VA examiner remains masked to treatment assignment. Best corrected visual acuity is done before any other ocular procedures are performed.

35 **[0142]** Intraocular Pressure (IOP): Intraocular pressure of the study eye is measured at every visit using Goldmann applanation tonometry or Tono pen™. The same method of IOP measurement is used throughout the study for each individual patient. Intraocular pressure is measured pre-dose (bilateral) by the masked physician (or designee), and at approximately 30 minutes post-dose (study eye) by the unmasked physician (or designee).

40 **[0143]** Slit Lamp Examination: Patients' anterior eye structure and ocular adnexa is examined bilaterally pre-dose at each study visit using a slit lamp by the masked investigator.

[0144] Gonioscopy: Patients are evaluated for the development of neovascularization of the iridocorneal angle by gonioscopy in conjunction with slit lamp biomicroscopy. The examination is performed in the study eye only before the application of mydriatic agents or if frank rubeosis is present.

45 **[0145]** Indirect Ophthalmoscopy: Patients' posterior pole and peripheral retina are examined by indirect ophthalmoscopy at each study visit pre-dose (bilateral) by the masked investigator and post-dose (study eye) by the unmasked investigator. Post-dose evaluation is performed immediately after injection (active drug or sham).

[0146] Fundus Photography (FP)/Fluorescein Angiography (FA): The anatomical state of the retinal vasculature and the DRSS level is evaluated by FA and FP.

50 **[0147]** Spectral Domain Optical Coherence Tomography (SD-OCT): Retinal characteristics are evaluated at every visit using SD-OCT. Images are captured and transmitted for both eyes. Images are sent to an independent reading center where they are read by masked readers. All OCTs are electronically archived at the study sites as part of the source documentation. Optical coherence tomography technicians are certified by the reading center to ensure consistency and quality in image acquisition. Every effort is made to ensure that OCT technicians at the study site remain masked to treatment assignment.

55 **[0148]** Visual Field Testing: Visual field testing is assessed in the study eye using the Humphrey Visual Field Analyzer by sites who have access to this machine. Technicians are certified to ensure consistency and quality testing procedures. Every effort is made to ensure that visual field technicians at the study site remain masked to treatment assignment.

Adverse events (AEs)

[0149] Overall safety was assessed by evaluation of treatment-emergent adverse events (TEAEs), physical examinations, electrocardiograms (ECGs), vital signs, and clinical safety laboratory tests (hematology, blood chemistry, hemoglobin A1c [HbA1c], and urinalysis) at various time points.

[0150] A TEAE is defined as an event (or an exacerbation of a preexisting event during the treatment period) that is observed or reported after the first administration of study drug, and no later than 30 days after last administration of study drug (active or sham injection).

[0151] The investigator (or designee) records all AEs that occur from the time the informed consent is signed until the end of study. All serious adverse events (SAEs), regardless of assessment of causal relationship to study drug must be reported within 24 hours.

[0152] Other events requiring reporting within 24 hours include symptomatic overdose of study drug (accidental or intentional overdose of at least 2 times the intended dose of study drug within the intended therapeutic window, if associated with an AE) and pregnancy.

[0153] The severity of AEs will be graded according to the following scale:

Mild: Does not interfere in a significant manner with the patient's normal functioning level. It may be an annoyance. Prescription drugs are not ordinarily needed for relief of symptoms, but may be given because of personality of the patient.

[0154] Moderate: Produces some impairment of functioning but is not hazardous to health. It is uncomfortable or an embarrassment. Treatment for symptom may be needed.

[0155] Severe: Produces significant impairment of functioning or incapacitation and is a definite hazard to the patient's health. Treatment for symptom may be given and/or patient hospitalized.

Results and conclusions

[0156] The proportion of patients with ≥ 2 -step improvement in the DRSS was significantly greater in the IAI groups vs sham. See Figures 8, 19, 21 and 22. Patients also achieved ≥ 3 -step improvement in the IAI groups vs. Sham. See Figures 9 and 32.

[0157] IAI reduced the number of patients who developed a VTC and CI-DME. At 24 and 52 weeks, the proportion of patients experiencing a VTC and/or DME is summarized in Figures 10 and 23. A Kaplan-Meier analysis of the probability of developing a VTC or CI-DME in patients in each treatment group over time is set forth in Figures 24 and 25. A vision threatening complication (VTC) is progression to proliferative diabetic retinopathy (PDR) and anterior segment neovascularization (ASNV).

[0158] The changes in best corrected visual acuity (BCVA) experienced by patients in each treatment group at 24 and 52 weeks are summarized Figures 11 and 26. The changes to central retinal thickness of each treatment group is summarized in Figures 12 and 27.

[0159] Efficacy outcomes were similar in the 2Q16 (Group 1) and the 2Q8 (Group 2) groups.

[0160] Ocular treatment emergent adverse events at 24 and 52 weeks (TEAEs) (Figures 13 and 28, respectively), ocular serious TEAEs at 24 and 52 weeks (Figures 14 and 29, respectively), intra-ocular inflammation at 24 and 52 weeks (Figures 15 and 30, respectively), and Anti-Platelet Trialists' Collaboration (APTC) events at 24 and 52 weeks (Figures 16 and 31, respectively) and deaths at 24 weeks (Figure 17) are provided. At 52 weeks, there was a total of 7 deaths (7 in the sham treatment group and 1 in the q8w treatment group). (APTC: See Antithrombotic Trialists' Collaboration. Collaborative overview of randomized trial of antiplatelet therapy - II: Maintenance of vascular graft or arterial patency by antiplatelet therapy. Br Med J 1994; 308:168-171; and Antithrombotic Trialists' Collaboration. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. Br Med J 2002; 324:71-86).

SEQUENCES

[0161]

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SEQUENCE LISTING

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<120> Use of a VEGF Antagonist to Treat Angiogenic Eye Disorders

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<130> 10409P1-WO

<140> PCT/US2018/063025

EP 3 716 992 B1

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55			450				455									

Claims

1. A VEGF antagonist for use in a method for treating or preventing diabetic retinopathy in a patient in need of such treatment or prevention, wherein said patient does not suffer from center involved diabetic macular edema, said method comprising administering, to an eye of the patient,
5 5 monthly doses followed by one or more secondary doses every 8 weeks of about 2 mg of VEGF antagonist that is a VEGF receptor-based chimeric molecule comprising (1) an immunoglobulin-like (Ig) domain 2 of a VEGFR1 and (2) Ig domain 3 of VEGFR2, and (3) a multimerizing component.
- 10 2. The VEGF antagonist for use in the method of claim 1 wherein:
 - (a) the VEGF antagonist
 - (i) comprises (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2; (2) a VEGFR2 component comprising amino acids 130-231 of SEQ ID NO:2; and (3) a multimerization component comprising amino acids 232-457 of SEQ ID NO:2;
 - (ii) is aflibercept; or
 - (iii) is conbercept, and/or
 - 20 (b) the VEGF antagonist is administered by intravitreal injection.
3. The VEGF antagonist for use in the method of claims 1 or 2 wherein:
 - (a) the patient suffers from nonproliferative diabetic retinopathy;
 - 25 (b) the patient suffers from moderately severe or severe nonproliferative diabetic retinopathy;
 - (c) the patient suffers from nonproliferative diabetic retinopathy which is **characterized by** a Diabetic Retinopathy Severity Scale level of 47 or 53;
 - (d) the patient has a baseline best-corrected visual acuity (BCVA) ETDRS letter score of 69 or greater; and/or
 - 30 (e) the vision of the patient is **characterized by** a Snellen visual acuity of 20/40 or better.
4. The VEGF antagonist for use in the method of any one of claims 1-3, wherein:
 - (a) the patient is human; or
 - 35 (b) the patient is a human 18 years of age or older.
5. The VEGF antagonist for use in the method of any one of claims 1-4 wherein the patient does not suffer from:
 - retinal neovascularization;
 - anterior segment neovascularization (ASNV);
 - 40 - vitreous hemorrhage; and/or
 - tractional retinal detachment.
6. The VEGF antagonist for use in the method of any one of claims 1 to 5 wherein:
 - 45 (a) the patient has nonproliferative diabetic retinopathy without diabetic macular edema; and/or
 - (b) the patient:
 - (i) has diabetes;
 - (ii) has a hemoglobin A1c of about 8.5
 - 50 (iii) has an ETDRS BCVA score of about 82;
 - (iv) has a central retinal thickness of about 247 μ m;
 - (v) has a diabetic retinopathy severity score of 47 or 53; and/or
 - (vi) is about 56 years of age.
- 55 7. The VEGF antagonist for use in the method of any one of claims 1 to 6 wherein the patient is administered the VEGF antagonist for at least 24 weeks and achieves one or more of:
 - (i) at least a 2 step improvement from baseline in diabetic retinopathy severity scale (DRSS) score;

- (ii) at least a 3 step improvement from baseline in diabetic retinopathy severity scale (DRSS) score;
 - (iii) an improvement in best corrected visual acuity of at least about 1.9 letters;
 - (iv) does not experience a reduction in best corrected visual acuity of any more than 4 letters;
 - (v) does not develop diabetic macular edema;
 - 5 (vi) does not experience a vision threatening complication;
 - (vii) does not develop proliferative diabetic retinopathy;
 - (viii) does not develop anterior segment neovascularization; and/or
 - (ix) experiences a reduction in central retinal thickness of about 19 μ m.
- 10 8. The VEGF antagonist for use in the method of any one of claims 1-7 wherein the VEGF antagonist is aflibercept.

Patentansprüche

- 15 1. VEGF-Antagonist für die Verwendung in einem Verfahren zum Behandeln oder Vorbeugen von diabetischer Retinopathie bei einem Patienten, der einer solchen Behandlung oder Vorbeugung bedarf, wobei der Patient nicht an einem zentral involvierten diabetischen Makulaödem leidet, wobei das Verfahren das Verabreichen an ein Auge des Patienten von
- 20 5 monatlichen Dosen, gefolgt von einer oder mehreren sekundären Dosen alle 8 Wochen von etwa 2 mg VEGF-Antagonist umfasst, der ein chimäres Molekül auf VEGF-Rezeptorbasis ist, umfassend (1) eine immunoglobulinähnliche (Ig) Domäne 2 eines VEGFR1 und (2) Ig-Domäne 3 von VEGFR2, und (3) eine multimerisierende Komponente.
2. VEGF-Antagonist für die Verwendung in dem Verfahren nach Anspruch 1, wobei:
- 25 (a) der VEGF-Antagonist
- (i) (1) eine VEGFR1-Komponente, umfassend die Aminosäuren 27 bis 129 von SEQ ID NO: 2; (2) eine VEGFR2-Komponente, umfassend die Aminosäuren 130-231 von SEQ ID NO: 2; und (3) eine Multimerisierungskomponente umfasst, umfassend die Aminosäuren 232-457 von SEQ ID NO: 2;
- 30 (ii) Aflibercept ist; oder
- (iii) Conbercept ist, und/oder
- (b) der VEGF-Antagonist durch intravitreale Injektion verabreicht wird.
- 35 3. VEGF-Antagonist für die Verwendung in dem Verfahren nach Anspruch 1 oder 2, wobei:
- (a) der Patient an nichtproliferativer diabetischer Retinopathie leidet;
- (b) der Patient an einer mäßig schweren oder schweren nichtproliferativen diabetischen Retinopathie leidet;
- 40 (c) der Patient an einer nichtproliferativen diabetischen Retinopathie leidet, die durch einen Wert des Schweregrads der diabetischen Retinopathie von 47 oder 53 gekennzeichnet ist;
- (d) der Patient einen ETDRS-Ausgangsbuchstabenwert für die bestkorrigierte Sehschärfe (BCVA) von 69 oder mehr aufweist; und/oder
- (e) das Sehvermögen des Patienten durch eine Snellen-Sehschärfe von 20/40 oder besser gekennzeichnet ist.
- 45 4. VEGF-Antagonist für die Verwendung in dem Verfahren nach einem der Ansprüche 1-3, wobei:
- (a) der Patient ein Mensch ist; oder
- (b) der Patient ein Mensch im Alter von 18 Jahren oder älter ist.
- 50 5. VEGF-Antagonist für die Verwendung in dem Verfahren nach einem der Ansprüche 1-4, wobei der Patient nicht leidet an:
- retinaler Neovaskularisation;
 - Neovaskularisation des anterioren Segments (ASNV);
 - 55 - Glaskörperblutung; und/oder
 - traktionelle Netzhautablösung.
6. VEGF-Antagonist für die Verwendung in dem Verfahren nach einem der Ansprüche 1 bis 5, wobei:

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- (a) der Patient eine nichtproliferative diabetische Retinopathie ohne diabetisches Makulaödem hat; und/oder
(b) der Patient:

- (i) Diabetes hat;
(ii) ein Hämoglobin A1c von etwa 8,5 hat
(iii) einen ETDRS BCVA-Wert von etwa 82 hat;
(iv) eine zentrale Netzhautdicke von etwa 247 µm hat;
(v) einen Wert des Schweregrads der diabetischen Retinopathie von 47 oder 53 hat; und/oder
(vi) etwa 56 Jahre alt ist.

7. VEGF-Antagonist für die Verwendung in dem Verfahren nach einem der Ansprüche 1 bis 6, wobei dem Patient der VEGF-Antagonist wenigstens 24 Wochen lang verabreicht wird und eine oder mehrere erreicht von:

- (i) wenigstens einer 2-stufigen Verbesserung des Werts des Schweregrads der diabetischen Retinopathie (DRSS) gegenüber dem Ausgangswert;
(ii) wenigstens einer 3-stufigen Verbesserung des Werts des Schweregrads der diabetischen Retinopathie (DRSS) gegenüber dem Ausgangswert;
(iii) einer Verbesserung der bestkorrigierten Sehschärfe um wenigstens etwa 1,9 Buchstaben;
(iv) keine Reduzierung der bestkorrigierten Sehschärfe um mehr als 4 Buchstaben erfährt;
(v) kein diabetisches Makulaödem entwickelt;
(vi) keine das Sehvermögen bedrohende Komplikation erfährt;
(vii) keine proliferative diabetische Retinopathie entwickelt;
(viii) keine Neovaskularisation des anterioren Segments entwickelt; und/oder
(ix) eine Reduzierung der zentralen Netzhautdicke von etwa 19 µm erfährt.

8. VEGF-Antagonist für die Verwendung in dem Verfahren nach einem der Ansprüche 1-7, wobei der VEGF-Antagonist Aflibercept ist.

Revendications

1. Antagoniste du VEGF pour l'utilisation dans un procédé pour le traitement ou la prévention de rétinopathie diabétique chez un patient ayant besoin d'un tel traitement ou d'une telle prévention, dans lequel ledit patient ne souffre pas d'œdème maculaire diabétique atteignant le centre, ledit procédé comprenant l'administration, à un œil du patient, de 5 doses mensuelles, suivies par une ou plusieurs doses secondaires toutes les 8 semaines, d'environ 2 mg d'antagoniste du VEGF qui est une molécule chimérique à base de récepteur du VEGF comprenant (1) un domaine 2 analogue à immunoglobuline (Ig) d'un VEGFR1 et (2) domaine 3 Ig de VEGFR2, et (3) un composant multimérisant.

2. Antagoniste du VEGF pour l'utilisation dans le procédé selon la revendication 1, dans lequel :

- (a) l'antagoniste du VEGF

- (i) comprend (1) un composant VEGFR1 comprenant des acides aminés 27 à 129 de SEQ ID n° : 2 ; (2) un composant VEGFR2 comprenant des acides aminés 130 à 231 de SEQ ID n° : 2 ; et (3) un composant de multimérisation comprenant des acides aminés 232 à 457 de SEQ ID n° : 2 ;
(ii) est aflibercept ; ou
(iii) est conbercept, et/ou

- (b) l'antagoniste du VEGF est administré par injection intravitréenne.

3. Antagoniste du VEGF pour l'utilisation dans le procédé selon les revendications 1 ou 2, dans lequel :

- (a) le patient souffre de rétinopathie diabétique non proliférative ;
(b) le patient souffre de rétinopathie diabétique non proliférative modérément sévère ou sévère ;
(c) le patient souffre de rétinopathie diabétique non proliférative qui est **caractérisée par** un niveau d'échelle de sévérité de rétinopathie diabétique de 47 ou 53 ;
(d) le patient a un résultat de lettres ETDRS de meilleure acuité visuelle corrigée (MAVC) de ligne de base de 69 ou plus ; et/ou

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(e) la vision du patient est **caractérisée par** une acuité visuelle Snellen de 20/40 ou mieux.

4. Antagoniste du VEGF pour l'utilisation dans le procédé selon l'une quelconque des revendications 1 à 3, dans lequel :

- 5 (a) le patient est humain ; ou
(b) le patient est un humain dont l'âge est de 18 ans ou plus.

5. Antagoniste du VEGF pour l'utilisation dans le procédé selon l'une quelconque des revendications 1 à 4 dans lequel le patient ne souffre pas de :

- 10 - néovascularisation rétinienne ;
- néovascularisation du segment antérieur (NVSA) ;
- hémorragie vitréenne ; et/ou
15 - décollement de rétine tractionnel.

6. Antagoniste du VEGF pour l'utilisation dans le procédé selon l'une quelconque des revendications 1 à 5 dans lequel :

- (a) le patient a une rétinopathie diabétique non proliférative sans œdème maculaire diabétique ; et/ou
20 (b) le patient :

- (i) a du diabète ;
(ii) a une hémoglobine A1c d'environ 8,5
(iii) a un résultat de MAVC ETD RS d'environ 82 ;
(iv) a une épaisseur rétinienne centrale d'environ 247 µm ;
25 (v) a un résultat de sévérité de rétinopathie diabétique de 47 ou 53 ; et/ou
(vi) a un âge d'environ 56 ans.

7. Antagoniste du VEGF pour l'utilisation dans le procédé selon l'une quelconque des revendications 1 à 6, dans lequel l'antagoniste du VEGF est administré au patient pendant au moins 24 semaines et accomplit un ou plusieurs de :

- 30 (i) au moins 2 niveaux d'amélioration par rapport à la ligne de base dans le résultat d'échelle de sévérité de rétinopathie diabétique (DRSS) ;
(ii) au moins 3 niveaux d'amélioration par rapport à la ligne de base dans le résultat d'échelle de sévérité de rétinopathie diabétique (DRSS) ;
35 (iii) une amélioration de la meilleure acuité visuelle corrigée d'au moins environ 1,9 lettres ;
(iv) ne subit pas de réduction de meilleure acuité visuelle corrigée de plus de 4 lettres ;
(v) ne développe pas d'œdème maculaire diabétique ;
(vi) ne subit pas de complication menaçant la vision ;
(vii) ne développe pas de rétinopathie diabétique proliférative ;
40 (viii) ne développe pas de néovascularisation du segment antérieur ; et/ou
(ix) subit une réduction d'épaisseur rétinienne centrale d'environ 19 µm.

8. Antagoniste du VEGF pour l'utilisation dans le procédé selon l'une quelconque des revendications 1 à 7, dans lequel l'antagoniste du VEGF est aflibercept.

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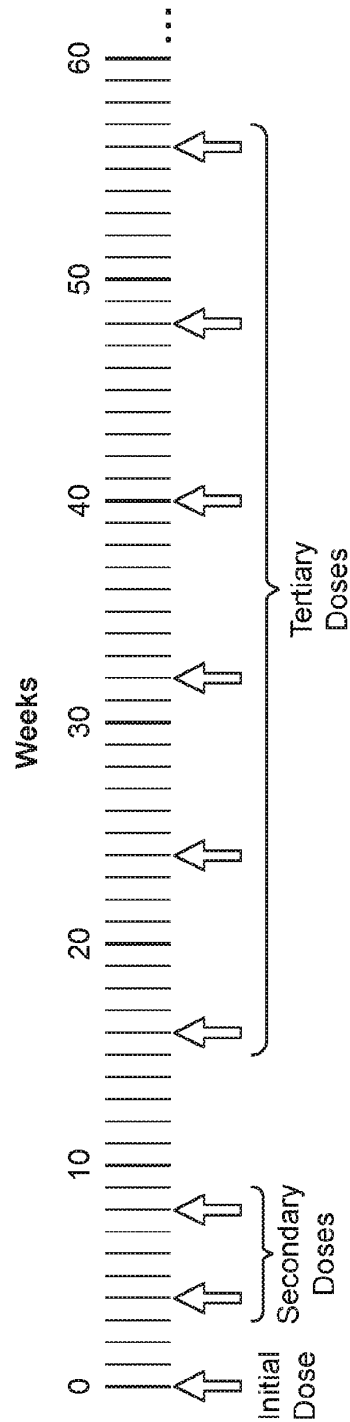


FIG. 1

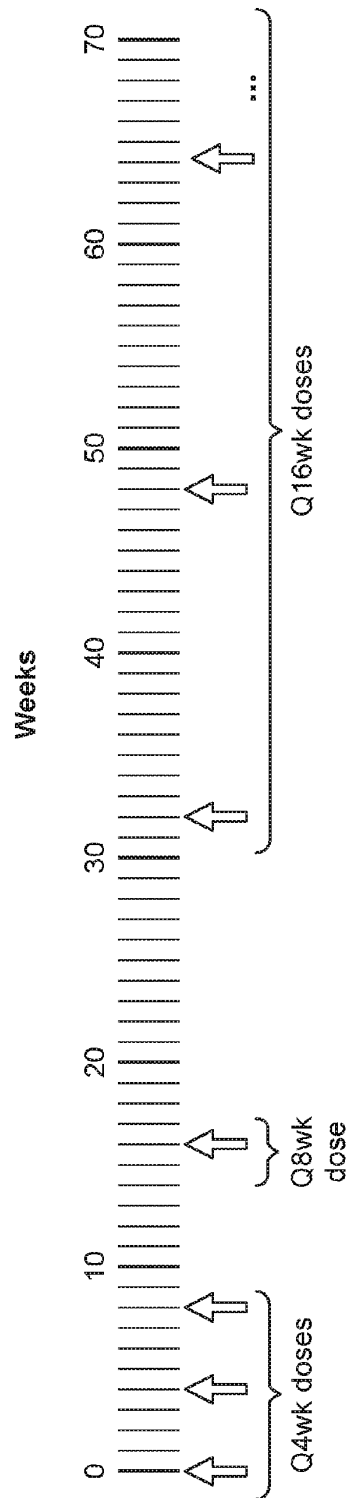


FIG. 2

Study Design

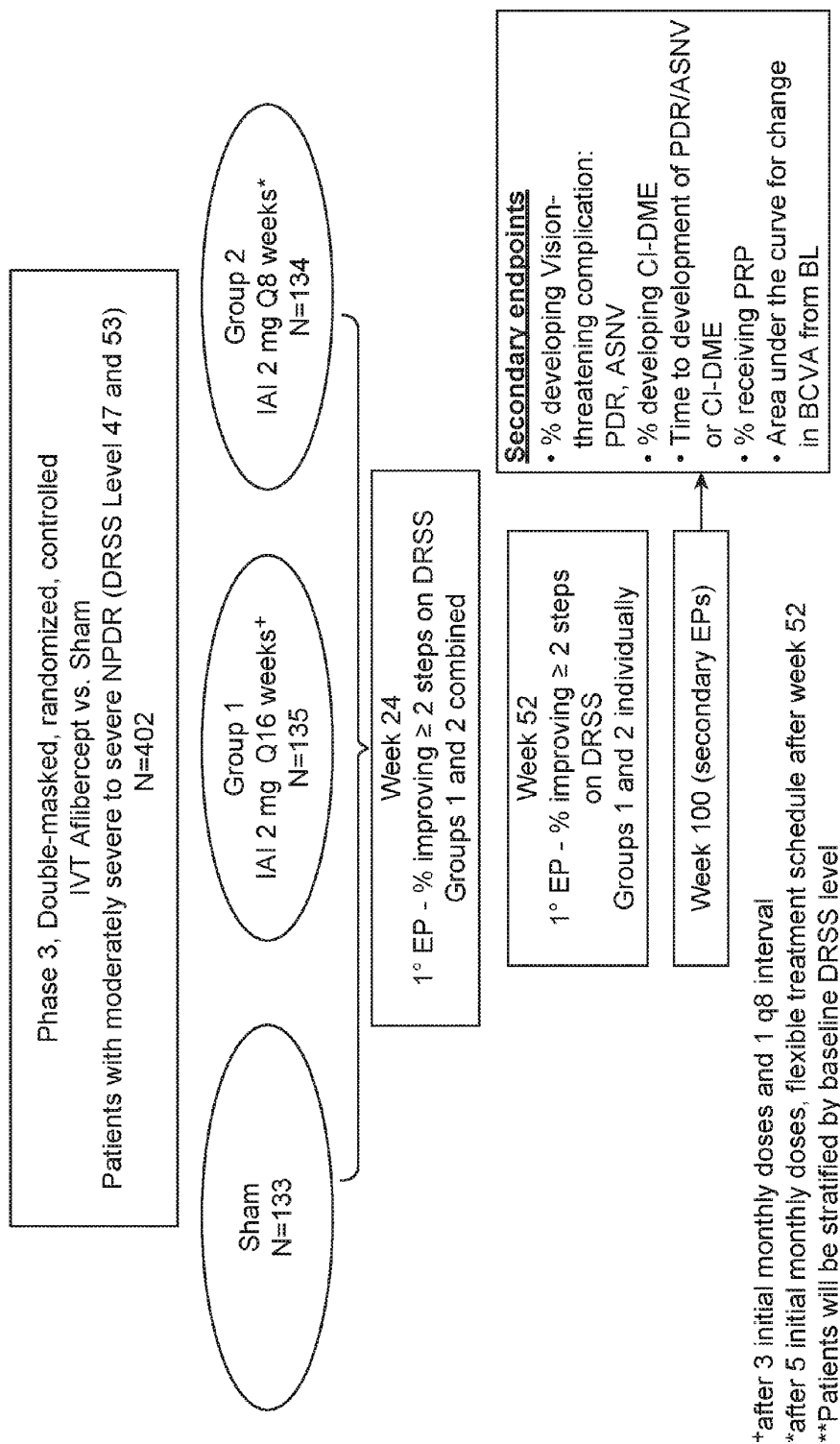
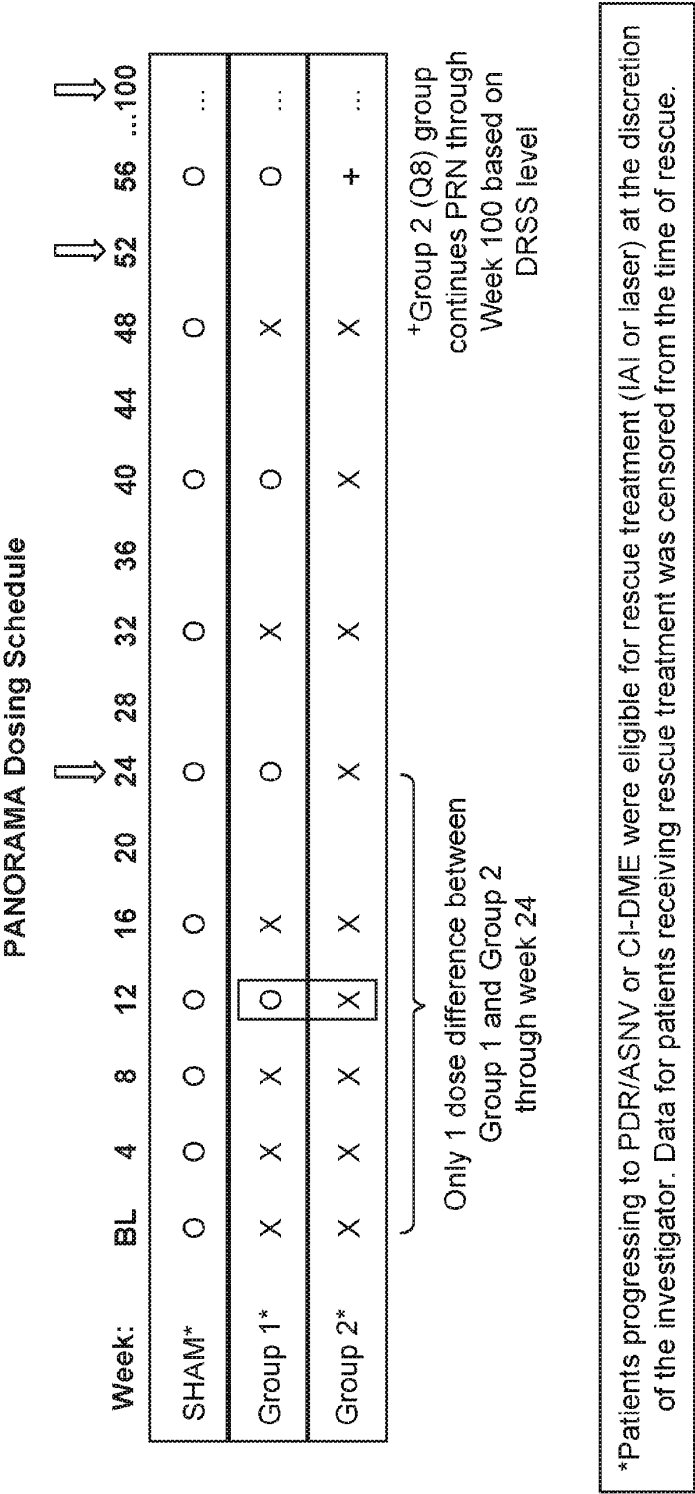


FIG. 3



X=active injection, O=sham injection

FIG. 4

Disposition and Baseline Demographics

	— Sham	---- Group 1	— Group 2	--- All IAI	— Total
Number of Patients who Completed at Week 24	119 (89.5%)	129 (95.6%)	132 (98.5%)	261 (97.0%)	380 (94.5%)
N (FAS/SAF)	133	135	134	269	402
Age (years (SD))	55.8 (10.31)	55.4 (11.13)	55.8 (10.19)	55.6 (10.66)	55.7 (10.53)
Women # (%)	64 (48.1%)	60 (44.4%)	53 (39.6%)	113 (42.0%)	177 (44.0%)
Race # (%)					
White	107 (80.5%)	99 (73.3%)	104 (77.6%)	203 (75.5%)	310 (77.1%)
Black or African American	13 (9.8%)	16 (11.9%)	12 (9.0%)	28 (10.4%)	41 (10.2%)
Asian	4 (3.0%)	12 (8.9%)	7 (5.2%)	19 (7.1%)	23 (5.7%)
Other	9 (6.8%)	8 (5.9%)	11 (8.2%)	19 (7.1%)	28 (7.0%)
Hemoglobin A1C (%)	8.5 (1.54)	8.6 (1.69)	8.4 (1.64)	8.5 (1.66)	8.5 (1.62)
Duration of Diabetes (years (SD))	15.5 (9.34)	13.7 (8.61)	14.0 (9.69)	13.8 (9.15)	14.4 (9.24)
Diabetes Type 2	123 (92.5%)	121 (89.6%)	124 (92.5%)	245 (91.1%)	368 (91.5%)

Group 1: 3 monthly doses followed by 1 q8 intervals, Group 2: 5 monthly doses

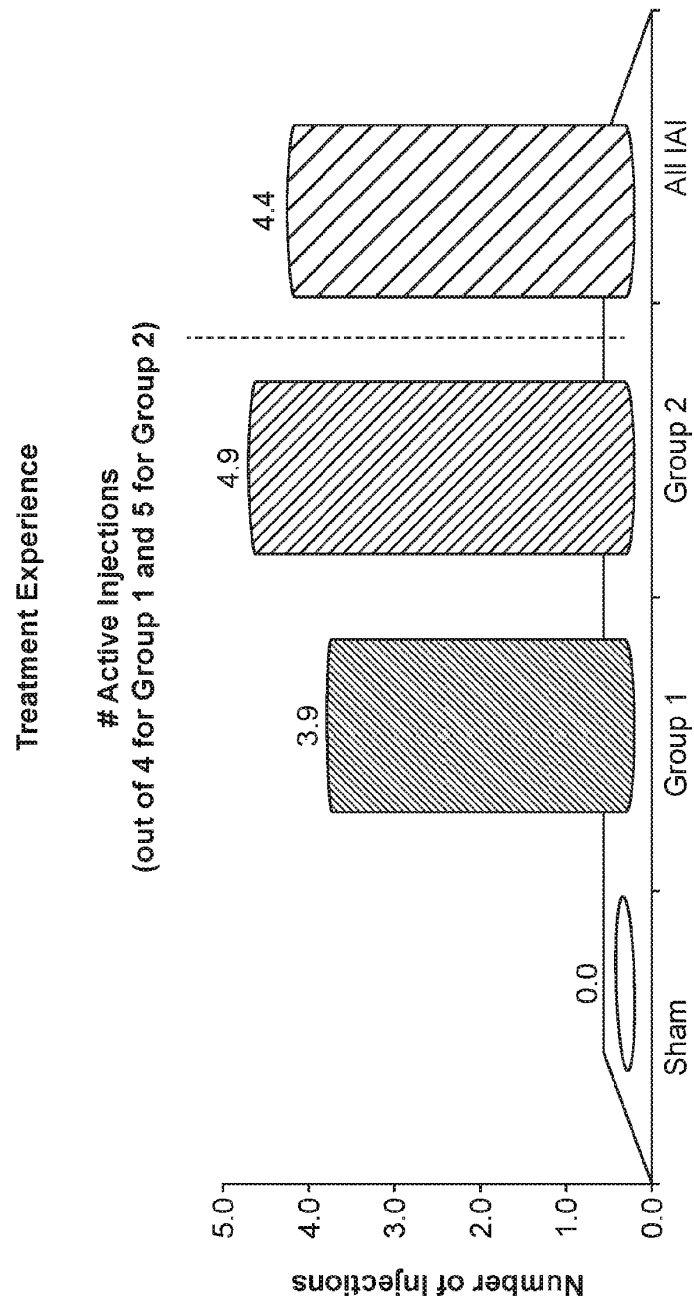
FIG. 5

Baseline Disease Characteristics

	— Sham	---- Group 1	— Group 2	--- All IAI	— Total
N (FAS/SAF)	133	135	134	269	402
ETDRS BCVA (letters) Mean (SD) Snellen Equivalent	82.7 (6.03) 20/25	82.2 (6.63) 20/25	82.3 (5.15) 20/25	82.3 (5.93) 20/25	82.4 (5.96) 20/25
CRT(microns) Mean (SD)	249.4 (38.41)	246.0 (34.34)	246.8 (31.59)	246.4 (32.94)	247.4 (34.82)
Diabetic Retinopathy Severity Score (DRSS)					
Level 47	99 (74.4%)	102 (75.6%)	101 (75.4%)	203 (75.5%)	302 (75.1%)
Level 53	34 (25.6%)	33 (24.4%)	33 (24.6%)	66 (24.5%)	100 (24.9%)

Group 1: 3 monthly doses followed by 1 q8 intervals, Group 2: 5 monthly doses

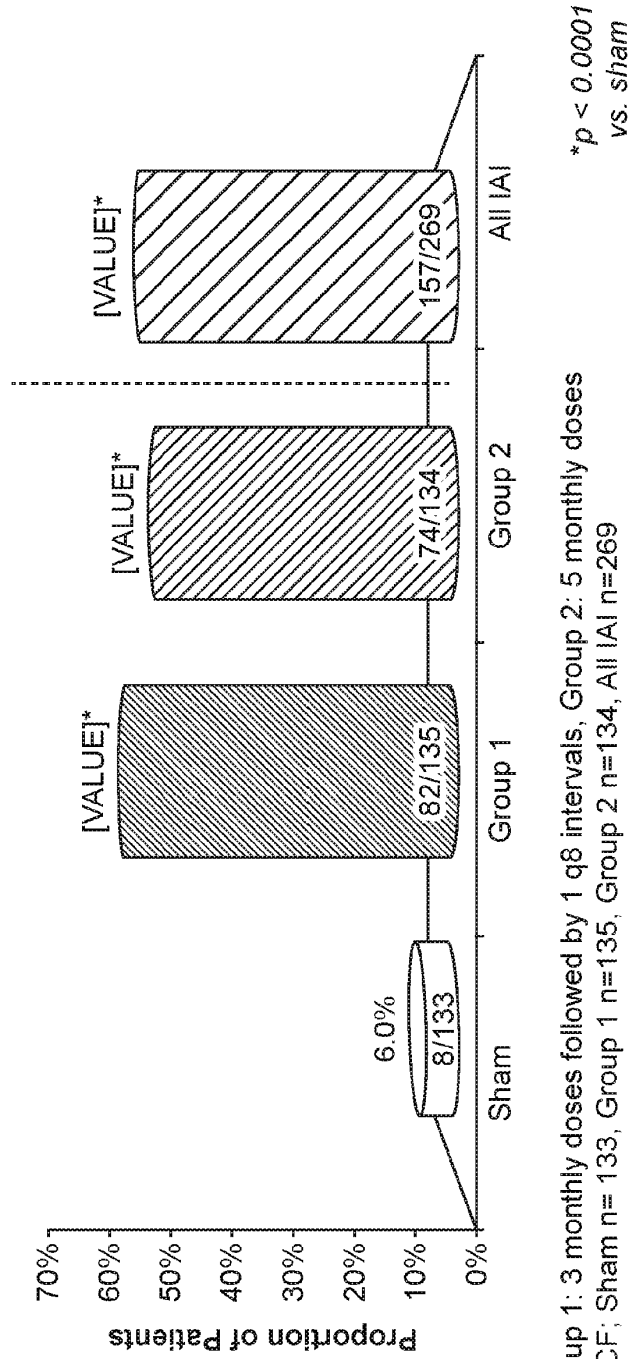
FIG. 6



Group 1: 3 monthly doses followed by 1 q8 intervals, Group 2: 5 monthly doses
Sham n= 133, Group 1 n=135, Group 2 n=134, All IAI n=269

FIG. 7

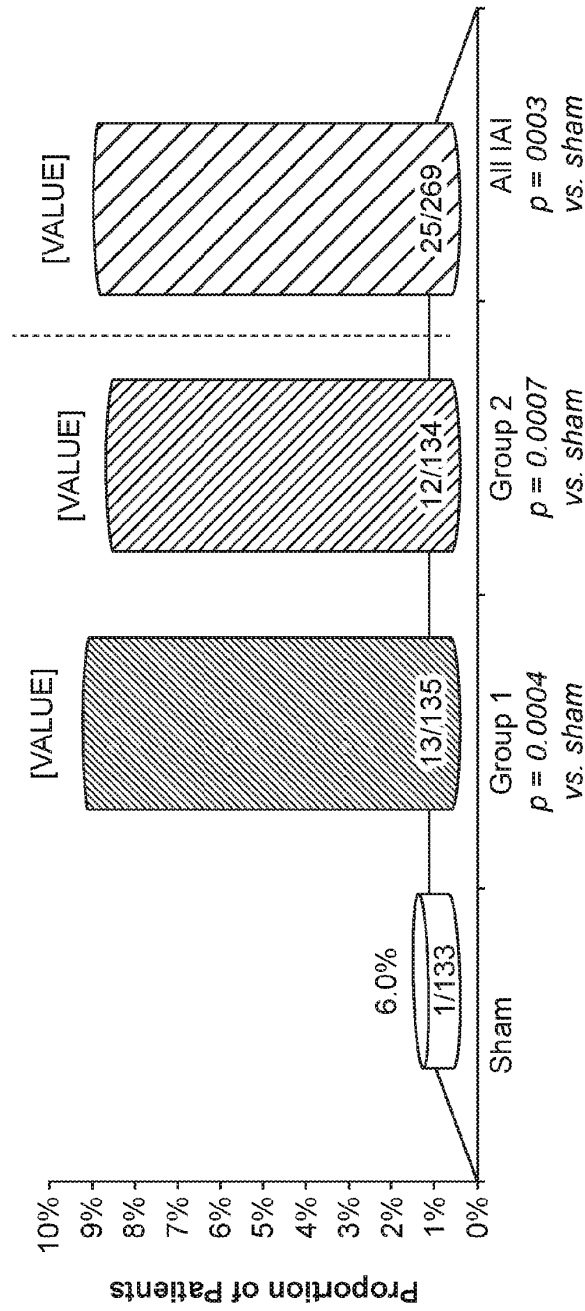
Proportion of Patients with ≥ 2 -Step Improvement from Baseline in DRSS



Group 1: 3 monthly doses followed by 1 q8 intervals, Group 2: 5 monthly doses
 LOCF; Sham n= 133, Group 1 n=135, Group 2 n=134, All IAI n=269

FIG. 8

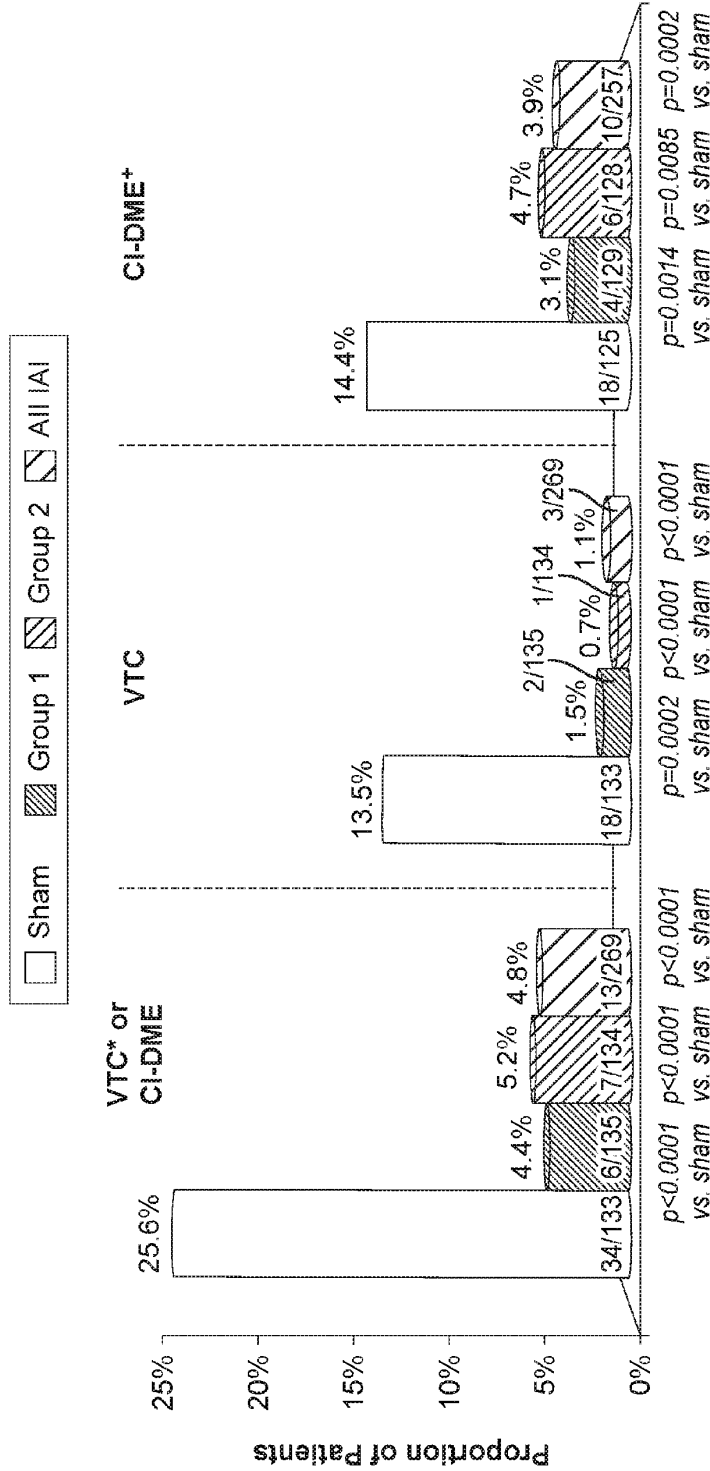
Proportion of Patients with ≥3-Step Improvement
from Baseline in DRSS



Group 1: 3 monthly doses followed by 1 q8 intervals, Group 2: 5 monthly doses
Sham n= 133, Group 1 n=135, Group 2 n=134, All IAI n=269

FIG. 9

Proportion of Patients Developing a
Vision Threatening Complication or CI-DME through Week 24



Group 1: 3 monthly doses followed by 1 q8 intervals, Group 2: 5 monthly doses
*VTC = Vision threatening complication, PDR/ASNV
+CI-DME evaluable set excludes patients who at baseline had CRT>300µm and
had qualitative evidence of CI-DME as assessed by the reading center.

FIG. 10

Mean Change in BCVA through Week 24

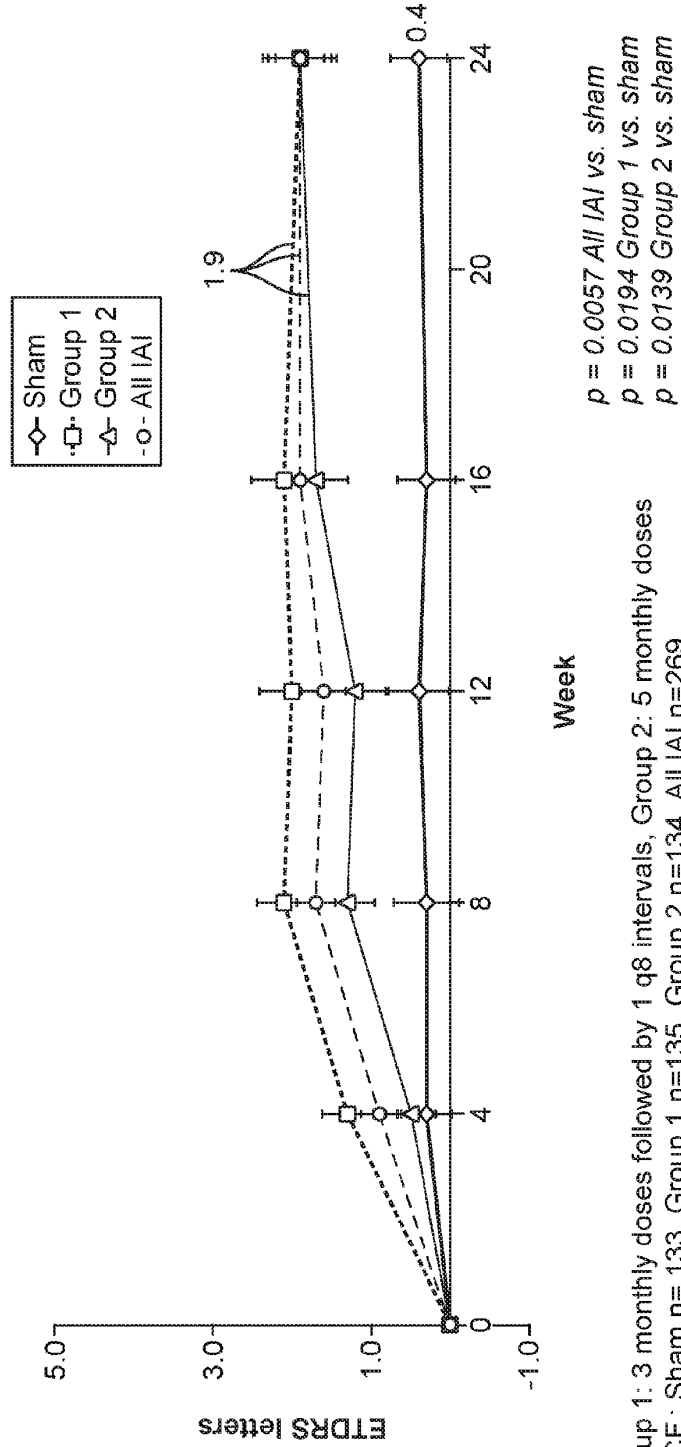
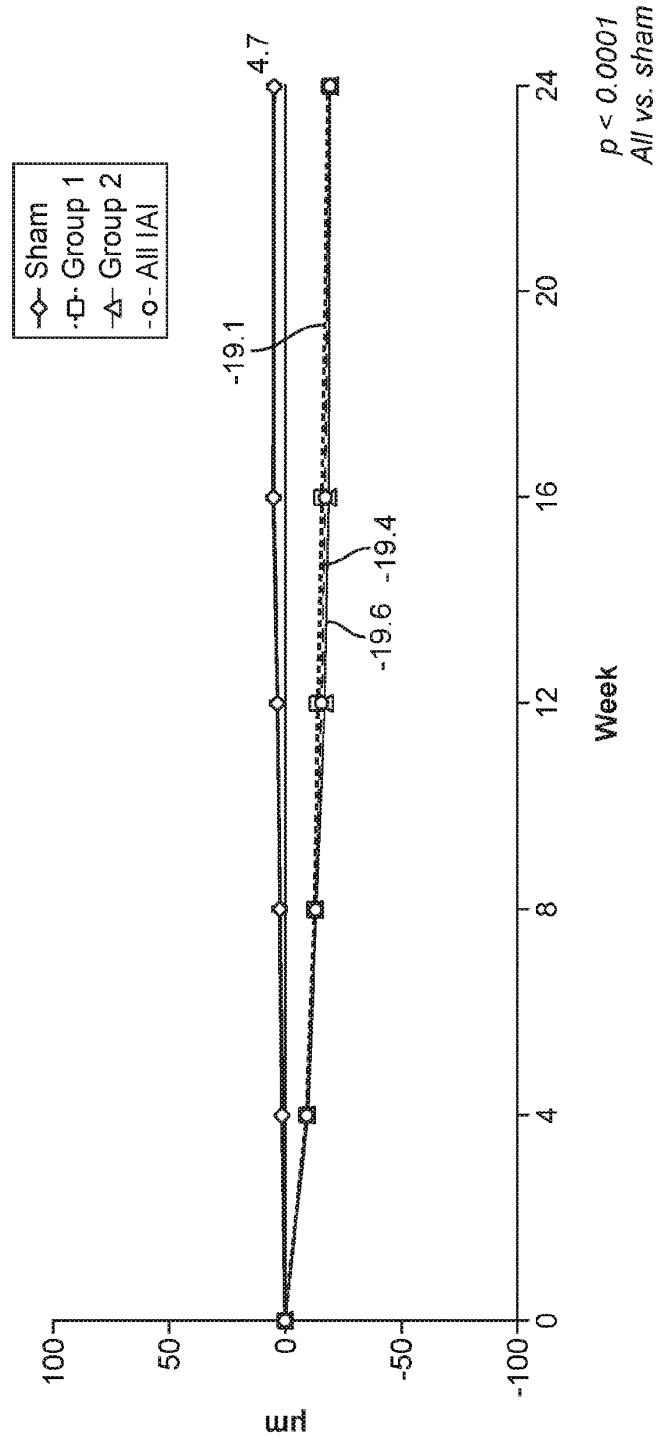


FIG. 11

Mean Change in CRT through Week 24



Group 1: 3 monthly doses followed by 1 q8 intervals, Group 2: 5 monthly doses
LOCF; Sham n= 133, Group 1 n=135, Group 2 n=134, All IAI n=269

FIG. 12

Ocular TEAEs in Study Eye through Week 24
(≥3%)

	— Sham	--- All IAI
N (FAS/SAF)	133	269
Number of Patients ≥ 1 AE, n (%)	44 (33.1%)	77 (28.6%)
Eye disorders	42 (31.6%)	76 (28.3%)
Conjunctival haemorrhage	4 (3.0%)	32 (11.9%)
Vitreous floaters	1 (0.8%)	14 (5.2%)
Diabetic retinal oedema	18 (13.5%)	11 (4.1%)
Eye pain	2 (1.5%)	11 (4.1%)
Diabetic retinopathy	4 (3.0%)	1 (0.4%)

FIG. 13

Ocular Serious TEAEs in Study Eye through Week 24

	— Sham	--- All IA
N (FAS/SAF)	133	269
Number of Patients with ≥ 1 AE, n (%)	1 (0.8%)	0
Iris neovascularisation	1 (0.8%)	0

FIG. 14

Ocular Inflammation in Study Eye through Week 24

	— Sham	--- All IAI
N (FAS/SAF)	133	269
# of injection	0	1182
Vitreous cells	0	1 (0.4%) (0.08% per injection)

FIG. 15

APTC Events through Week 24

	— Sham	--- All AI
N (FAS/SAF)	133	269
Number of Patients with ≥ 1 AE, n (%)	2 (1.5%)	1 (0.4%)
Non Fatal Stroke	0	1 (0.4%)
Cerebrovascular accident	0	1 (0.4%)
Vascular Death	2 (1.5%)	0
Cardiac arrest	1 (0.8%)	0
Myocardial infarction	1 (0.8%)	0

FIG. 16

Deaths through Week 24

Treatment Group	Patient Age /Sex /Race	Days from First Treatment to Death	Days from Last Treatment to Death	AE Preferred Term with Fatal Outcome	Rescue/ FE Treatment
Sham	43/F/W	52	23	Acute respiratory failure Pulmonary hypertension	No rescue/ No FE treatment
Sham	65/M/W	73	13	Cardiac arrest	No rescue/ No FE treatment
Sham	73/M/W	86	4	Myocardial infarction	No rescue/ 2 FE treatments

FIG. 17

**International Clinical Diabetic Retinopathy Disease
Severity Scale Detailed Table**

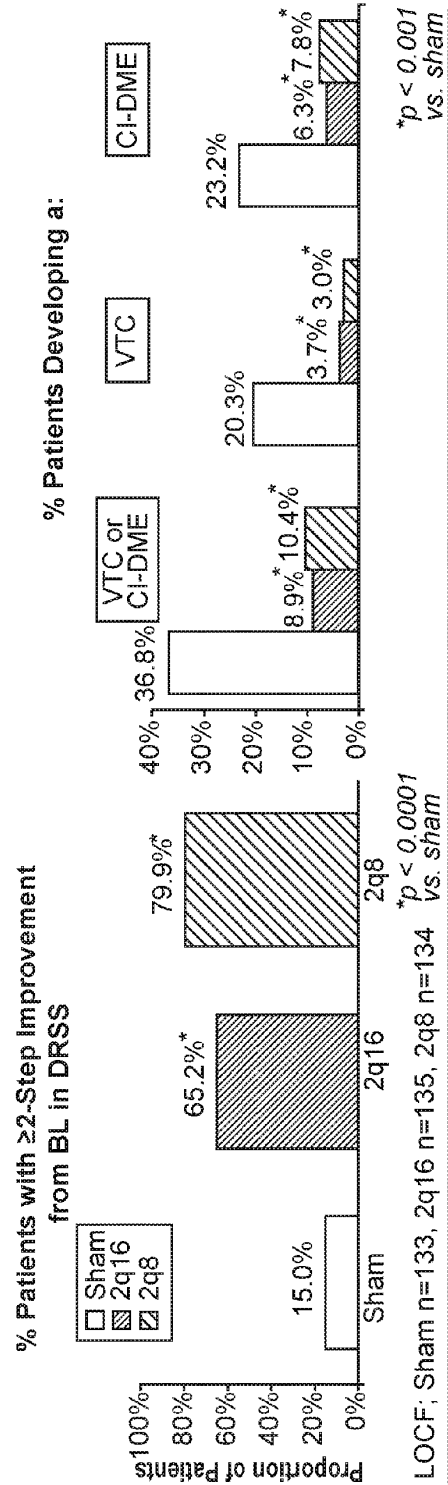
LEVEL	SEVERITY
10	DR absent
20	Microaneurysms only
35	Mild NPDR
43	Moderate NPDR
47	Moderately severe NPDR
53	Severe NPDR
61	Mild PDR
65	Moderate PDR
71	High Risk PDR
75	High Risk PDR
81	Advanced PDR, fundus partially obscured, center of macula attached
85	Advanced PDR, posterior fundus obscured or center of macula detached
90	Cannot grade, even sufficiently for level 81 or 85

ETDRS Group, #12 1991

DRSS grading scale was derived from the ETDRS and is used to grade severity of DR and describe the change in severity over time.

FIG. 18

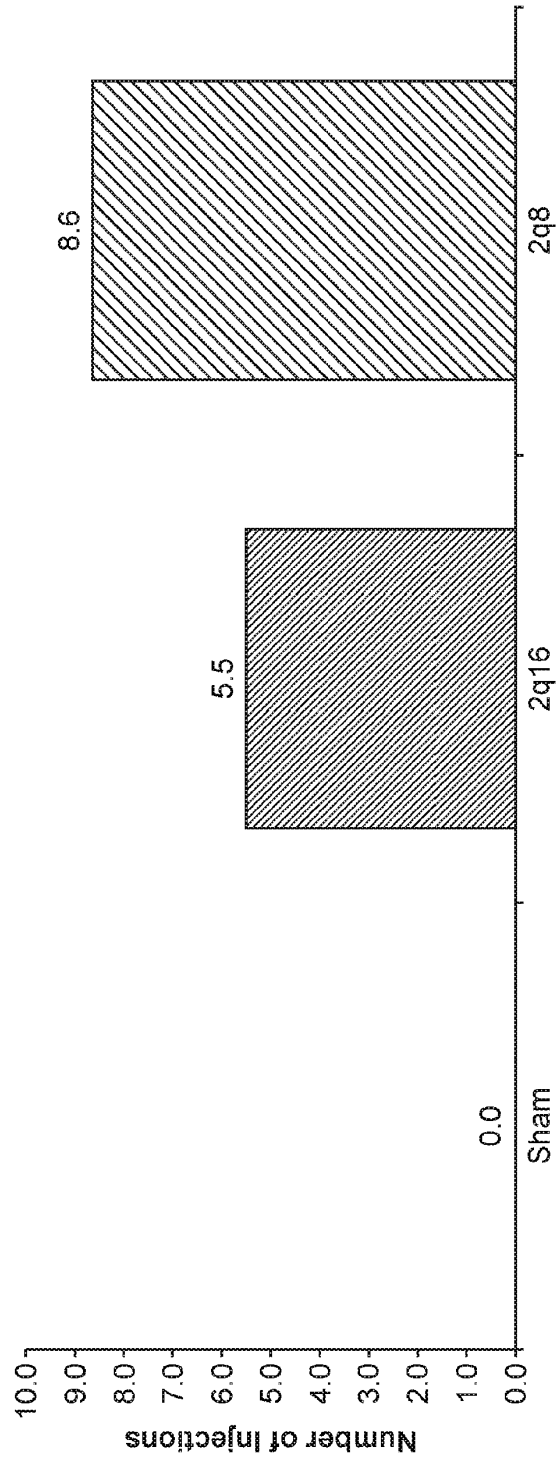
PANORAMA 52 Week Results



- The proportion of patients with ≥ 2 -step DRSS improvement was significantly greater with aflibercept vs sham
 - Rate was greater in 2q8 group compared with 2q16
- Vision threatening complications (PDR/ASNV) and CI-DME occurred in a substantially greater proportion of sham patients
 - Rates were similar between the 2q16 and 2q8 treatment groups
- No new safety signals identified

FIG. 19

Treatment Experience through Week 52
Active Injections
(out of 6 for 2q16 and 9 for 2q8)



Sham n=133, 2q16 n=135, 2q8 n=134

FIG. 20

Proportion of Patients with ≥ 2 -Step Improvement
from Baseline in DRSS at Week 52

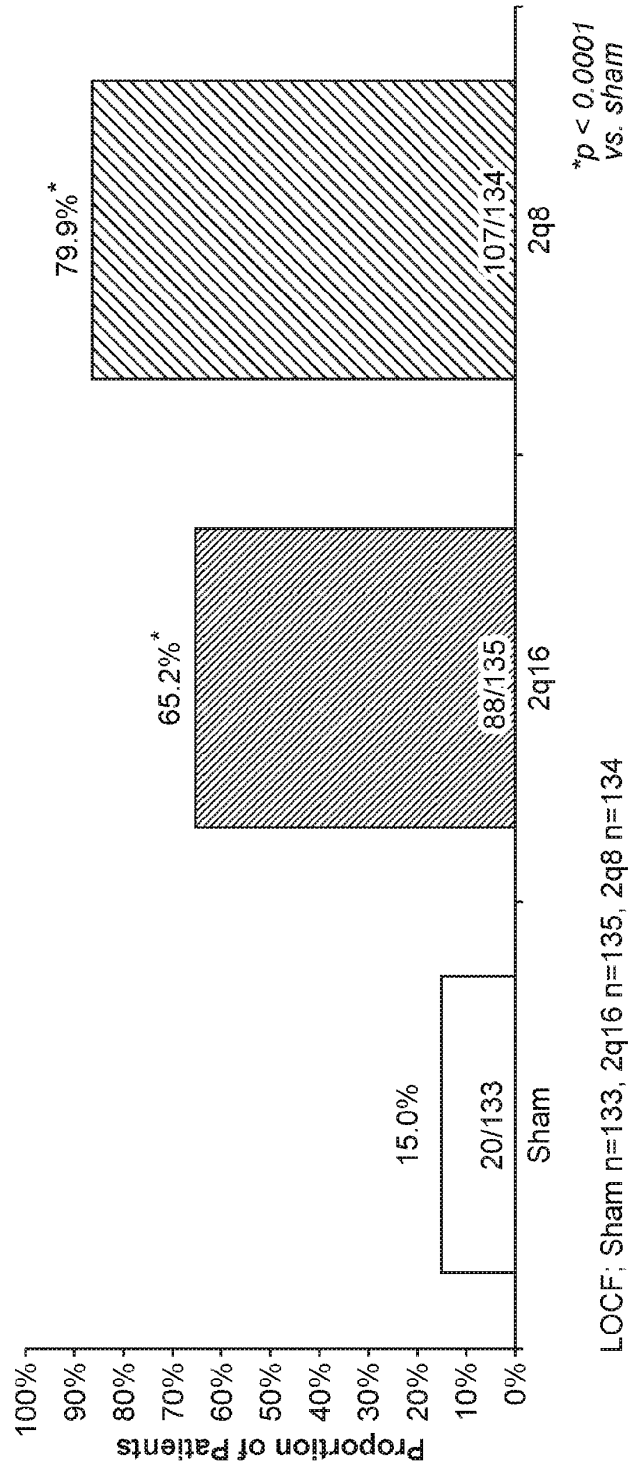
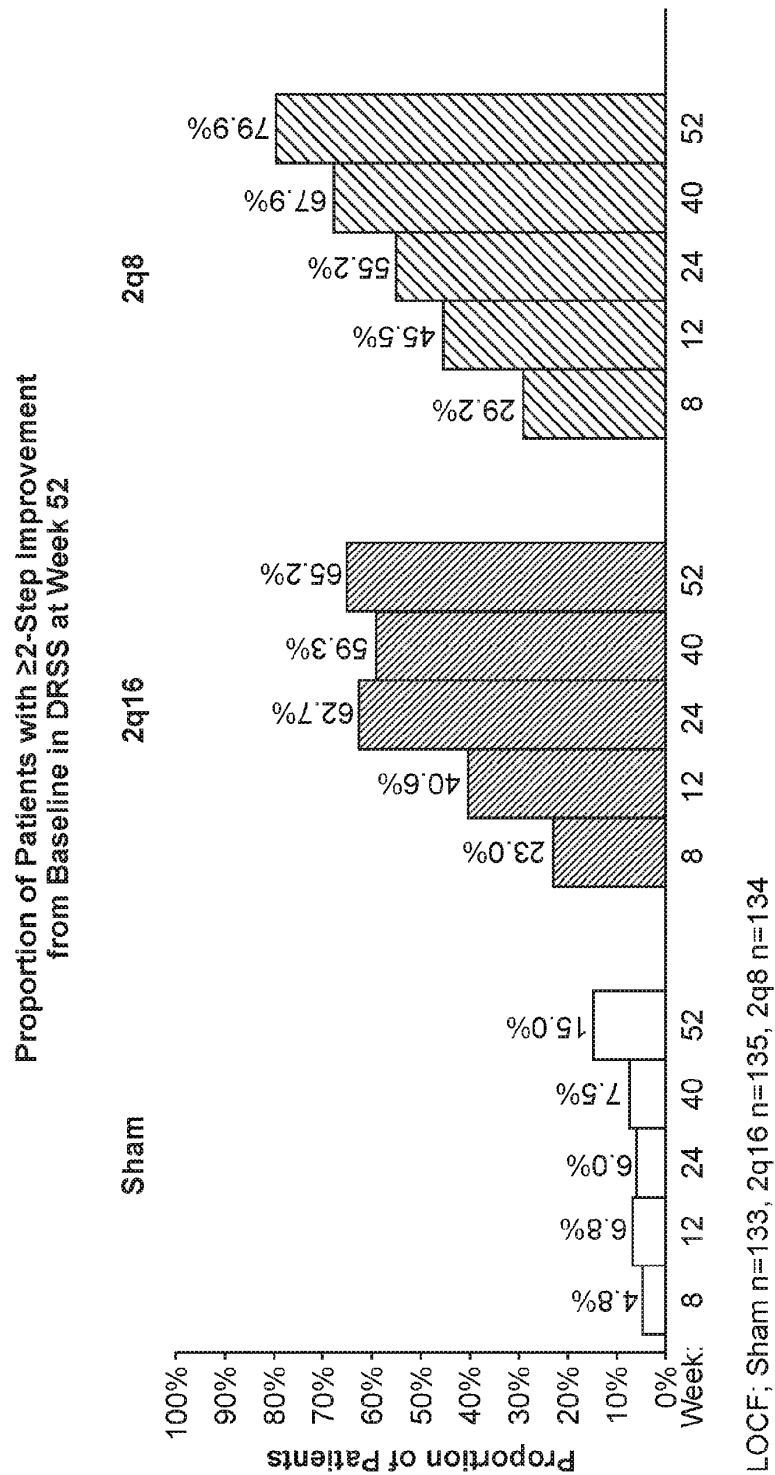


FIG. 21



Proportion of Patients Developing a Vision Threatening Complication (VTC) or Center Involved (CI)-DME through Week 52

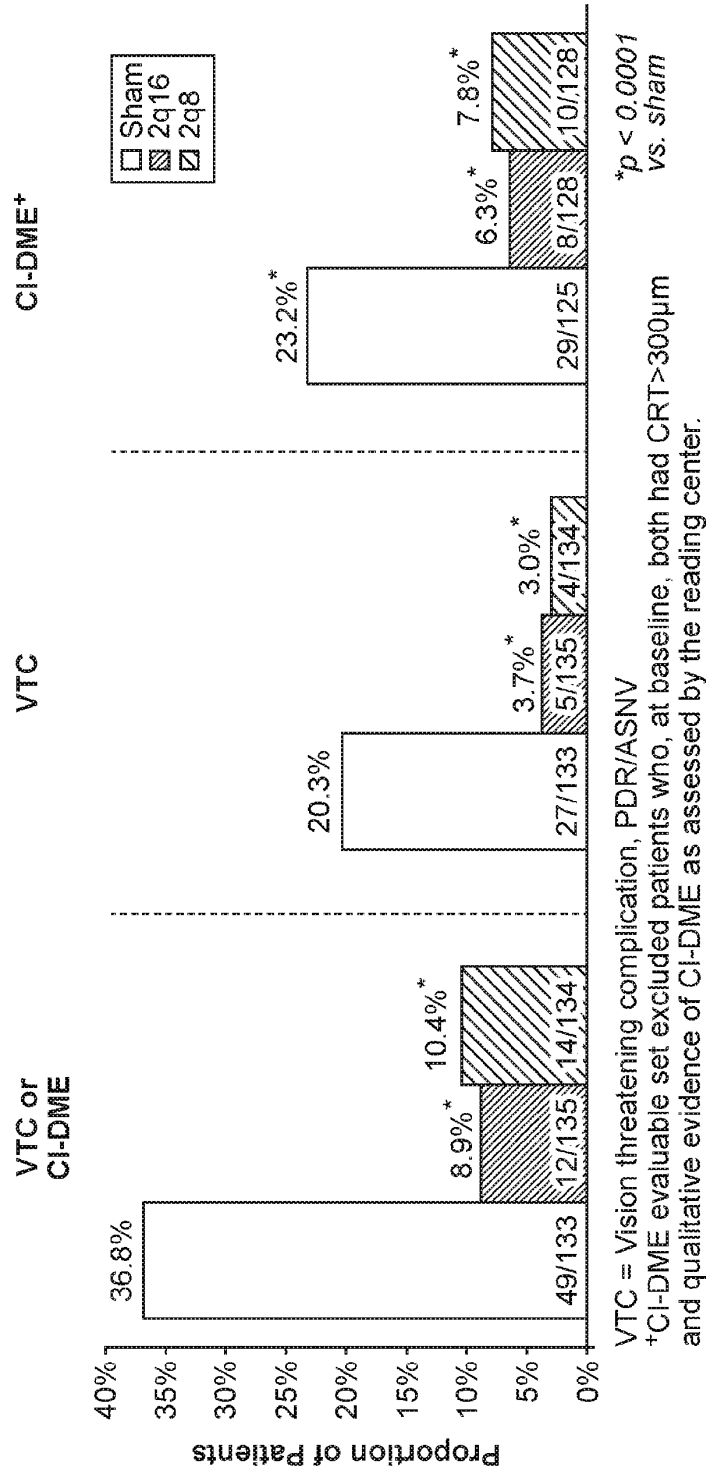
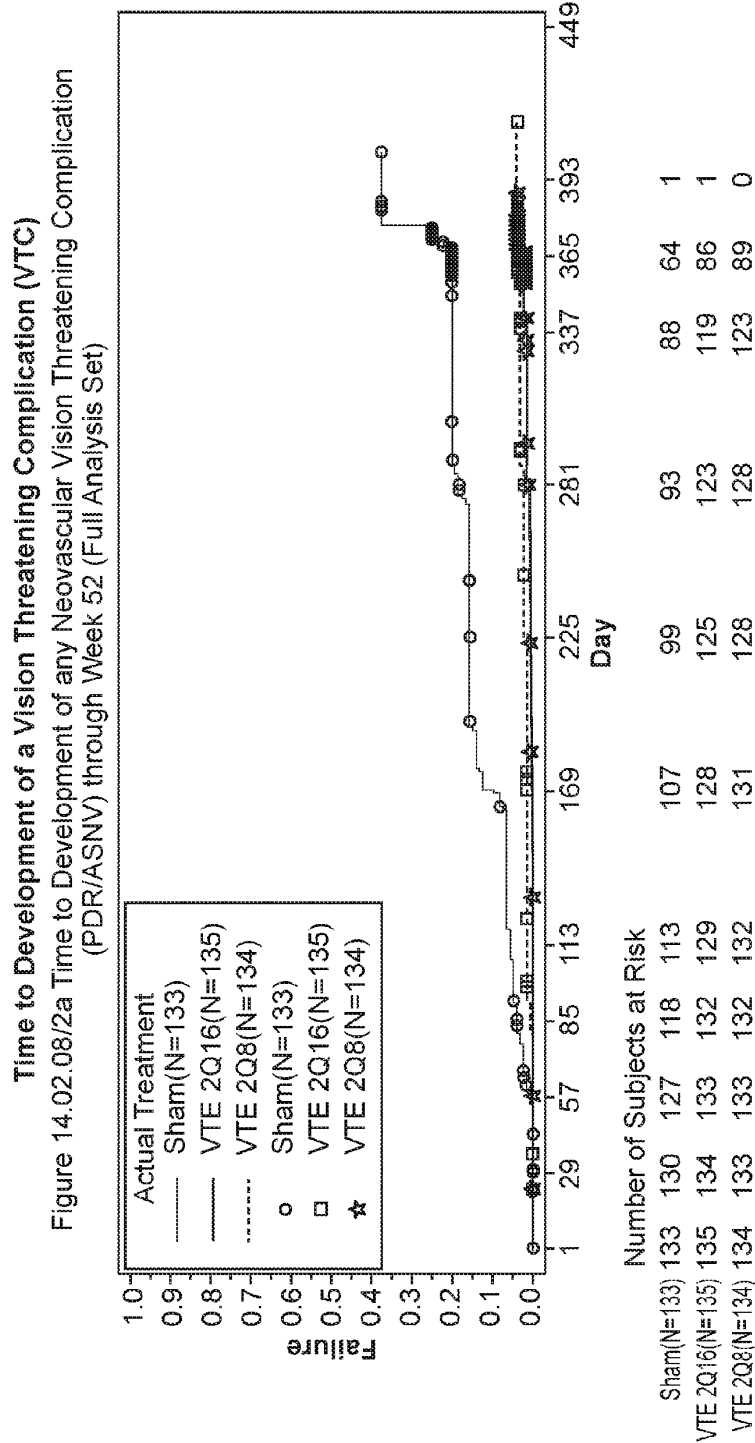
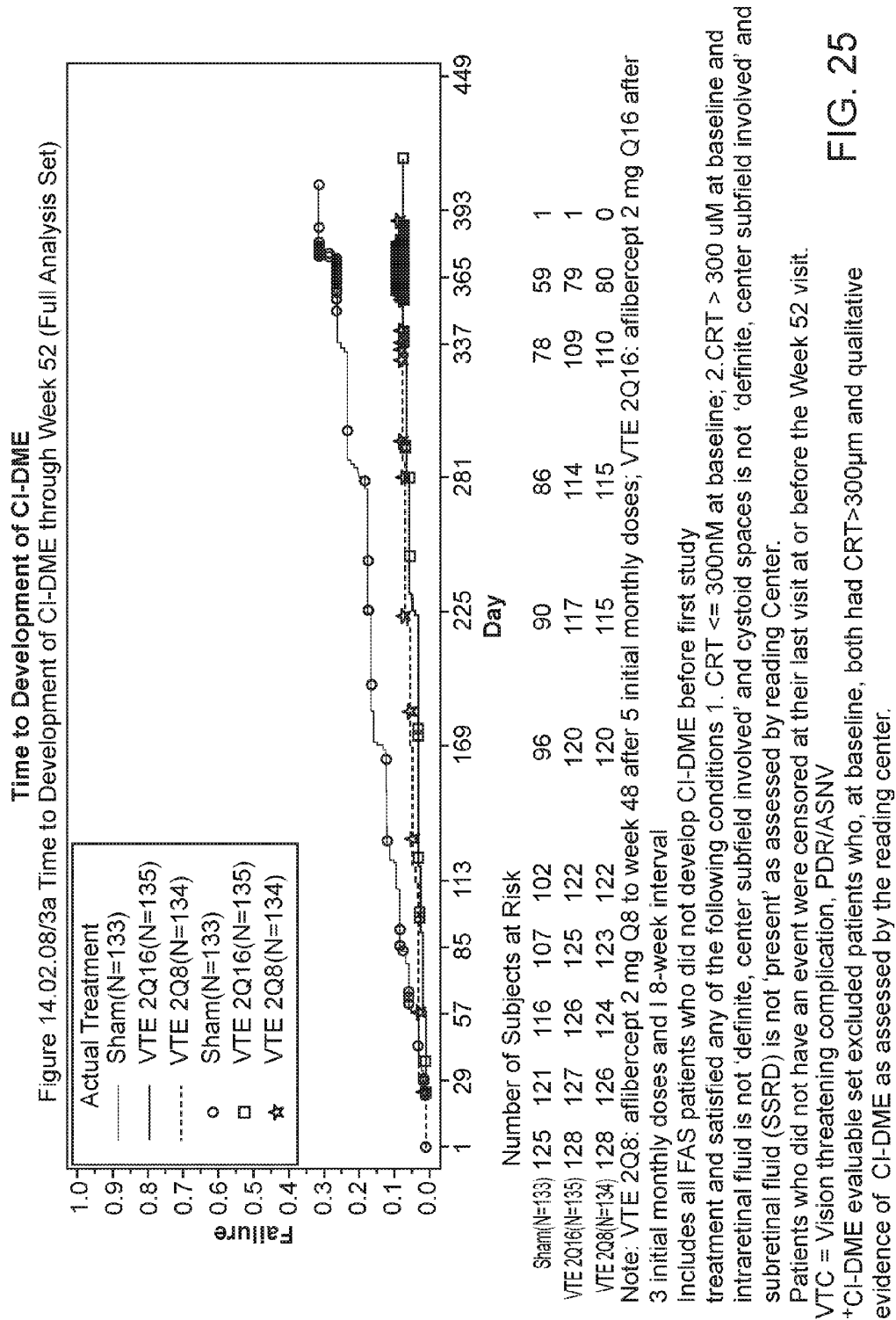


FIG. 23



Note: VTE 2Q8: aflibercept 2 mg Q8 to week 48 after 5 initial monthly doses; VTE 2Q16: aflibercept 2 mg Q16 after 3 initial monthly doses and 1 8-week interval
Patients who did not have an event were censored at their last visit at or before the Week 52 visit.
VTC = Vision threatening complication, PDR/ASNV
+CI-DME evaluable set excluded patients who, at baseline, both had CRT>300µm and qualitative evidence of CI-DME as assessed by the reading center.

FIG. 24



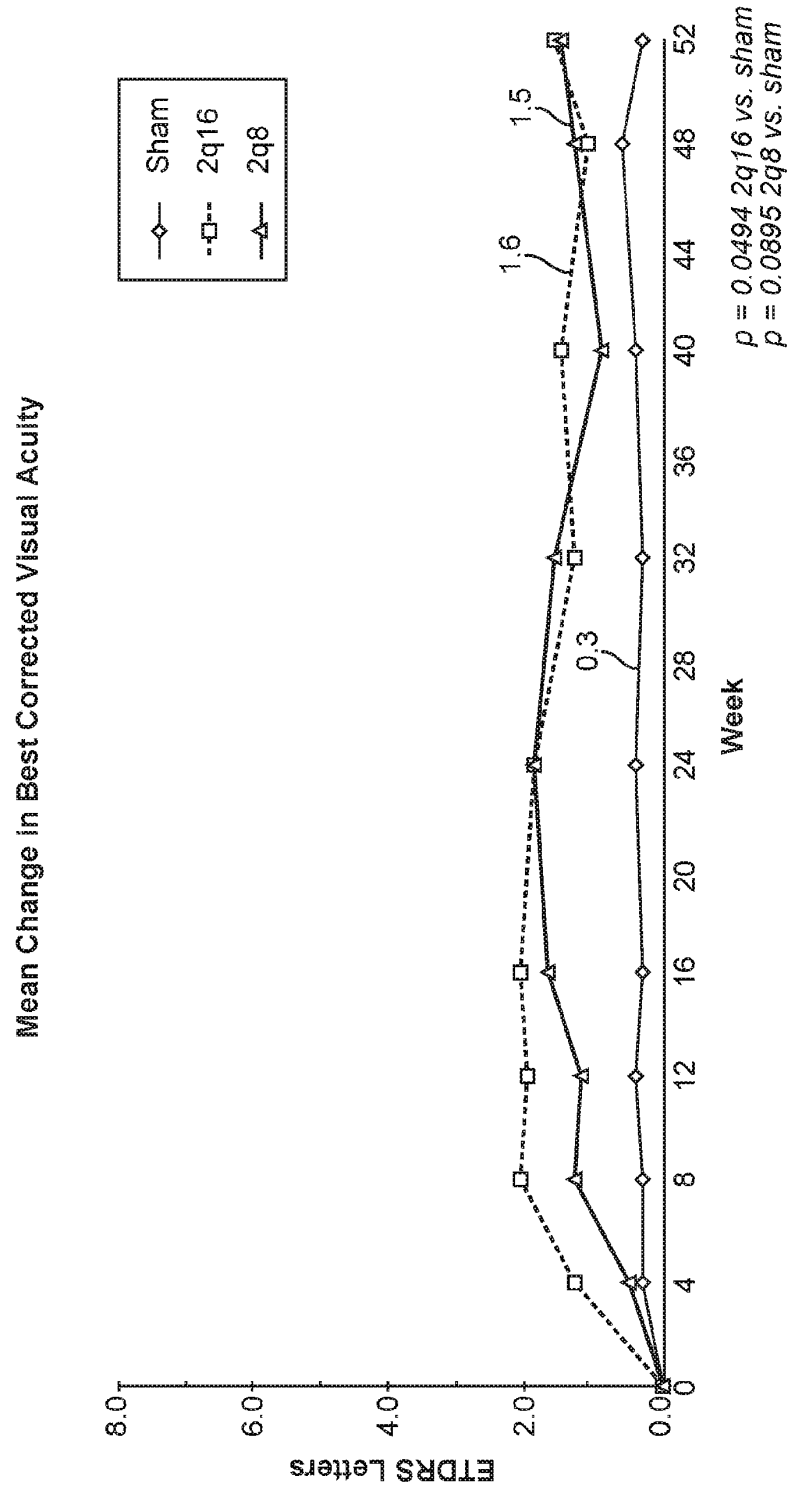


FIG. 26

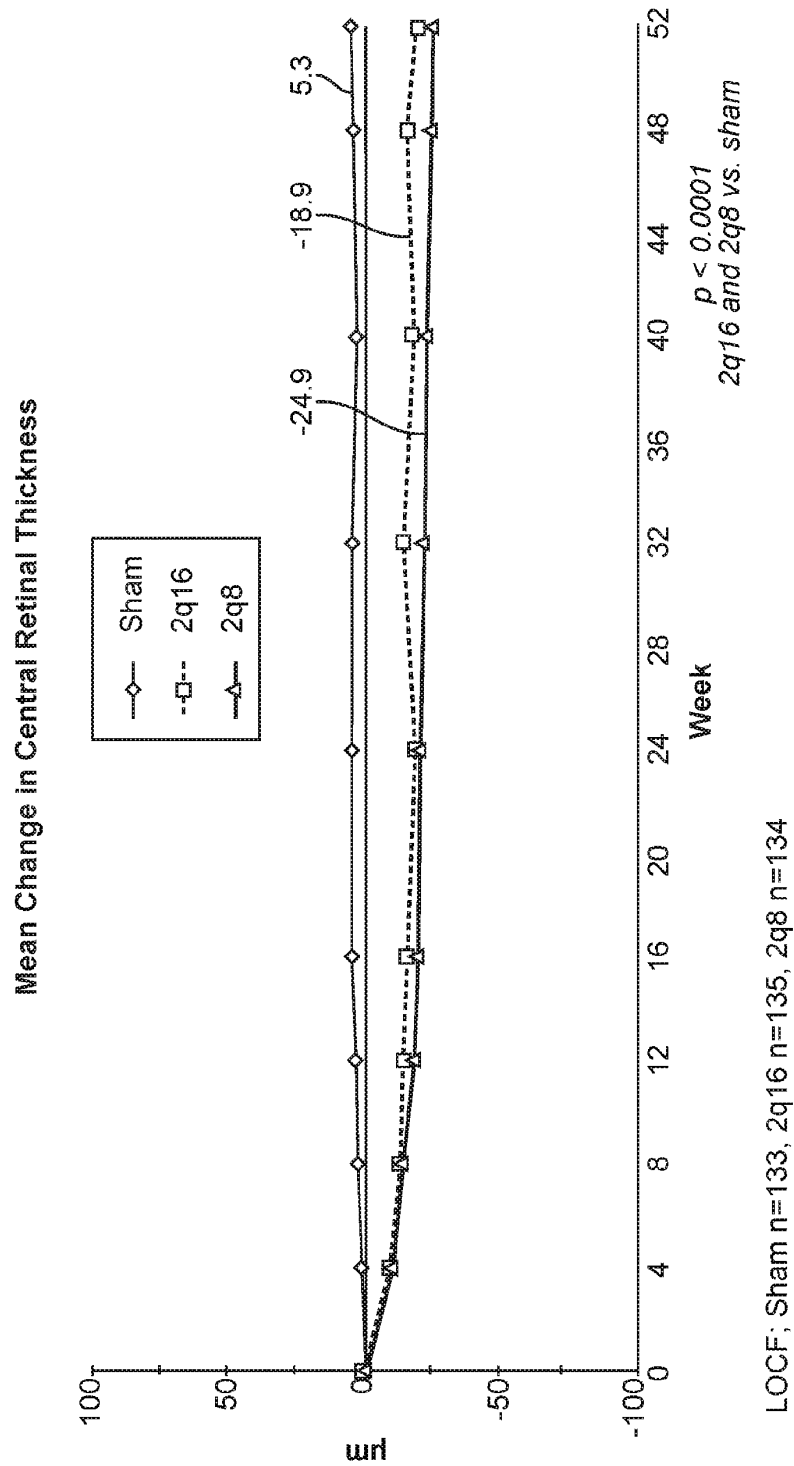


FIG. 27

Ocular TEAEs in Study Eye through Week 52 (≥3%)

	— Sham	---- 2q16	— 2q8
N (FAS/SAF)	133	135	134
Number of Patients ≥ 1 AE, n (%)	67 (50.4%)	58 (43.0%)	60 (44.8%)
Eye disorders	64 (48.1%)	57 (42.2%)	59 (44.0%)
Conjunctival haemorrhage	7 (5.3%)	16 (11.9%)	23 (17.2%)
Diabetic retinal oedema	32 (24.1%)	8 (5.9%)	12 (9.0%)
Vitreous floaters	3 (2.3%)	6 (4.4%)	12 (9.0%)
Eye pain	4 (3.0%)	10 (7.4%)	5 (3.7%)
Retinal exudates	5 (3.8%)	5 (3.7%)	7 (5.2%)
Blepharitis	1 (0.8%)	2 (1.5%)	6 (4.5%)
Vitreous detachment	1 (0.8%)	4 (3.0%)	4 (3.0%)
Cataract	1 (0.8%)	3 (2.2%)	4 (3.0%)
Dry eye	4 (3.0%)	3 (2.2%)	4 (3.0%)
Diabetic retinopathy	13 (9.8%)	2 (1.5%)	3 (2.2%)
Visual impairment	0	1 (0.7%)	4 (3.0%)

FIG. 28

Ocular Serious TEAEs in Study Eye through Week 52

	— Sham	---- 2q16	— 2q8
N (FAS/SAF)	133	135	134
# of Patients with ≥ 1 AE, n (%)	1 (0.8%)	0	1 (0.7%)
Visual acuity reduced	0	0	1 (0.7%)
Vitreous haemorrhage	0	0	1 (0.7%)
Iris neovascularisation	1 (0.8%)	0	0

FIG. 29

Ocular Inflammation in Study Eye through Week 52

<div>— Sham<div>---- 2q16</div><div>— 2q8</div></div>		
N (FAS/SAF)	133	134
# of injections	0	1158
# of Patients with ≥ 1 AE, n (%)	0	1 (0.7%) (0.09% per injection)
Anterior chamber flare	0	1 (0.7%)
Iritis	0	0

1 additional event of vitreal cells was included in the IOI table, but it was determined not to be an IOI event

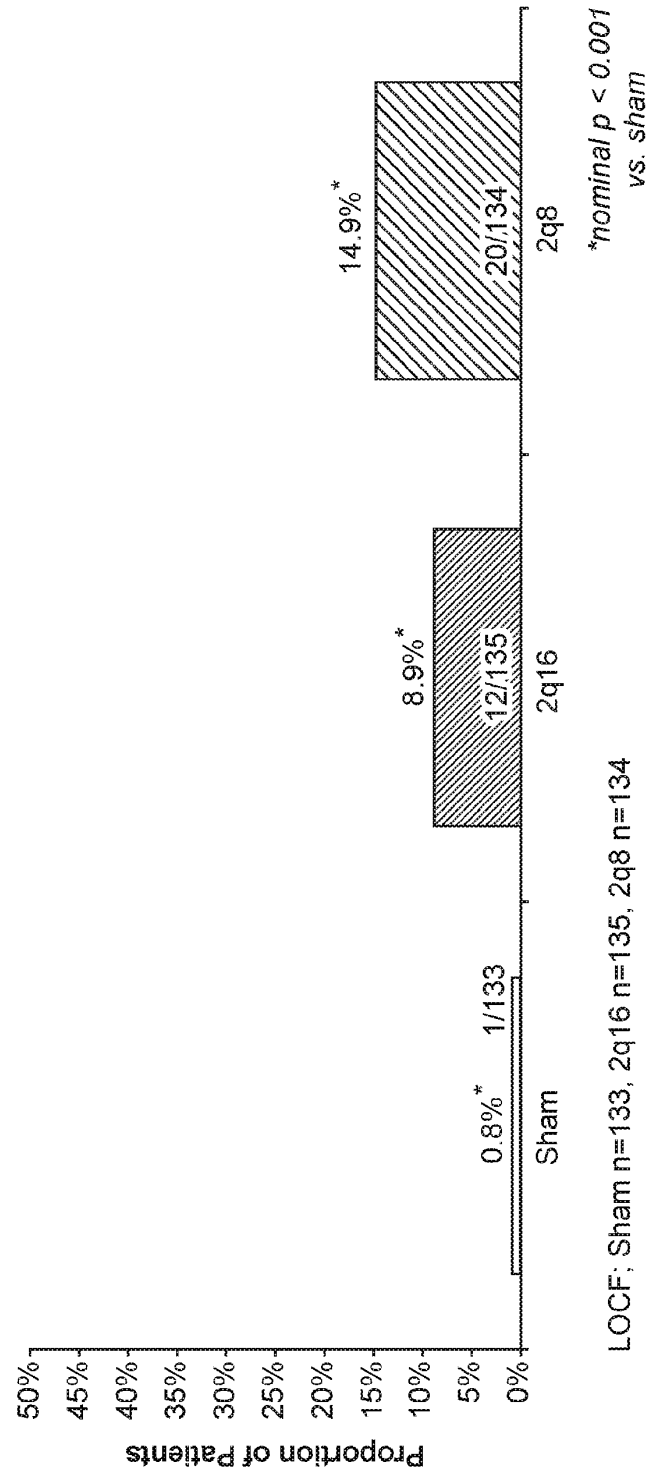
FIG. 30

APTC Events through Week 52

	— Sham	----- 2q16	— 2q8
N (FAS/SAF)	133	135	134
Number of Patients with at Least One Such AE, n (%)	5 (3.8%)	4 (3.0%)	2 (1.5%)
Non Fatal Stroke	2 (1.5%)	3 (2.2%)	1 (0.7%)
Non Fatal MI	0	1 (0.7%)	0
Vascular Death	3 (2.3%)	0	1 (0.7%)

FIG. 31

Proportion of Patients with ≥ 3 -Step Improvement
from Baseline in DRSS at Week 52



LOCF; Sham n=133, 2q16 n=135, 2q8 n=134

FIG. 32

REFERENCES CITED IN THE DESCRIPTION

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(54) **PHARMACEUTICAL COMPOSITION CONTAINING ELEMENE, PREPARATION METHOD THEREFOR, AND USE THEREOF**

(57) The application refers to a pharmaceutical composition, containing elemene, a protein carrier, and oil for injection, having improved safety and stability.

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Description**FIELD OF THE INVENTION**

5 **[0001]** The present invention relates to the field of pharmaceutical formulations, in particular, to a pharmaceutical composition comprising elemene and preparation methods and use thereof.

BACKGROUND OF THE INVENTION

10 **[0002]** Elemene is a volatile oily compound extracted from plants such as *Curcuma Wenyujin* Y. H. Chen et C. ling and *Cymbopogon citratus* ((DC.)) Stapf. This compound appears as a pale yellow or yellow clear liquid with a pungent fennel smell. Currently, elemene is clinically used as a mixture containing α -elemene, β -elemene, δ -elemene and γ -elemene, which is widely used in malignant pleural effusion, lung cancer, gastrointestinal tumors and other superficial tumors. Since the elemene available in market and its formulations contain Cremophor EL and other excipients are highly irritating, most patients will suffer from serious phlebitis, fever, local pain, allergic reaction or mild digestive tract reaction, as well as pyrexia and other adverse reactions after the use of the drugs available in market. In the actual clinical medication process, due to the high incidence of phlebitis and higher recurrence rate thereof, the speed of intravenous infusion should be strictly controlled, resulting in a long infusion time.

15 **[0003]** Elemene is almost insoluble in water and has strong lipid solubility, especially in organic solvents such as petroleum ether, diethyl ether and the like. Its molecular formula is $C_{15}H_{24}$ and only consists of hydrocarbons. Due to its unique chemical structure and physicochemical properties, there are great difficulties and obstacles in its preparation of pharmaceutical formulations. In order to increase the solubility of elemene, the Cremophor EL and other excipients were added in drug prescription available in market during the preparation of elemene into injection, resulting in problems of serious hemolysis and great irritation. In addition, although the marketing of paclitaxel albumin nano-formulation provides a new form of pharmaceutical formulation for solving clinical side effects, due to the physicochemical properties of elemene, which is oily at room temperature, it is extremely challenging to formulate elemene into albumin formulations. Therefore, there is currently no corresponding research or development.

SUMMARY OF THE INVENTION

30 **[0004]** The present application provides a pharmaceutical composition comprising elemene, an oil for injection and a protein carrier, which is able to effectively reduce one or more side effects of pharmaceutical compositions administered into the human body.

35 **[0005]** In one or more embodiments of the present application, the protein carrier used in the pharmaceutical composition includes a protein, and any suitable protein may be used. Examples of suitable protein include, but are not limited to, albumin, immunoglobulin, including but not limited to IgA, lipoprotein, apolipoprotein B, alpha-acid glycoprotein, beta-2-macroglobulin, thyroglobulin, transferrin protein, fibronectin, factor VII, factor VIII, factor IX, factor X and analogues. The protein carrier may be natural or synthetic. In some embodiments, the protein carrier is a non-blood protein such as casein, alpha-lactalbumin, and beta-lactoglobulin. In some embodiments, the protein carrier includes albumin, such as human serum albumin (HSA), bovine serum albumin, and the like. Human serum albumin is a highly soluble globulin, Mr65K, composed of 585 amino acids. HSA is the most abundant protein in plasma and constitutes 70-80% of the colloidal osmotic pressure in human plasma.

40 **[0006]** In one or more embodiments of the present application, the "oil for injection" used in the pharmaceutical composition includes, but are not limited to, ethyl oleate, benzyl benzoate, medium chain triglyceride (MCT) and vegetable oil, among which the vegetable oil is selected from one or more of soybean oil, safflower oil, cottonseed oil, corn oil, sunflower oil, peanut oil, and olive oil. In one or more embodiments of the present application, the oil for injection is soybean oil.

45 **[0007]** In one or more embodiments of the present application, the pharmaceutical composition may be formulated into particles, wherein the particle size of the particles is less than 180 nm, for example, about 70-170 nm, preferably about 70-150 nm.

50 **[0008]** In one or more embodiments of the present application, the composition comprises nanoparticles of any shape (for instance, spherical or non-spherical shape), and in some embodiments, the average or intermediate diameter of the particles is no greater than about 180 nm. In some embodiments, the average or intermediate diameter of the particles is from about 50 nm to about 180 nm. In some embodiments, the average or intermediate diameter of the particles is from about 60 nm to about 180 nm. In some embodiments, the average or intermediate diameter of the particles is from about 70 nm to about 170 nm. In some embodiments, the average or intermediate diameter of the particles is from about 70 nm to about 170 nm. In some embodiments, the average or intermediate diameter of the particles is from about 70 nm to about 170 nm. In some embodiments, the average or intermediate diameter of the particles is from about 70 nm to about 170 nm. In some embodiments, the average or intermediate diameter of the particles is about 60 nm, 70 nm,

80 nm, 90 nm, 100 nm, 110 nm, 120 nm, 130 nm, 140 nm, or 150 nm. In some implementations, the particles are sterile and filterable.

[0009] In one or more embodiments of the present application, the elemene in the pharmaceutical composition is selected from one or more of α -elemene, β -elemene, γ -elemene and δ -elemene. In one or more embodiments of the present application, the elemene in the pharmaceutical composition is β -elemene.

[0010] In one or more embodiments of the present application, the ratio of the albumin carrier, the elemene and the oil for injection in the pharmaceutical composition is the weight ratio of the feed.

[0011] In one or more embodiments of the present application, the weight ratio of the protein carrier to the elemene in the pharmaceutical composition is 0.520; for example, 0.5-10.

[0012] In one or more embodiments of the present application, the weight ratio of the oil for injection to the elemene in the pharmaceutical composition is 0.3510; for example, 0.5 to 10.

[0013] In one or more embodiments of the present application, the pharmaceutical composition further contains a lyoprotectant. The lyoprotectant is selected from one or more of glucose, sucrose, maltose, lactose, mannose, trehalose, glycine and dextran, preferably trehalose or sucrose. In one or more embodiments, wherein the ratio of the weight of the lyoprotectant to the volume of the solution of the pharmaceutical composition is 3:100-20:100 g/mL, preferably 5:100-10:100 g/mL.

[0014] In one or more embodiments of the present application, the pharmaceutical composition further contains one or more of isoosmotic adjusting agent, antioxidant, preservative, and pH regulator. The isoosmotic adjusting agent is one or more of glycerol, sorbitol, mannitol or glucose; the pH regulator is one or more of sodium hydroxide, sodium citrate, citric acid, phosphoric acid, acetic acid or hydrochloric acid; the preservative is one or more of hydroxybenzene alkyl esters, benzoic acid, sodium benzoate, sorbic acid, chlorhexidine acetate, benzalkonium bromide; the antioxidant is one or more of sodium sulfite, sodium bisulfite, sodium metabisulfite, sodium thiosulfate, ascorbic acid, butylated hydroxyanisole, 2,6-di-tert-butylated hydroxytoluene, and vitamin E.

[0015] In one or more embodiments of the present application, there is also provided a pharmaceutical composition that no organic solvents is used during the preparation process thereof.

[0016] In one or more embodiments of the present application, the pharmaceutical composition may be formulated into particles, wherein the particle size of the particles is less than 180 nm, for example, about 70-150 nm.

[0017] In one or more embodiments of the present application, wherein in the pharmaceutical composition, the weight ratio of the soybean oil to the elemene is about 0.35-3, and the weight ratio of the human serum albumin to the elemene is about 0.5-20; preferably, the weight ratio of the soybean oil to the elemene is about 0.5-3, the weight ratio of the human serum albumin to the elemene is about 0.5-2.5; or in the pharmaceutical composition, the weight ratio of the soybean oil to the elemene is about 0.35-1.5, and the weight ratio of the human serum albumin to the elemene is about 1.5-10.

[0018] In one or more embodiments of the present application, the weight ratio of soybean oil to elemene is about 0.5, and the weight ratio of human serum albumin to elemene is about 1.5.

[0019] In one or more embodiments of the present application, the weight ratio of soybean oil to elemene is about 0.75, and the weight ratio of human serum albumin to elemene is about 5.

[0020] In one or more embodiments of the present application, the weight ratio of soybean oil to elemene is about 1.5, and the weight ratio of human serum albumin to elemene is about 3-5.

[0021] In one or more embodiments of the present application, there is also provided a pharmaceutical composition that contains an organic solvent during the preparation process. Wherein the organic solvent is selected from one or more of chloroform, dichloromethane, tertiary butanol, isopropanol, ethyl acetate, ethanol, tetrahydrofuran, dioxane, acetonitrile, acetone, dimethyl sulfoxide, dimethylformamide, and methylpyrrolidone. Wherein the liposoluble organic solvent is a mixed solvent of dichloromethane and ethanol.

[0022] Wherein the volume ratio of dichloromethane to ethanol is 1:1-8, preferably 1:4.

[0023] The pharmaceutical composition may be formulated into particles, and the particle size of the particles is about 70-150 nm.

[0024] In the pharmaceutical composition, the weight ratio of the soybean oil to the elemene is about 2.4-10, and the weight ratio of the human serum albumin to the elemene is about 0.5-10; preferably, in the pharmaceutical composition, the weight ratio of the soybean oil to the elemene is about 2.4, the weight ratio of the human serum albumin to the elemene is about 3.

[0025] One or more embodiments of the present application provide a method for preparing the pharmaceutical composition of the present application, which comprises the following steps:

- (1) mixing elemene and an oil for injection evenly, optionally adding an organic solvent, to obtain a solution of the elemene and the oil for injection; dissolving a protein carrier in water to obtain a protein carrier solution;
- (2) mixing the two solutions obtained in step (1) to form an emulsion;
- (3) homogenizing the emulsion in step (2) under a high pressure, and optionally evaporating the emulsion under a

reduced pressure to remove the organic solvent, to obtain a nanoparticle solution.

[0026] One or more embodiments of the present application provide a method for preparing the pharmaceutical composition of the present application, which comprises the following steps:

- (1) mixing elemene and an oil for injection evenly;
- (2) taking an albumin and dissolving the albumin in water;
- (3) mixing the both to form an emulsion;
- (4) homogenizing the emulsion in step (3) under a high pressure to obtain a solution of albumin nanoparticles.

[0027] In one or more embodiments of the present application, in step (2), a shearing or ultrasonic method is used for mixing; and in step (3), a method of high-pressure homogenization or micro-jet homogenization is used for homogenization.

[0028] In one or more embodiments of the present application, step (3) may further comprise: flushing the pipeline of the homogenizer with an appropriate amount of trehalose aqueous solution to replace the homogeneous solution remaining in the homogenizer, and then flushing the pipeline of the homogenizer with an appropriate amount of ultrapure water to replace the residual trehalose aqueous solution, combining the trehalose aqueous solution with the homogeneous solution, mixing evenly, and then filtering.

[0029] In one or more embodiments of the present application, wherein the shearing may be performed by shearing with a mixer.

[0030] In one or more embodiments of the present application, wherein the speed of shearing with the mixer is 5000 rpm-10,000 rpm.

[0031] In one or more embodiments of the present application, wherein the shearing time of the mixer is 1 min-20 min, preferably for example 2 min-10 min or, more preferably 1 min-10 min.

[0032] In one or more embodiments of the present application, wherein the shearing is preferably performed multiple times with the mixer, preferably more than 2 times.

[0033] In one or more embodiments of the present application, preferably the first time is shearing at 5000 rpm for 1 min, and the second time is shearing at 10,000 rpm for 5 min.

[0034] In one or more embodiments of the present application, the pressure of the high-pressure homogenization is 200-1,600 bar, preferably for example 250-1,550 bar.

[0035] In one or more embodiments of the present application, wherein the time of the high-pressure homogenization is 1-10 min, for example, preferably 2-10 min, such as further preferably 5-8 min.

[0036] In one or more embodiments of the present application, wherein the high-pressure homogenization is preferably performed multiple times, preferably for example more than 2 times.

[0037] In one or more embodiments of the present application, the pressure of the micro-jet homogenization is 20,000 psi.

[0038] In one or more embodiments of the present application, wherein the time of the micro-jet homogenization is 30 min.

[0039] One or more embodiments of the present application further provide a method for preparing the pharmaceutical composition of the present application, which uses an organic solvent in the preparation process.

[0040] In one or more embodiments of the present application, the method for preparing the pharmaceutical composition of the present application comprises the following steps:

- (1) dissolving elemene and an oil for injection in a liposoluble organic solvent;
- (2) taking an albumin and dissolving the albumin in water;
- (3) mixing the two solutions obtained in step (1) and (2) to form an emulsion;
- (4) homogenizing the emulsion in step (3);
- (5) evaporating the emulsion in step (4) under a reduced pressure to remove the organic solvent, to obtain a solution of albumin nanoparticles.

[0041] In one or more embodiments of the present application, the organic solvent in step (1) is selected from one or more of chloroform, dichloromethane, tertiary butanol, isopropanol, ethyl acetate, ethanol, tetrahydrofuran, dioxane, acetonitrile, acetone, dimethyl sulfoxide, dimethylformamide, methylpyrrolidone; for example, a mixed solvent of dichloromethane and ethanol, and the volume ratio of ethanol to dichloromethane in the mixed solvent is 1:1-8, for example 1:4.

[0042] In one or more embodiments of the present application, in the step (3), a shearing or ultrasonic method may be used for mixing; and in the step (4), a high-pressure or micro-jet method may be used for homogenization.

[0043] In one or more embodiments of the present application, wherein the shearing may be performed by shearing with a mixer.

- [0044]** In one or more embodiments of the present application, wherein the speed of shearing with the mixer is 5,000-10,000 rpm.
- [0045]** In one or more embodiments of the present application, wherein the shearing time of the mixer is 1 min-20 min, for example 2-10 min.
- 5 **[0046]** In one or more embodiments of the present application, wherein the shearing is preferably performed multiple times with the mixer, preferably more than 2 times.
- [0047]** In one or more embodiments of the present application, preferably the first time is shearing at 5,000 rpm for 1 min, and the second time is shearing at 10,000 rpm for 5 min.
- [0048]** In one or more embodiments of the present application, wherein the pressure of the high-pressure homogenization is 200-1,600 bar, for example 250-1,550 bar.
- 10 **[0049]** In one or more embodiments of the present application, wherein the time of high-pressure homogenization is 1-10 min, for example 2-10 min, such as further preferably 5-8 min.
- [0050]** In one or more embodiments of the present application, wherein the high-pressure homogenization is preferably performed multiple times, for example more than 2 times.
- 15 **[0051]** In one or more embodiments of the present application, the pressure of the micro-jet homogenization is 20,000 psi.
- [0052]** In one or more embodiments of the present application, wherein the time of the micro-jet homogenization is 30 min.
- [0053]** In one or more embodiments of the present application, the method for preparing the pharmaceutical composition of the present application further comprises the step of lyophilizing the obtained solution of albumin nanoparticles.
- 20 **[0054]** One or more embodiments of the present application further provide use of the pharmaceutical composition of the present application in a medicament for the prevention or treatment of cancer.
- [0055]** In one or more embodiments of the present application, the cancer involved in the present application includes adrenocortical carcinoma, cryptogenic myeloid metaplasia, AIDS-related cancer, anal cancer, appendiceal cancer, astrocytoma, basal cell carcinoma, cholangiocarcinoma, bladder cancer, bone cancer, glioma, ependymoma, oligodendroglioma, meningioma, craniopharyngioma, hemangioblastoma, medulloblastoma, neuroectodermal tumor, visual pathway and hypothalamic glioma and glioblastoma, bronchial adenoma, carcinoid tumor, central nervous system lymphoma, cervical cancer, colon cancer, colorectal cancer, chronic myeloproliferative disorder, endometrial cancer, ependymoma, Ewing's tumor family, eye cancer, gallbladder cancer, gastric cancer, gastrointestinal carcinoid tumor, gastrointestinal stromal tumor, germ cell carcinoma, gestational trophoblastic tumor, head and neck cancer, liver cancer, laryngeal cancer, leukemia, lip and oral cancer, lung cancer, lymphoma, medulloblastoma, melanoma, mesothelioma, metastatic neck squamous cell carcinoma, multiple endocrine neoplasia syndrome, myelodysplastic syndrome, myelodysplastic/myeloproliferative disease, carcinoma of nasal cavity and sinuses, neuroblastoma, neuroendocrine cancer, oropharyngeal cancer, nasopharyngeal cancer, brain tumor, bone metastasis cancer, intestinal cancer, esophageal cancer, ovarian cancer, pancreatic cancer, skin cancer, parathyroid carcinoma, penile carcinoma, peritoneal cancer, pharyngeal carcinoma, pheochromocytoma, pineoblastoma and supratentorial primitive neuroectodermal tumor, pituitary tumor, pleuropulmonary blastoma, lymphoma, primary central nervous system lymphoma, pulmonary lymphangiomyomatosis, rectal cancer, kidney cancer, renal pelvis and ureter cancer, rhabdomyosarcoma, salivary gland cancer, small intestine cancer, squamous epithelial cell carcinoma, testicular cancer, throat cancer, thymoma and thymic carcinoma, thyroid cancer, urethral cancer, vaginal cancer.
- 40 **[0056]** In one or more embodiments of the present application, the cancer involved in the present application includes lung cancer, liver cancer, esophageal cancer, nasopharyngeal cancer, brain tumor, bone metastasis cancer, gastric cancer, intestinal cancer, uterine cancer, cervical cancer, germ cell carcinoma, endometrial cancer, gestational trophoblastic tumor, breast cancer, skin cancer, lymphoma, leukemia, malignant melanoma.
- 45 **[0057]** In one or more embodiments of the present application, the brain tumor includes glioma, brain stem glioma, cerebellar or cerebral astrocytoma, malignant glioma, ependymoma, oligodendroglioma, meningioma, craniopharyngioma, hemangioblastoma, medulloblastoma, visual pathway and hypothalamic glioma or glioblastoma; the cerebellar or cerebral astrocytoma is fibrous cell astrocytoma or diffuse astrocytoma or anaplastic (malignant) astrocytoma.
- [0058]** In one or more embodiments of the present application, the pharmaceutical composition of the present application may be used in combination with radiotherapy and chemotherapy for lung cancer, liver cancer, esophageal cancer, nasopharyngeal cancer, brain tumor, bone metastasis and other malignant tumors to enhance the curative effect, reduce the toxic and side effects of radiotherapy and chemotherapy, and may also be used for interventional, intracavitary chemotherapy and the treatment of cancerous hydrothorax and ascite.
- 50 **[0059]** In one or more embodiments of the present application, the compounds of the present application may be used in combination with other drugs such as anticancer drugs, wherein the anticancer drugs include temozolomide, paclitaxel, docetaxel, taxane, gemcitabine, anti-VEGF drugs, PD-1 antibody drugs; wherein, anti-VEGF drugs include AVASTIN, ranibizumab, aflibercept or conbercept; wherein, PD-1 antibody drugs include nivolumab, pembrolizumab, toripalimab, sintilimab, cemiplimab, atezolizumab, avelumab, or durvalumab.
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[0060] One or more embodiments of the present application provide a method for the treatment of human disease, comprising the administration of a therapeutically effective amount of the pharmaceutical composition of the present application. The pharmaceutical composition is administered by parenteral, by inhalation, intraperitoneal, intravesical, intramuscular, intravenous, intratracheal, subcutaneous, intraocular, intrathecal, transdermal, rectal or vaginal.

5 **[0061]** In one or more embodiments of the present application, the pharmaceutical composition of the present application may be formulated into a pharmaceutical formulation suitable for intravenous administration, a pharmaceutical formulation for parenteral administration, a pharmaceutical formulation for gastrointestinal administration, an aerosol, a pharmaceutical formulation for vaginal administration or other suitable pharmaceutical formulation, the pharmaceutical formulation is a solid formulation, a liquid formulation or a gas formulation.

10 **[0062]** In one or more embodiments of the present application, the pharmaceutical composition of the present application may be used as a liquid formulation for injection, which comprises elemene and a pharmaceutically acceptable carrier, wherein the pharmaceutically acceptable carrier includes an albumin carrier and an oil for injection; wherein the composition may be formulated into particles, wherein the particle size of the particles is 70-150 nm; and in the composition, the weight ratio of the albumin carrier to the elemene is about 0.5-20, and the weight ratio of the oil for injection and the elemene is about 0.35-10.

15 **[0063]** In one or more embodiments of the present application, the liquid formulation of the present application may be a stable aqueous suspension reconstituted from a sterile lyophilized powder.

[0064] One or more embodiments of the present application provide a sealed container, which may be a unit-dose container or a multi-dose container, comprising the pharmaceutical composition of the present application. The sealed container may be a prefilled syringe. The pharmaceutical composition is a liquid composition or a dry composition. The pharmaceutical composition is lyophilized and sterile.

20 **[0065]** In one or more embodiments of the present application, the nano-formulation or nanoparticles described herein may be present in the form of a dry formulation (for instance, a lyophilized composition) or suspended in a biocompatible medium. The suitable biocompatible medium includes, but is not limited to, water, aqueous buffered medium, saline, buffered saline, optionally buffered amino acid solution, optionally buffered protein solution, optionally buffered sugar solution, optionally buffered vitamin solution, optionally buffered synthetic polymer solution, lipid-containing emulsion, and the like.

[0066] Unless expressly stated otherwise, an "individual" as used herein is a mammal including, but not limited to, a primate, human, bovine, equine, feline, canine, or rodent.

30 **[0067]** As used herein, "treatment" is a method of obtaining beneficial or desired outcomes, including clinical outcomes. For the purposes of the present invention, beneficial or desired clinical outcomes include, but are not limited to, any one or more of the following: reducing one or more symptoms resulting from the disease, reducing the severity of the disease, stabilizing the disease (for instance, preventing or delaying the exacerbation of the disease), preventing or delaying the spread (e.g. metastasis) of disease, preventing or delaying the occurrence or recurrence of disease, preventing or slowing the progression of the disease, improving the state of the disease, providing remission (whether partial or total) of the disease, reducing the dose of one or more other drugs necessary to treat a disease, delaying the progression of the disease, increasing quality of life, and/or prolonging survival. In some embodiments, the composition reduces the severity of one or more symptoms associated with cancer, as compared to corresponding symptoms in the same subject prior to treatment or compared to corresponding symptoms in other subjects who do not receiving the composition, by at least about any one of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100%. "Treatment" also includes alleviation of the pathological consequences of cancer. The methods of the present invention contemplate any one or more of these therapeutic aspects.

40 **[0068]** As used herein, "pharmaceutically acceptable" or "pharmacologically compatible" means a substance that is not biological or otherwise undesirable may be incorporated into a pharmaceutical composition administered to a patient, for instance, without causing any significant undesired biological effect or without interacting in a deleterious manner with any other ingredient contained in the composition. The pharmaceutically acceptable carrier or excipient preferably meets the standards required for toxicology and manufacturing testing.

[0069] Reference herein to "about" a value or parameter includes (and describes) embodiments that refer to the value or parameter itself. For example, description referring to "about X" includes description of "X".

50 **[0070]** There are a variety of suitable formulations for the pharmaceutical composition of the present invention. The following formulations and methods are exemplary only and not limiting.

[0071] Formulations for oral administration may be made of (a) a liquid solution, for instance, an effective amount of the active ingredient dissolved in a diluent such as water, saline or orange juice, (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient in solid or granular form, (c) a suspension in an appropriate liquid, and (d) a suitable emulsion. Tablet forms may include one or more of lactose, mannitol, corn starch, potato starch, microcrystalline cellulose, arabic gum, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid and other excipients, colorants, diluents, buffers, wetting agents, preservatives, flavoring agents and pharmacologically compatible excipients. Lozenge forms may contain the active ingredient in spices, usually sucrose and

arabic gum or tragacanth, and troches containing the active ingredient in an inert matrix, such as gelatin and glycerin, or sucrose and arabic gum, emulsions, gels, etc., in addition to the active ingredient, excipients known in the art are also contained.

5 **[0072]** Formulations for parenteral administration include an aqueous and nonaqueous, isotonic sterile injection solution, which may contain antioxidants, buffers, bacteriostatic agents, and solutes for rendering the formulation isotonic with the blood of the intended recipient, as well as an aqueous and non-aqueous sterile suspension which may include suspending agents, solubilizers, thickening agents, stabilizers and preservatives. The formulation may be presented in unit-dose or multi-dose sealed containers, such as ampoules and vials, and may be stored in a freeze-drying (lyophilizing) condition immediately before use, requiring only the addition of a sterile liquid excipient, such as water, for injection.

10 Extemporaneous injection solutions and suspensions may be prepared from some kinds of the sterile powders, granules and tablets as described above.

[0073] Aerosols comprise the pharmaceutical composition of the present invention, the pharmaceutical composition includes an aqueous and nonaqueous, isotonic sterile solution which may contain antioxidants, buffers, bacteriostatic agents, and solutes, as well as an aqueous and non-aqueous sterile suspension which may include suspending agents, solubilizers, thickening agents, stabilizers and preservatives; the pharmaceutical composition may be prepared as an aerosol formulation alone or in combination with other suitable components for administration by inhalation. These aerosol formulations may be placed in pressurized acceptable propellants such as difluoromethane, propane, nitrogen, and the like. They may also be formulated as a non-pressure formulation of drugs, for example in a nebulizer or atomizer.

15 **[0074]** Other suitable formulations are also possible, for example suppositories may be prepared by using various bases such as emulsified bases or water-soluble bases. Formulations for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations, containing suitable carriers known in the art in addition to the active ingredient.

BRIEF DESCRIPTION OF THE DRAWINGS

25 **[0075]**

Figure 1 shows the hydratization diameter distribution of β -elemene albumin nanoparticles;
 Figure 2 shows the potential diagram of β -elemene albumin nanoparticles;
 30 Figure 3 shows the transmission electron microscope image of β -elemene albumin nanoparticles;
 Figure 4 shows the stability investigation of particle size of different lyophilized samples after reconstitution;
 Figure 5 shows the content stability investigation of different lyophilized samples after storage;
 Figure 6 shows the survival of tumor-bearing mice in each group;
 Figure 7 shows the results of the hemolytic experiment;
 35 Figure 8 shows the tumor growth curve of the orthotopic tumor model in U87R1 mice;
 Figure 9 shows the survival curve of the orthotopic tumor model in U87R1 mice.

DETAILED DESCRIPTION OF THE INVENTION

40 **[0076]** The following embodiments are further illustrations of the invention and are not a limitation of the scope of the invention. The present invention is further described in detail below with reference to the examples, but those skilled in the art should understand that the present invention is not limited to these examples and the preparation methods as used. Moreover, those skilled in the art can make equivalent substitutions, combinations, improvements or modifications to the present invention according to the description of the present invention, but these will all be included in the scope of the present invention.

45 **[0077]** The soybean oil involved in the present invention comes from soybean oil (SIO, injection grade, Lot19C4520/Lot18C4816); the human serum albumin (HSA) involved in the present invention comes from human serum albumin (20%, Baxter, LotA1U133A) or (20%, Grifols, LotALAFD03042).

50 **[0078]** The commonly used detection methods of the present invention include particle size and particle size distribution, content determination, hemolysis experiment, etc., and the details are as follows:

Particle size and particle size distribution

55 **[0079]** The particle size and the particle size distribution were determined with a particle size analyzer (MALVERN ZETASIZER NANO ZS90). 25 mL of sample was taken, 1 mL of ultrapure water was added to dilute it for detection, detection angle: 90°; dispersion medium: water; temperature: 25°C.

[0080] The particle size was the Z-average value (the average particle size of the sample) measured by the particle size analyzer, and the particle size distribution was the PDI value measured by the particle size analyzer.

Content determination

[0081] It was determined by HPLC (General Rule 0512 of the Fourth Part of Chinese Pharmacopoeia 2015 Edition).

[0082] Chromatographic column was CHIRALPAK® IG (4.6×150 mm, 5 mm), with acetonitrile-water (55:45) as mobile phase, at a flow rate of 1.0 mL/min. The injection volume was 20 μL, the column temperature was 30°C, and the detection wavelength was 210 nm.

[0083] Preparation of reference substance solution (0.2 mg/mL) (external standard method): about 10 mg of β-elemene reference substance was precisely weighed into a 50 mL volumetric flask with a small amount of acetonitrile added, acetonitrile was added to be dissolved and diluted to volume scale, the mixture was shaken well to obtain the reference substance solution.

[0084] Preparation of test solution: the cap was removed from 1 bottle of this product and the product was precisely weighed (m_1), an appropriate amount of water was added (to make the ratio of API to water of 20 mg:1 mL), precisely weighed (m_2) and reconstituted by shaking. About 500 mg of the reconstituted solution (about equivalent to API 10 mg) (m_3) was precisely weighed and placed in a 50 mL volumetric flask, about 1.5 mL of water was added first, and then acetonitrile was slowly added to 80% of the full range, sonicated for 10 min, cooled down, and acetonitrile was added again to dilute to volume scale, the mixture was shaken well, and filtered through a nylon filter with a pore size of 0.45 μm, to obtain the test solution. After the reconstituted solution was used, the vial was washed with water and dried, and the empty vial was precisely weighed (m_4).

[0085] 20 μL was precisely measured, injected into a liquid chromatograph, and the chromatogram was record. The content of β-elemene ($C_{15}H_{24}$) in the test sample was calculated by the peak area according to the external standard method.

[0086] Calculation formula was:

$$\text{Content}(\%) = C_R \times \frac{A_x \times 50 \times (m_2 - m_4)}{A_R \times m_3 \times (m_1 - m_4)} \times 100\%$$

in the formula,

A_x : API peak area in the test solution

A_R : peak area of the reference substance

C_R : concentration of the reference substance

Method for the determination of hemolysis value

[0087] Preparation of blood cell suspension: a few milliliters of blood was taken from rabbits (male New Zealand rabbits, laboratory-suitable rearing for 1 week, body weight 2.5 kg-3 kg, blood was taken from the heart), and put in a beaker, the blood was stirred with a glass rod or bamboo stick to remove fibrinogen and get defibrinated blood. 0.9% sodium chloride solution was added in an amount of about 10 times, mixed evenly, centrifuged at 1,500 rpm/min for 15 min, the supernatant was discarded, and the precipitated red blood cells were washed 2-3 times with 0.9% sodium chloride solution according to the above method, until the supernatant was not red. The obtained red blood cells were prepared into 2% blood cell suspension with 0.9% sodium chloride solution for later use.

[0088] Sample preparation: according to the sample content, a proper amount was taken and diluted successively with normal saline to obtain 1 mg/mL sample (actual content).

[0089] Reaction observation: each drug was added in the following manner, and after mixing, the mixture was immediately placed in a water bath at 37°C for incubation, and the hemolysis was observed after 3 hours.

[0090] Drug group: 2.5 mL of 2% blood cell suspension, 2.2 mL of normal saline, and 0.3 mL of drug were successively added to a test tube.

[0091] Positive group: 2.5 mL of 2% blood cell suspension and 2.5 mL of distilled water were successively added to a test tube.

[0092] Negative group: 2.5 mL of 2% blood cell suspension and 2.5 mL of normal saline were successively added to a test tube.

[0093] Determination of OD value: 100 μL of the supernatant was pipetted into a 96-well plate, and the OD value was measured at a wavelength of 540 nm.

[0094] The calculation method of hemolysis value: (OD value of the sample - OD value of the negative group) / (OD value of the positive group - OD value of the negative group).

Test Example 1

[0095] The preparation of β -elemene albumin nanoparticles included the following steps: 1) 200 mg of elemene and 600 mg of soybean oil were weighted, and dissolved in 2 mL of dichloromethane to obtain an oil phase. 2) 650 mg of human serum albumin was dissolved in water for injection to obtain an aqueous solution of human serum albumin with a mass volume percentage of 3.6% (g/mL), that is, to obtain an aqueous phase. 3) Under the action of water bath ultrasound, the oil phase was added dropwise to the aqueous phase, and sonicated by a probe for 8 min (250 W) to produce a primary emulsion. 4) The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1300 bar, and the homogenization was performed for 9 times. The organic solvent was removed from the homogenized system by rotary evaporation, and a 0.22 μ m microporous membrane was used for filtration and sterilization to obtain an aqueous solution of elemene albumin nanoparticles, and the metered volume was adjusted to 10 mg/mL with water for injection. 5) 3% sucrose was added to the obtained elemene albumin nanoparticles, and lyophilized. The lyophilization parameters were: pre-freezing at -45°C for 5h; starting the vacuum pump and keeping the vacuum degree at about 10 Pa, temperature programming, drying at -40°C for 4h, drying at -30°C for 10h, and then drying at -20°C, -10°C, 0°C, 5°C and 10°C for 4h respectively. The lyophilized samples prepared above were reconstituted in pure water, and the particle size, potential and microscopic morphology of the nanoparticles were investigated by the following methods.

[0096] The Malvern particle size analyzer was preheated for 30 min before measurement, 50 μ L of aqueous solution of the elemene albumin nanoparticles was taken, diluted to 1 mL with water, shaken slightly (to avoid bubbles), and placed in a Malvern Nano-ZS90 particle size dish or a potential dish to measure particle size and Zeta potential. The measurement temperature was 25°C, the equilibration time was 120 s, and the rest of the items were kept at the default values of the instrument, and the data was collected and processed by the Malvern V2.2 workstation. The particle size diagram and Zeta potential of the elemene albumin nanoparticles obtained were shown in Figure 1 and Figure 2. As shown in Figure 1 and 2, the particle size of the elemene albumin nanoparticles was about 110 nm, PDI was 0.127, and the potential was about -18 mV.

[0097] The aqueous solution of the elemene albumin nanoparticles was aspirated by a pipette and dropped on a copper mesh. After natural evaporation, 1% phosphotungstic acid solution was dropped on the copper mesh in the same way, and evaporated naturally, a few drops of pure water was aspirated to rinse the surface of the copper mesh carefully. After evaporation, the morphology was observed by transmission electron microscope as shown in Figure 3, the nanoparticles were spherical, regular in shape, relatively uniform in particle size, with a particle diameter of about 100 nm.

Test Example 2

[0098] Elemene albumin nanoparticles was prepared according to the method of Test Experiment 1, wherein the oil for injection was employed with the same amount as those of soybean oil, corn oil, camellia oil, and medium chain triglyceride (MCT) respectively to prepare elemene albumin nanoparticles using different oils for injection as raw materials. The particle size of the prepared nanoparticles was determined by a laser particle size analyzer. After lyophilization, the drug content of each group of formulations before and after lyophilization was determined respectively, and the drug loss rate during lyophilization was calculated.

[0099] The preparation of elemene albumin nanoparticles without soybean oil included the following steps: 1) 200 mg of elemene was weighed and dissolved in 2 mL of dichloromethane to obtain an oil phase. 2) 650 mg of human serum albumin was dissolved in water for injection to obtain an aqueous solution of human serum albumin with a mass volume percentage of 3.6% (g/mL), that is, to obtain an aqueous phase. 3) Under the action of water bath ultrasound, the oil phase was added dropwise to the aqueous phase, and sonicated by a probe for 8 min (250 W) to produce a primary emulsion. 4) The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1300 bar, and the homogenization was performed for 9 times. The organic solvent was removed from the homogenized system by rotary evaporation, and a 0.22 μ m microporous membrane was used for filtration and sterilization to obtain an aqueous solution of elemene albumin nanoparticles, and the metered volume was adjusted to 10 mg/mL with water for injection. 5) 3% sucrose was added to the obtained elemene albumin nanoparticles, and lyophilized according to the lyophilization parameters of Test Example 1. The drug content of a certain volume of the formulation before and after lyophilization was determined respectively, and the drug loss rate during lyophilization was calculated.

[0100] In addition, the groups of soybean oil, corn oil, camellia oil, and medium chain triglyceride only comprising no lyoprotectant of 3% sucrose were prepared according to the above methods, and the drug content of each certain volume of the formulations before and after lyophilization was determined respectively, and the drug loss rate during lyophilization was calculated. The results are shown in Table 1.

Table 1: Drug loss rate of samples prepared with different oils for injection after lyophilization

Lyophilization loss rate	Soybean oil	Corn oil	Camellia oil	MCT	None
3% sucrose contained	7.1%	10.4%	9.2%	11.8%	31.4%
Sucrose free	9.5%	13.3%	11.1%	13.0%	39.5%

[0101] Experiments show that the lyophilization loss rates of albumin nanoparticles prepared by mixing oil for injection with elemene were reduced in different degrees compared with nanoparticles prepared without oil for injection.

Test Example 3

[0102] According to the method of Test Example 1, the amount of soybean oil was adjusted, the elemene albumin nanoparticles were prepared by feeding according to the mass ratio of β -elemene/soybean oil of 5:1, 1:1, 1:3, 1:5, 1:10, 1:20 and without oils for injection.

[0103] The particle size of the prepared nanoparticles was determined by a laser particle size analyzer. After adding 3% sucrose and lyophilizing, the lyophilized samples were placed in a room temperature dryer. The samples were taken out periodically and reconstituted, and the changes of particle size and main drug content of the lyophilized samples within 30 days were determined. Specific detection parameters were as follows: instrument model: MALVERN ZETA-SIZER NANO ZS90, detection angle: 90°; detection mode: automatic; detection times: 3; detection type: nanoparticles; refractive index of the sample to be determined: 1.340; dispersion medium: water; temperature: 25°C; viscosity: 0.8872cp; refractive index of dispersion medium: 1.330; equilibration time: 120s; type of particle size dish : DTS0012.

[0104] The results were shown in Figure 4 and Figure 5. The particle size of the formulation without soybean oil during the preparation process was more than 200 nm after lyophilization. With the prolongation of storage time, the particle size of the sample after reconstitution showed a trend of increasing gradually, and the drug content showed a trend of decreasing. Gradually increasing the proportion of soybean oil in the formulation, the particle size of the lyophilized samples after reconstitution was all less than 200 nm, the stability of particle size was better, and the stability of the main drug content was also gradually enhanced. Wherein, when the mass ratio of soybean oil/elemene was in the range of 1-10, the prepared lyophilized sample of nanoparticles had no aggregation phenomenon within 30 days at room temperature, the particle size was 100-150 nm, the PDI was less than 0.3, and the content of the main drug of β -elemene changed little. When the amount of elemene was 1 part and the amount of soybean oil was less than 1 part, the prepared nanoparticles did not show a good enough stability.

Test Example 4

[0105] To investigate the therapeutic effect of the elemene albumin nanoparticles on mouse brain glioma, the experimental method was as follows.

[0106] Tumor modeling: the well-grown C6 cells in logarithmic phase were digested with trypsin, centrifuged, and the upper waste solution was discarded. The cells were washed with PBS, centrifuged, and the upper waste solution was discarded, and the cells were resuspended in PBS to make the final C6 cell density about 1×10^6 cells/5 mL. Healthy male Kunming mice (28-30 g) were anesthetized by intraperitoneal injection of an appropriate amount of 4% chloral hydrate. After shaving the hair on the top of the head, 75% ethanol was applied for disinfection, and a small incision was made along the sagittal midline of the head with sterile ophthalmic scissors, the tissue membrane was quickly removed with a cotton swab moistened with 10% hydrogen peroxide, the skull was separated, and the hydrogen peroxide was washed away with a cotton swab moistened with PBS. The head of the mouse was fixed on a brain stereotaxic device, located at a position 1.8 mm to the right and 0.6 mm to the back of the bregma, a small hole was drilled at the locating point with the needle of a 2 mL syringe, which should not be too deep to avoid bleeding. 5 mL of the above-mentioned C6 cells with a density of 1×10^6 cells/5 mL was aspirated with a micro-injection needle. The needle was inserted by 4 mm and withdrawn by 1 mm along the vertical direction of the small hole to leave a space for cells, and the cell suspension was slowly injected into the brain. The microneedle was taken out after remaining in the position of the small hole for 5 min. The wound was sutured with surgical thread, and finally the sutured surface was smeared with a cotton swab moistened with double antibody.

[0107] On the 10th day after tumor inoculation, the tumor-bearing mice were divided into normal saline group (NS), commercially available elemene liposomes (Lip, Dalian Jingang, approval number: Guoyaozhunzi H10960114), elemene albumin nanoparticles (NPs) prepared in Test Example 1, with 12 mice in each group. The dosage of administration was 30 mg/kg, once every 2 days, for a total of 4 times. The survival time of each mouse was recorded, and the survival curve was made.

[0108] The investigation results of survival were shown in figure 6. The results of the experiment showed that the C6 glioma-bearing mice administered with the elemene albumin nanoparticles exhibited longer survival. The average survival of the normal saline group was 21 days, the average survival of the elemene liposome group was 28 days, and the average survival of the elemene albumin nanoparticles group was 41 days. Therefore, the elemene albumin nanoparticles prepared in the experiment have an effect of prolonging the survival of C6-bearing glioma mice.

Test Example 5

[0109] Hemolysis experiment:
 10 New Zealand rabbits (male, laboratory-suitable housed for 1 week, body weight 2.5 kg-3 kg) were divided into two groups of commercially available elemene injection (Ailineng, CSPC Yuanda Pharmaceutical Co., Ltd., batch number 17050302) and elemene albumin nanoparticles (NPs) prepared in Test Example 1, with 2 mice in each group, the dosage of administration was 50 mg/kg. The two formulations were respectively prepared into medicinal solutions having a concentration of 6 mg/mL with glucose solution, and then the rabbits were administered by intravenous infusion.
 15 **[0110]** Within 10-15 minutes after administration, about 1 mL of venous blood was drawn from the ear vein and placed in a centrifuge tube coated with heparin sodium, centrifuged at 3500 r/min for 15 minutes, and the supernatant was taken to observe the color.
[0111] The color of the obtained plasma was shown in figure 7. The result showed that the New Zealand rabbits administered with the elemene albumin nanoparticles did not show hemolysis, demonstrating little irritation and high safety.

Test Example 6

1.1 Preparation of experimental samples

Sample 1

[0112] 3.33 g of β -elemene and 8 g of soybean oil were weighed and mixed to obtain an oil phase. 50 mL (10g) of 20% human serum albumin was measured, diluted evenly by adding an appropriate amount of ultrapure water, and the metered volume was adjusted to 333 mL to obtain an aqueous solution of human serum albumin with a mass volume percentage of 3% (g/mL), namely an aqueous phase. The aqueous phase was poured into the oil phase, and the mixer (FLUKO, FA30/30F) was used for shearing at 10,000 rpm for 10 min to form a primary emulsion. The obtained primary emulsion was transferred to a micro-jet homogenizer (Microfluidizer, M-110-EH) for homogenization, at a homogenization pressure of 20,000 psi for 30 min. The obtained emulsion was filtered by 0.45 μ m and 0.22 μ m PVDF plate filters, and the metered volume was adjusted, and 3% (g/mL) of sucrose was added as a lyoprotectant. The homogeneous solution added with the lyoprotectant was lyophilized, and after lyophilization, the lyophilized powder was reconstituted with sterilized ultrapure water. Particle size was 175.97 nm detected by a particle size analyzer, PDI was 0.188.

Sample 2

[0113] 3.33 g of β -elemene and 8 g of soybean oil were weighed, dissolved in an organic solvent, and mixed to obtain an oil phase. The organic solvent was a mixed solvent of 26.67 mL dichloromethane and 6.67 mL anhydrous ethanol. 50 mL (10 g) of 20% human serum albumin was measured, diluted evenly by adding an appropriate amount of ultrapure water, and the metered volume was adjusted to 333 mL to obtain an aqueous solution of human serum albumin with a mass volume percentage of 3% (g/mL), namely an aqueous phase. The aqueous phase solution was poured into the oil phase, and the mixer (FLUKO, FA30/30F) was used for shearing at 10,000 rpm for 10 min to form a primary emulsion. The obtained primary emulsion was transferred to a micro-jet homogenizer (Microfluidizer, M-110-EH) for high-pressure homogenization, at a homogenization pressure of 20,000 psi for 30 min. After homogenization, the organic solvent was removed by a thin film evaporator (Shanghai DEA) from the organic solvent-containing emulsion, and the parameters were: temperature 30°C, vacuum degree 4000 pa, liquid feeding speed 100 rpm, scraper speed 50 rpm. The obtained emulsion was filtered by 0.45 μ m and 0.22 μ m PVDF plate filters, and the metered volume was adjusted, and 3% (g/mL) of sucrose was added as a lyoprotectant. The homogeneous solution added with the lyoprotectant was lyophilized, and after lyophilization, the lyophilized powder was reconstituted with sterilized ultrapure water. Particle size was 78.34 nm detected by a particle size analyzer, PDI was 0.207.

[0114] **Sample 3 (Elemene injection (trade name: Ailineng)):** CSPC Yuanda (Dalian) Pharmaceutical Co., Ltd., batch number: 19061502; particle size was 14.74 nm detected by a particle size analyzer, PDI: 0.035.

1.2 PK and mouse brain tissue distribution of elemene albumin nanoparticles

[0115] Kunming mice (male, SPF grade, body weight 20-25 g) were used for the experiment. The dosage of administration was 50 mg/kg, administered through the tail vein. Brain tissue samples were collected at different time periods and stored at -20°C in time for the detection of drug content in brain tissue. There were a total of 45 mice for three samples, 3 Kunming mice for each dosage form at each time point. The eyeballs were removed for blood collection at 5min, 15min, 30min, 1h, and 2h after administration, and brain tissues were collected to detect drug content in brain tissues. GC-MS was used to quantitatively analyze β -elemene in biological samples of plasma and brain tissue of the Kunming mice. The results of the study on the half-life in brain tissue and the brain tissue exposure are shown in Table 2.

Table 2 Results of studies on half-life and brain tissue exposure

	Sample 1	Sample 2	Sample 3
Particle size (nm)	175.97	78.34	14.7
Half-life in brain tissue (min)	41.8	57.5	33.6
Brain drug exposure (mg/L*min)	2808.7	3008.6	2812.7

Test Example 7

Preparation and stability investigation of sample 4

[0116] 1 g of β -elemene and 3 g of soybean oil were weighed and mixed to obtain an oil phase. 15 mL (3 g) of 20% human serum albumin was measured, diluted evenly by adding 85 mL of ultrapure water, to obtain an aqueous solution of human serum albumin with a mass volume percentage of 3% (g/mL), namely an aqueous phase. The aqueous phase solution was poured into the oil phase, and the mixer was used for shearing at 5000 rpm for 1 min, and at 10,000 rpm for 5 min, to form a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization at a homogenization pressure of 250-300 bar for 2 min, and at 1350-1400 bar for 9 min. After homogenization, the total volume of the homogeneous solution was measured with a measuring cylinder, which was about 50 mL. 5 g trehalose was added, shaken well to be dissolved, and filtered with a 0.45 μ m microporous membrane to obtain a solution of elemene albumin nanoparticles. The particle size was 141.0 nm detected by a particle size analyzer, PDI was 0.162. The homogeneous solution added with trehalose was lyophilized, and after lyophilization, the lyophilized powder was reconstituted with sterilized ultrapure water. The particle size was 144.07 nm detected by a particle size analyzer, PDI was 0.123. The loss rate of elemene content before and after lyophilization was 1.27%.

Preparation and stability investigation of sample 5

[0117] 4 g of β -elemene and 2 g of soybean oil were weighed and mixed to obtain an oil phase. 30 mL (6 g) of 20% human serum albumin was measured, diluted evenly by adding 170 mL of ultrapure water, to obtain an aqueous solution of human serum albumin with a mass volume percentage of 3% (g/mL), namely an aqueous phase. The aqueous phase solution was poured into the oil phase, and the mixer was used for shearing at 5000 rpm for 1 min, and at 10,000 rpm for 10 min, to form a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization at a homogenization pressure of 250-300 bar for 2 min, and at 1350-1400bar for 24 min. After homogenization, 150 mL of homogeneous solution was measured with a measuring cylinder, 15 g trehalose was added, shaken well to be dissolved, and filtered with a 0.45 μ m microporous membrane to obtain a solution of elemene albumin nanoparticles. The particle size was 132.4 nm detected by particle size analyzer, PDI was 0.133. The homogeneous solution added with trehalose was lyophilized, and after lyophilization, the lyophilized powder was reconstituted with sterilized ultrapure water. The particle size was 123.68 nm detected by a particle size analyzer, PDI was 0.144. The loss rate of elemene content after lyophilization was 1.58%.

Preparation and stability investigation of sample 6

[0118] 1 g of β -elemene and 2 g of egg yolk lecithin were weighed, 50 mL of ultrapure water was added, and the mixer was used for shearing at 5000 rpm for 1 min, and shearing at 10,000 rpm for 5 min. Then the congealed fat adhered to the bottle wall was scraped into the solution, and sheared at 15,000rpm for 3 min again until no obvious undispersed phospholipid was found, and a primary emulsion was obtained. The primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization at a homogenization pressure of 250-300 bar for 2 min, and at 1300-1350

bar for 12 min. After homogenization, the homogeneous solution was filtered with a 0.45 μm filter membrane, and 50 mL of 2% HSA solution (containing 1 g of HSA) was added to the filtered homogeneous solution, and homogenized at 280 bar for 1 cycle; and the homogeneous solution was discharged, 40 mL was measured with a measuring cylinder, 4 g trehalose was added, shaken well to be dissolved, and filtered with a 0.45 μm microporous membrane to obtain a solution of albumin nanoparticles. The particle size was 150.86 nm detected by a particle size analyzer, PDI was 0.114. The homogeneous solution added with trehalose was lyophilized, and after lyophilization, the lyophilized powder was reconstituted with sterilized ultrapure water. The particle size was 181.56 nm detected by a particle size analyzer, PDI was 0.251. The loss rate of elemene content after lyophilization was 16.47%.

10 Preparation and stability investigation of sample 7

[0119] 1 g of β -elemene, 2 g of egg yolk lecithin and 0.5g of TPGS were weighed, 50 mL of ultrapure water was added, and the mixer was used for shearing at 5000 rpm for 1 min, and shearing at 10,000 rpm for 5 min. Then the congealed fat adhered to the bottle wall was scraped into the solution, and sheared at 15,000rpm for 3 min again until no obvious undispersed phospholipid was found, and a primary emulsion was obtained. The primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization at a homogenization pressure of 250-300 bar for 2 min, and at 1300-1350 bar for 12 min. After homogenization, the solution was filtered with a 0.22 μm filter membrane, and 50 mL of 2% HSA solution (containing 1 g of HSA) was added to the filtered homogeneous solution, homogenized at 140 bar for 1 cycle; and the homogeneous solution was discharged, 40 mL was measured with a measuring cylinder, 4 g of trehalose was added, shaken well to be dissolved, and filtered with a 0.45 μm microporous membrane to obtain a solution of albumin nanoparticles. The particle size was 93.11 nm detected by a particle size analyzer, PDI was 0.131. The homogeneous solution added with trehalose was lyophilized, and after lyophilization, the lyophilized powder was reconstituted with sterilized ultrapure water. The particle size was 811.70 nm detected by a particle size analyzer, PDI was 0.313. The loss rate of elemene content after lyophilization was 82.64%.

25 Example 1

[0120] 200 mg of β -elemene and 600 mg of soybean oil were weighed, dissolved in 2 mL of an organic solvent, and mixed by vortex to obtain an oil phase. The organic solvent is dichloromethane, or ethyl acetate or a mixture of dichloromethane and ethanol. 650 mg of human serum albumin was taken and dissolved in 18 mL of water for injection to obtain an aqueous solution of human serum albumin with a mass volume percentage of 3.6% (g/mL), that is, to obtain an aqueous phase. Under the action of water bath ultrasound, the oil phase was added dropwise to the aqueous phase, and sonicated by a probe for 8 min (250 W) to produce a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1000 bar, and the homogenization times was 16 times. The organic solvent was removed by rotary evaporation after homogenization, and a 0.22 μm microporous membrane was used for filtration and sterilization to obtain an aqueous solution of elemene albumin nanoparticles.

Table 3 Investigation results of single factor for organic solvents

Number	Organic solvent	Particle size (d.nm)	PDI
1	Without organic solvent	142.1	0.094
2	Ethyl acetate	122.0	0.164
3	Dichloromethane	108.8	0.129
4	Dichloromethane/ethanol (4:1)	98.5	0.130

[0121] The experimental results showed that, no matter whether β -elemene and soybean oil were dissolved in organic solvents or not during the preparation process, β -elemene albumin formulations with suitable particle size were prepared. Among various organic solvents, the nanoparticles prepared by dichloromethane or a mixed solvent of dichloromethane and ethanol had smaller particle size.

55 Example 2

[0122] 200 mg of β -elemene and 600 mg of soybean oil were weighed, dissolved in a mixed solvent of dichloromethane and ethanol (dichloromethane:ethanol=4:1, 2 mL), and mixed by vortex to obtain an oil phase with a volume of 2 mL. 650 mg of human serum albumin was taken and dissolved in water for injection to obtain an aqueous solution of human

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serum albumin with a mass volume percentage of 3.6% (g/mL), and then 100 mg of polyethylene glycol-12-hydroxysuccinate was added, to obtain an aqueous phase. Under the action of water bath ultrasound, the oil phase was added dropwise to the aqueous phase, and sonicated by a probe for 8 min (250 W) to produce a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1000 bar, and the homogenization times was 16 times. After homogenization, the organic solvent was removed by rotary evaporation, and a 0.22 μ m microporous membrane was used for filtration and sterilization to obtain an aqueous solution of elemene albumin nanoparticles. The measured particle size was 116.4 nm, PDI was 0.118.

Example 3

[0123] 200 mg of β -elemene and 400 mg of medium and long chain fatty glyceride were weighed, dissolved in a mixed solvent of dichloromethane and ethanol (dichloromethane:ethanol=4:1, 2 mL), and mixed by vortex, to obtain an oil phase with a volume of 2 mL. 900 mg of human serum albumin was taken and dissolved in 18 mL of water for injection to obtain an aqueous solution of human serum albumin with 5.0% of mass volume percentage (g/mL), that is, to obtain an aqueous phase. Under the action of water bath ultrasound, the oil phase was added dropwise to the aqueous phase, and sonicated by a probe for 8 min (250 W) to produce a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1000 bar, and the homogenization times was 16 times. After homogenization, the organic solvent was removed by rotary evaporation, and a 0.22 μ m microporous membrane was used for filtration and sterilization to obtain an aqueous solution of elemene albumin nanoparticles. The measured particle size was 105.3 nm, PDI was 0.116.

Example 4

[0124] 800 mg of β -elemene and 2400 mg of sesame oil were weighed, dissolved in a mixed solvent of dichloromethane and ethanol (dichloromethane:ethanol=4:1, 10 mL), and mixed by vortex to obtain an oil phase. 4000 mg of human serum albumin was taken and dissolved in 100 mL of water for injection to obtain an aqueous solution of human serum albumin with 4% of mass volume percentage (g/mL), that is, to obtain an aqueous phase. The oil phase was added dropwise to the aqueous phase, and a tissue masher was used at 10,000 rpm for shearing for 10 min to prepare a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1000 bar, and the homogenization times was 20 times. After homogenization, the organic solvent was removed by rotary evaporation, and a 0.22 μ m microporous membrane was used for filtration and sterilization to obtain an aqueous solution of elemene albumin nanoparticles. The measured particle size was 121.3 nm, PDI was 0.133.

Example 5

[0125] 800 mg of β -elemene and 2400 mg of olive oil were weighed, successively added to a dichloromethane/ethanol mixed solvent (dichloromethane:ethanol=4:1, 5 mL), and mixed by vortex to obtain an oil phase. 4000 mg of human serum albumin was taken and dissolved in water for injection to obtain an aqueous solution of human serum albumin with a mass volume percentage of 4% (g/mL), to obtain an aqueous phase. The oil phase was added dropwise to the aqueous phase, and a tissue masher was used at 12000 rpm for shearing for 10 min to prepare a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1400 bar, and the homogenization times was 20 times. The organic solvent was removed from the homogenized system by rotary evaporation. The rotary-evaporated medicinal solution was filtered and sterilized via a 0.22 μ m microporous membrane to obtain an aqueous solution of elemene albumin nanoparticles. 3% sucrose was added and lyophilized, capped after lyophilization, and stored at 4°C. The lyophilized samples were taken and reconstituted in water for injection, and the measured particle size was 112.6 nm, PDI was 0.101.

Example 6

[0126] 200 mg of β -elemene and 600 mg of soybean oil were weighed, dissolved in a mixed solvent of dichloromethane and ethanol (dichloromethane:ethanol=4:1, 10 mL), and mixed by vortex to obtain an oil phase. 4000 mg of human serum albumin was dissolved in water for injection to obtain an aqueous solution of human serum albumin with 10% of mass volume percentage (g/mL), that is, to obtain an aqueous phase. Under the action of water bath ultrasound, the oil phase was added dropwise to the aqueous phase, and sonicated by a probe for 8 min (250 W) to produce a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1000 bar, and the homogenization times was 16 times. After homogenization, the organic solvent was removed by rotary evaporation, and a 0.22 μ m microporous membrane was used for filtration

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and sterilization to obtain an aqueous solution of elemene albumin nanoparticles. The measured particle size was 140.3 nm, PDI was 0.164.

Example 7

[0127] 200 mg of β -elemene and 1000 mg of soybean oil were weighed, dissolved in a mixed solvent of dichloromethane and ethanol (dichloromethane:ethanol=4:1, 5 mL), and mixed by vortex to obtain an oil phase. 650 mg of human serum albumin was taken and dissolved in water for injection to obtain an aqueous solution of human serum albumin with 3.6% of mass volume percentage (g/mL), that is, to obtain an aqueous phase. Under the action of water bath ultrasound, the oil phase was added dropwise to the aqueous phase, and sonicated by a probe for 8 min (250 W) to produce a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1000 bar, and the homogenization times was 16 times. After homogenization, the organic solvent was removed by rotary evaporation, and a 0.22 μ m microporous membrane was used for filtration and sterilization to obtain an aqueous solution of elemene albumin nanoparticles. The measured particle size was 125.3 nm, PDI was 0.148.

Example 8

[0128] 800 mg of β -elemene and 2400 mg of soybean oil were weighed, dissolved in a mixed solvent of dichloromethane and ethanol (dichloromethane:ethanol=4:1, 10 mL), and mixed by vortex to obtain an oil phase. 4000 mg of human serum albumin was dissolved in water for injection to obtain an aqueous solution of human serum albumin with 4% of mass volume percentage (g/mL), that is, to obtain an aqueous phase. The oil phase was added dropwise to the aqueous phase, and a tissue masher was used at 10000 rpm for shearing for 10 min to prepare a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1000 bar, and the homogenization times was 20 times. After homogenization, the organic solvent was removed by rotary evaporation, and a 0.22 μ m microporous membrane was used for filtration and sterilization to obtain an aqueous solution of elemene albumin nanoparticles. The measured particle size was 119.3 nm, PDI was 0.143. The prepared nanoparticle solution was taken and configured to a drug concentration of 10 mg/mL, an appropriate amount of lyoprotectant was added, and the amount of the lyoprotectant was 3% (g/mL), and lyophilized. After lyophilization, water for injection was added to reconstitute, and the particle size was determined. The results are shown in the following table.

Table 4 Particle size of elemene albumin nanoparticles with different lyoprotectants after lyophilization

Lyoprotectant	None	Glucose	Sucrose	Trehalose	Mannitol	Lactose	Maltose
Particle size (nm)	187.4	160.7	153.5	167.4	172.1	166.3	164.5
PDI	0.233	0.165	0.125	0.167	0.133	0.178	0.196

[0129] The experimental results showed that, during the lyophilization process of elemene albumin nanoparticles, various lyoprotectants were added to obtain lyophilized powders with loose structure and uniform texture, which could be reconstituted by adding water, and the particle size after reconstitution did not exceed 200 nm. Wherein, the nanoparticles obtained by reconstituting the sample by lyophilizing with sucrose as the lyoprotectant had the smallest particle size.

Example 9

[0130] 500 mg of β -elemene and 1200 mg of medium and long chain fatty glyceride were weighed, dissolved in a mixed solvent of dichloromethane and ethanol (dichloromethane:ethanol=4:1, 5 mL), and mixed by vortex, to obtain an oil phase. 1500 mg of human serum albumin was taken and dissolved in 50 mL of water for injection to obtain an aqueous solution of human serum albumin with 3% of mass volume percentage (g/mL), to obtain an aqueous phase. The oil phase was added dropwise to the aqueous phase, and sonicated by a probe for 8 min (250 W) to produce a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1400 bar, and the homogenization times was 10 times. After homogenization, the organic solvent was removed by rotary evaporation, and a 0.22 μ m microporous membrane was used for filtration and sterilization, the filtrate was diluted and configured to a drug concentration of 15 mg/mL, 3% sucrose was added, lyophilized, capped, and stored at 4°C. The lyophilized sample was taken and reconstituted in water for injection, and the particle size was determined. The measured particle size was 103.5 nm, PDI was 0.123.

Example 10

[0131] 800 mg of β -elemene and 2400 mg of sesame oil were weighed, successively added to a dichloromethane/isopropanol mixed solvent (dichloromethane:isopropanol=4:1, 5 mL), and mixed by vortex to obtain an oil phase. 4000 mg of human serum albumin was dissolved in water for injection to obtain an aqueous solution of human serum albumin with a mass volume percentage of 4% (g/mL), to obtain an aqueous phase. The oil phase was added dropwise to the aqueous phase, and a tissue masher was used at 10,000 rpm for shearing for 10 min to prepare a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1000 bar, and the homogenization times was 20 times. The organic solvent was removed from the homogenized system by rotary evaporation. The rotary-evaporated medicinal solution was filtered and sterilized via a 0.22 μ m microporous membrane to obtain an aqueous solution of elemene albumin nanoparticles. The aqueous solution was diluted and configured to a drug concentration of 10 mg/mL, and 3% sucrose was added for lyophilization, capped after lyophilization, and stored at 4°C. The lyophilized sample was taken and reconstituted in water for injection, and the measured particle size was 115.2 nm, PDI was 0.102.

Example 11

[0132] 800 mg of β -elemene and 2400 mg of olive oil were weighed, successively added to a dichloromethane/tertiary butanol mixed solvent (dichloromethane:tertiary butanol=4:1, 5 mL), and mixed by vortex to obtain an oil phase. 4000 mg of human serum albumin was dissolved in water for injection to obtain an aqueous solution of human serum albumin with a mass volume percentage of 4% (g/mL), to obtain an aqueous phase. The oil phase was added dropwise to the aqueous phase, and a tissue masher was used at 10,000 rpm for shearing for 10 min to prepare a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1000 bar, and the homogenization times was 20 times. The organic solvent was removed from the homogenized system by rotary evaporation. The rotary-evaporated medicinal solution was filtered and sterilized via a 0.22 μ m microporous membrane to obtain an aqueous solution of elemene albumin nanoparticles. The aqueous solution was diluted and configured to a drug concentration of 10 mg/mL, and 3% sucrose was added for lyophilization, capped after lyophilization, and stored at 4°C. The lyophilized sample was taken and reconstituted in water for injection, and the measured particle size was 109.6 nm, PDI was 0.113.

Example 12

[0133] 800 mg of β -elemene and 2400 mg of sesame oil were weighed, successively added to a dichloromethane/tetrahydrofuran mixed solvent (dichloromethane:tetrahydrofuran=4:1, 5 mL), and mixed by vortex to obtain an oil phase with a volume of 5 mL. 4000 mg of human serum albumin was taken and dissolved in water for injection to obtain an aqueous solution of human serum albumin with a mass volume percentage of 4% (g/mL), to obtain an aqueous phase. The oil phase was added dropwise to the aqueous phase, and a tissue masher was used at 10,000 rpm for shearing for 10 min to prepare a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1000 bar, and the homogenization times was 20 times. The organic solvent was removed from the homogenized system by rotary evaporation. The rotary-evaporated medicinal solution was filtered and sterilized via a 0.22 μ m microporous membrane to obtain an aqueous solution of elemene albumin nanoparticles. 3% trehalose was added for lyophilization, capped after lyophilization, and stored at 4°C. The lyophilized sample was taken and reconstituted in water for injection, and the measured particle size was 112.6 nm, PDI was 0.101.

Example 13

[0134] 500 mg of β -elemene and 1200 mg of soybean oil were weighed, successively added to isopropanol (5 mL) and mixed by vortex to obtain an oil phase. 1500 mg of bovine serum albumin was taken and dissolved in water for injection to obtain an aqueous solution of bovine serum albumin with a mass volume percentage of 3% (g/mL), to obtain an aqueous phase. The oil phase was added dropwise to the aqueous phase, and sonicated by a probe for 8 min (250 W) to produce a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1400 bar, and the homogenization times was 10 times. The organic solvent was removed from the homogenized system by rotary evaporation. The rotary-evaporated medicinal solution was filtered and sterilized via a 0.22 μ m microporous membrane. The filtrate was diluted and configured to a drug concentration of 10 mg/mL, and 3% sucrose was added for lyophilization, capped after lyophilization, and stored at 4°C. The lyophilized samples were reconstituted in water for injection, and the measured particle size was 128.1 nm, PDI was 0.132.

Example 14

[0135] 500 mg of β -elemene and 1200 mg of soybean oil were weighed, successively added to tertiary butanol (5 mL) and mixed by vortex to obtain an oil phase. 1500 mg of bovine serum albumin was taken and dissolved in water for injection to obtain an aqueous solution of bovine serum albumin with a mass volume percentage of 3% (g/mL), to obtain an aqueous phase. The oil phase was added dropwise to the aqueous phase, and sonicated by a probe for 8 min (250 W) to produce a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1400 bar, and the homogenization times was 10 times. After homogenization, the organic solvent was removed by rotary evaporation. The rotary-evaporated medicinal solution was filtered and sterilized via a 0.22 μ m microporous membrane. The filtrate was diluted and configured to a drug concentration of 5 mg/mL, and 3% sucrose was added for lyophilization, capped after lyophilization, and stored at 4°C. The lyophilized sample was taken and reconstituted in water for injection, and the measured particle size was 116.8 nm, PDI was 0.113.

Example 15

[0136] 500 mg of β -elemene and 1200 mg of sesame oil were weighed, successively added to a dichloromethane/isopropanol mixed solvent (dichloromethane:isopropanol=4:1, 5 mL), and mixed by vortex to obtain an oil phase. 1500 mg of bovine serum albumin was dissolved in water for injection to obtain an aqueous solution of bovine serum albumin with a mass volume percentage of 3% (g/mL), to obtain an aqueous phase. The oil phase was added dropwise to the aqueous phase, and sonicated by a probe for 8 min (250 W) to produce a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1400 bar, and the homogenization times was 10 times. After homogenization, the organic solvent was removed by rotary evaporation. The rotary-evaporated medicinal solution was filtered and sterilized via a 0.22 μ m microporous membrane. The filtrate was diluted and configured to a drug concentration of 1 mg/mL, and 3% sucrose was added for lyophilization, capped after lyophilization, and stored at 4°C. The lyophilized sample was taken and reconstituted in water for injection, and the measured particle size was 121.8 nm, PDI was 0.111.

Example 16

[0137] 500 mg of β -elemene and 1200 mg of soybean oil were weighed, successively added to a dichloromethane/tertiary butanol mixed solvent (dichloromethane:tertiary butanol=4:1, 5 mL), and mixed by vortex to obtain an oil phase. 1500 mg of bovine serum albumin was dissolved in water for injection to obtain an aqueous solution of bovine serum albumin with a mass volume percentage of 3% (g/mL), to obtain an aqueous phase. The oil phase was added dropwise to the aqueous phase, and sonicated by a probe for 8 min (250 W) to produce a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1400 bar, and the homogenization times was 10 times. After homogenization, the organic solvent was removed by rotary evaporation. The rotary-evaporated medicinal solution was filtered and sterilized via a 0.22 μ m microporous membrane. The filtrate was diluted and configured to a drug concentration of 10 mg/mL, and 3% sucrose was added for lyophilization, capped after lyophilization, and stored at 4°C. The lyophilized sample was taken and reconstituted in water for injection, and the measured particle size was 121.1 nm, PDI was 0.109.

Example 17

[0138] 500 mg of β -elemene and 1200 mg of olive oil were weighed, successively added to a dichloromethane/ethanol mixed solvent (dichloromethane:ethanol=4:1, 5 mL), and mixed by vortex to obtain an oil phase. 1500 mg of bovine serum albumin was dissolved in water for injection to obtain an aqueous solution of bovine serum albumin with 3% of mass volume percentage (g/mL), to obtain an aqueous phase. The oil phase was added dropwise to the aqueous phase, and sonicated by a probe for 8 min (250 W) to produce a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1400 bar, and the homogenization times was 10 times. The organic solvent was removed from the homogenized system by rotary evaporation. The rotary-evaporated medicinal solution was filtered and sterilized via a 0.22 μ m microporous membrane. The filtrate was diluted and configured to a drug concentration of 12 mg/mL, and 3% sucrose was added for lyophilization, capped after lyophilization, and stored at 4°C. The lyophilized sample was taken and reconstituted in water for injection, and the measured particle size was 109.7 nm, PDI was 0.105.

Example 18

[0139] 500 mg of β -elemene and 1200 mg of soybean oil were weighed, successively added to a dichloromethane/tetrahydrofuran mixed solvent (dichloromethane:tetrahydrofuran=4:1, 5 mL), and mixed by vortex, to obtain an oil phase. 1500 mg of bovine serum albumin was dissolved in water for injection to obtain an aqueous solution of bovine serum albumin with 3% of mass volume percentage (g/mL), to obtain an aqueous phase. The oil phase was added dropwise to the aqueous phase, and sonicated by a probe for 8 min (250 W) to produce a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1400 bar, and the homogenization times was 10 times. The organic solvent was removed from the homogenized system by rotary evaporation. The rotary-evaporated medicinal solution was filtered and sterilized via a 0.22 μ m microporous membrane. The filtrate was diluted and configured to a drug concentration of 10 mg/mL, and 3% sucrose was added for lyophilization, capped after lyophilization, and stored at 4 °C. The lyophilized sample was taken and reconstituted in water for injection, and the measured particle size was 112.7 nm, PDI was 0.112.

Example 19

[0140] 200 mg of elemene and 600 mg of soybean oil were weighed, dissolved in a mixed solvent of dichloromethane and ethanol (dichloromethane:ethanol=4:1, 2 mL), and mixed by vortex, to obtain an oil phase. 650 mg of human serum albumin was dissolved in 18 mL of 0.5% sodium chloride solution to obtain a solution of human serum albumin with a mass volume percentage (g/mL) of 3.6%. Under the action of water bath ultrasound, the oil phase was added dropwise to the aqueous phase, and sonicated by a probe for 8 min (250 W) to produce a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer for high-pressure homogenization, the homogenization pressure was 1000 bar, and the homogenization times was 16 times. The organic solvent was removed from the homogenized system by rotary evaporation, and a 0.22 μ m microporous membrane was used for filtration and sterilization to obtain an aqueous solution of elemene albumin nanoparticles. The measured particle size was 126.1 nm, PDI was 0.122.

Example 20

[0141] 500 mg of β -elemene and 1500 mg of soybean oil were weighed, dissolved in 5 mL of dichloromethane/ethanol (dichloromethane:ethanol=8:1) mixed solvent to obtain an oil phase; and 2500 mg of human serum albumin was weighed and dissolved in 45 mL of water to obtain an aqueous phase. Under the action of ultrasound, the two phases were mixed evenly, and the obtained solution was transferred to a high-pressure homogenizer for high-pressure homogenization, at 400-500 bar for 4-6 times, and at 1300-1400 bar for 8-10 times. After homogenization, then the organic solvent was removed by rotary evaporation, the microporous membrane was used for filtration and sterilization, the bubbles in the formulation were removed by standing still for a moment, and a solution of elemene albumin nanoparticles was obtained. The measured particle size of the nanoparticles was 99.4 nm, PDI was 0.096. The obtained solution of elemene albumin nanoparticles was adjusted to a concentration of 10 mg/mL, 3% sucrose was added, lyophilized, and reconstituted by adding ultrapure water, the particle size was measured to be 101.6 nm, and the PDI was 0.113.

Example 21

[0142] The corresponding β -elemene, soybean oil, MCT (medium chain triglyceride) and surfactants (HS-15, namely macrogol 15-hydroxystearate; Tween 80; Poloxamer P188) were weighed according to the component amounts listed in Table 5 and mixed to obtain an oil phase. 20% human serum albumin (converted by mass as shown in Table 5) was taken, diluted by adding ultrapure water to a total volume of 50 mL and mixed evenly, to obtain an aqueous solution of human serum albumin, namely an aqueous phase. An appropriate amount of surfactant was added to the aqueous phase solution, and the aqueous phase solution was poured into the oil phase, and sheared with a mixer (IKA, T25) at 5,000 rpm for 1 min and at 10,000 rpm for 5 min to form a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer (ATS, AH-1500) for high-pressure homogenization at a homogenization pressure of 250-300 bar for 2 min, and at 1450-1550 bar for 6 min. After homogenization, the total volume of the homogeneous solution was measured with a measuring cylinder, trehalose of 10% (g/mL) was added, shaken well to be dissolved, and filtered with a 0.45 μ m microporous membrane to obtain a solution of elemene albumin nanoparticles. The particle size and PDI were detected, and the hemolysis value was determined. The specific results are shown in Table 5.

Table 5

Serial number	β -element/g	Soybean oil/g	MCT /g	HSA/g	Soybean oil/API (g/g)	HSA/API (g/g)	Surfactant	Hemolysis value	Particle size (nm)	PDI
1	0.5	0.6	0.6g	1.5	1.2	3	0.1g HS-15	31.6	182.7	0.156
2	0.5	0.6	0.6g	1.5	1.2	3	0.1g Tween 80	22.2	178.3	0.135
3	0.5	0.6	0.6g	1.5	1.2	3	0.1g Poloxamer P188	53.3	232.5	0.143
4	1	0.4	0.1g	1.5	0.4	1.5	/	6.4	128.3	0.167
5	1	0.6	0.15g	1.5	0.6	1.5	/	9.1	127.6	0.191
6	1	0.4	0.1g	5	0.4	5	/	13.8	103.4	0.162
7	1	0.6	0.15g	5	0.6	5	/	7	101.2	0.092

Example 22

[0143] β -elemene and soybean oil were weighed according to the component amounts listed in Table 6 and mixed evenly, to obtain an oil phase. Human serum albumin (converted by mass as shown in Table 6) was measured, diluted to a total volume of 50 mL by adding ultrapure water and mixed evenly, to obtain an aqueous solution of human serum albumin, namely an aqueous phase. The aqueous phase was poured into the oil phase, and sheared with a mixer (IKA, T25) at 5,000 rpm for 1 min and at 10,000 rpm for 5 min to form a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer (ATS, AH-1500) for high-pressure homogenization at a homogenization pressure of 250-300 bar for 2 min, and at 1450-1550 bar for 6min. After homogenization, the total volume of the homogeneous solution was measured with a measuring cylinder, trehalose was added at a mass/volume ratio of 10% (g/mL), shaken well to be dissolved, and filtered with a 0.45 μ m microporous membrane to obtain a solution of elemene albumin nanoparticles. The particle size and PDI were detected, and the hemolysis value was determined. The specific results are shown in Table 6.

Table 6

Serial number	β -elemene /g	Soybean oil /g	HSA /g	Soybean oil/API (g/g)	HSA/API (g/g)	Hemolysis value	Particle size (nm)	PDI
1	1	0.5	1.5	0.5	1.5	7.8	130.1	0.161
2	1	0.75	1.5	0.75	1.5	5.2	132	0.16
3	1	0.5	5	0.5	5	8.3	111	0.084
4	1	0.75	5	0.75	5	3.8	111.8	0.071
5	1	0.5	10	0.5	10	13.7	94.99	0.107
6	0.5	1.2	1.5	2.4	3	15.1	130.3	0.079
7	0.5	1.2	5	2.4	10	13.1	131.1	0.08
8	0.5	1.2	7.5	2.4	15	11.7	132.9	0.085
9	0.5	1.2	10	2.4	20	13.5	127.3	0.067
10	0.5	1.2	1.5	2.4	3	11	143.2	0.161
11	1	0.35	1.5	0.35	1.5	9	115.43	0.132

Example 23

[0144] 3.33 g of β -elemene and 8g of soybean oil were weighed and mixed to obtain an oil phase. 50 mL (10g) of 20% human serum albumin (HSA) was measured, diluted evenly by adding an appropriate amount of ultrapure water, to obtain an aqueous solution of human serum albumin with 3% of mass volume percentage (g/mL), namely an aqueous phase. The aqueous phase solution was poured into the oil phase, and the mixer (FLUKO, FA30/30F) was used for shearing at 10,000 rpm for 10 min to form a primary emulsion. The obtained primary emulsion was transferred to a micro-jet homogenizer (Microfluidizer, M-110-EH) for homogenization, at a homogenization pressure of 20,000 psi for 30 min. The homogenized solution was filtered by 0.45 μ m and 0.22 μ m PVDF plate filters, and the metered volume was adjusted, and different lyoprotectants (g/mL) corresponding to Table 7 was added respectively. The homogeneous solution added with the lyoprotectant was lyophilized, and after lyophilization, the lyophilized powder was reconstituted with sterilized ultrapure water. The particle size, PDI and hemolysis value were detected. The specific results are shown in Table 7.

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Table 7

Serial number	Prescription	Protectant	Hemolysis value	Particle size	PDI
1	3.33 g β -elemene 8g Soybean oil 10g HSA	3% Sucrose	13.4	175.97	0.188
2		10% Sucrose	13.4	138.76	0.119
3		3% Trehalose	11.3	170.91	0.192
4		10% Trehalose	14	138.39	0.158

Example 24

[0145] 3.33 g of β -elemene and 8 g of soybean oil were weighed, dissolved in an organic solvent, and mixed to obtain an oil phase. The organic solvent was a mixed solvent of 26.67 mL dichloromethane and 6.67 mL anhydrous ethanol. 50 mL (10g) of 20% human serum albumin (HSA) was measured, diluted evenly by adding an appropriate amount of ultrapure water, to obtain an aqueous solution of human serum albumin with a mass volume percentage of 3% (g/mL), namely an aqueous phase. The aqueous phase solution was poured into the oil phase, and the mixer (FLUKO, FA30/30F) was used for shearing at 10,000 rpm for 10 min to form a primary emulsion. The obtained primary emulsion was transferred to a micro-jet homogenizer (Microfluidizer, M-110-EH) for high-pressure homogenization, at a homogenization pressure of 20,000 psi for 30 min. After homogenization, the organic solvent was removed by a thin film evaporator from the organic solvent-containing emulsion, and the parameters were: temperature 30°C, vacuum degree 4000 pa, liquid feeding speed 100 rpm, scraper speed 50 rpm. The obtained emulsion was filtered by 0.45 μ m and 0.22 μ m PVDF plate filters, and the metered volume was adjusted, and 10% (g/mL) of trehalose was added as a lyoprotectant. The homogeneous solution added with the lyoprotectant was lyophilized, and after lyophilization, the lyophilized powder was reconstituted with sterilized ultrapure water. The detected particle size was 76.28, the PDI was 0.254 and the hemolysis value was 16.3.

Example 25

[0146] β -elemene and soybean oil were weighed according to the component amounts listed in Table 8, and dissolved in an organic solvent, the organic solvent was a mixed solvent of 1 mL of ethanol and 4 mL of dichloromethane, and mixed to obtain an oil phase. An appropriate amount of 20% human serum albumin (HSA) (converted by mass as shown in Table 8) was measured, diluted to a total volume of 50 mL by adding ultrapure water, namely an aqueous phase. The aqueous phase solution was poured into the oil phase, and sheared with a mixer (IKA, T25) at 5,000 rpm for 1 min and 10,000 rpm for 5 min to form a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer (ATS, AH-1500) for high-pressure homogenization at a homogenization pressure of 250-300 bar for 2 min, and at 1450-1550 bar for 6min. The organic solvent was removed with a rotary evaporator at 35°C, 150 rpm and a vacuum of 30 mbar. After completion, the total volume of the homogeneous solution was measured with a measuring cylinder, trehalose was added at a mass/volume ratio of 10% (g/mL), shaken well to be dissolved, and filtered with a 0.45 μ m microporous membrane to obtain a solution of elemene albumin nanoparticles.

Table 8

Serial number	β -elemene /g	Soybean oil /g	HSA /g	Soybean oil/API (g/g)	HSA/API (g/g)
1	0.5	0.1	1.5	0.2	3
2	0.5	1.2	1.5	2.4	3
3	0.5	2.5	1.5	5	3
4	0.5	5	1.5	10	3
5	0.5	1.2	2.5	2.4	5
6	0.5	1.2	5	2.4	10
7	0.5	1.2	10	2.4	20

[0147] The prepared samples were placed in an oven at 30°C, and sampled at 0h, 24h, and 48h to determine particle size, PDI and β -elemene content. The measurement results are shown in Table 9.

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Table 9

Serial number	0h		24h			48h		
	Particle size	PDI	Particle size	PDI	Content change (%)	Particle size	PDI	Content change (%)
1	326.9	0.07	348.5	0.177	-10.14	317.8	0.094	-18.84
2	79.11	0.14	80.22	0.229	-3.19	77.04	0.164	-5.32
3	99.59	0.174	101.6	0.203	-2.56	101.7	0.183	-3.85
4	121.8	0.194	125.4	0.211	0	119	0.216	-1.16
5	88.17	0.187	97.63	0.237	-2.35	99.19	0.219	-4.71
6	82.47	0.259	99.54	0.362	-3.70	102.6	0.271	-6.17
7	77.82	0.283	102.9	0.261	-2.0	104	0.22	-8.69

Example 26

[0148] β -elemene and soybean oil were weighed according to the component amounts listed in Table 10, to obtain an oil phase. An appropriate amount of 20% human serum albumin (HSA) (converted by mass as shown in Table 10) was measured, diluted to a total volume of 50 mL by adding ultrapure water, namely an aqueous phase. The aqueous phase solution was poured into the oil phase, and sheared with a mixer (IKA, T25) at 5,000 rpm for 1 min and at 10,000 rpm for 5 min to form a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer (ATS, AH-1500) for high-pressure homogenization at a homogenization pressure of 250-300 bar for 2 min, and at 1350-1450 bar for 6 min. After completion, the total volume of the homogeneous solution was measured with a measuring cylinder, trehalose was added at the mass/volume ratio of (g/mL) shown in Table 10, shaken well to be dissolved, and filtered with a 0.45 μ m microporous membrane to obtain a solution of elemene albumin nanoparticles.

Table 10

Serial number	β -elemene/ g	Soybean oil /g	HSA /g	Soybean oil/API (g/g)	HSA/API (g/g)	Protectant (g/mL)
1	1	1	1.5	1	1.5	10% Trehalose
2	1	1.5	1.5	1.5	1.5	10% Trehalose
3	1	2	1.5	2	1.5	10% Trehalose
4	1	2.5	1.5	2.5	1.5	10% Trehalose
5	1	3	1.5	3	1.5	10% Trehalose
6	1	0.5	0.5	0.5	0.5	10% Trehalose
7	1	0.5	1	0.5	1	10% Trehalose
8	1	0.5	1.5	0.5	1.5	10% Trehalose
9	1	0.5	2	0.5	2	10% Trehalose
10	1	0.5	2.5	0.5	2.5	10% Trehalose
11	1	0.5	1.5	0.5	1.5	20% Trehalose
12	1	0.5	1.5	0.5	1.5	15% Trehalose
13	1	0.5	1.5	0.5	1.5	5% Trehalose

[0149] The prepared samples were placed in an oven at 30°C, and sampled at 0h, 24h, and 48h to determine particle size, PDI and β -elemene content. The measurement results are shown in Table 11.

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Table 11

Serial number	0h		24h			48h		
	Particle size	PDI	Particle size	PDI	Content change (%)	Particle size	PDI	Content change (%)
1	140.3	0.127	143.7	0.147	-3.60	135.5	0.165	-5.76
2	146.7	0.204	154.2	0.226	-3.64	149.5	0.208	-4.24
3	141.7	0.213	144.6	0.251	-1.83	141.4	0.265	-2.44
4	131.8	0.192	135.9	0.229	-3.05	132.3	0.222	-1.83
5	105.8	0.31	117.4	0.36	-1.24	106.8	0.285	-1.24
6	152.3	0.188	154.5	0.122	-3.59	151.3	0.13	-3.59
7	143.2	0.108	144.5	0.139	-3.85	142.2	0.129	-5.13
8	123.2	0.059	125.6	0.121	-4.19	121.6	0.089	-5.39
9	117.3	0.15	117.3	0.132	-4.79	116.8	0.126	-7.19
10	154.1	0.145	159.2	0.185	-4.17	163.5	0.167	-6.55
11	133.7	0.095	134.1	0.122	-4.61	131.5	0.118	-5.93
12	139.9	0.097	134	0.115	-5.63	128.4	0.194	-6.88
13	130.6	0.132	122.2	0.115	-4.14	128.7	0.159	-5.33

Example 27

[0150] 2g of β -elemene and 1.5g of soybean oil were weighed and mixed to obtain an oil phase. 45 mL (9 g) of 20% human serum albumin was measured, namely an aqueous phase. The aqueous phase solution was poured into the oil phase, and sheared with a mixer (IKA, T25) at 5,000 rpm for 1 min and 10,000 rpm for 5 min to form a primary emulsion. The obtained primary emulsion was transferred to a high-pressure homogenizer (ATS, AH-1500) for high-pressure homogenization at a homogenization pressure of 250-300 bar for 2 min, and at 1450-1550 bar for 6 min, and the homogeneous solution was collected. The pipeline of the homogenizer was flushed with an aqueous solution of trehalose (25 mL of water + 10 g of trehalose) to replace the homogeneous solution remaining in the homogenizer, and then the pipeline of the homogenizer was flushed with 30 mL of ultrapure water to replace the residual aqueous solution of trehalose. The trehalose aqueous solution was combined with the homogeneous solution, mixed, and filtered through a 0.22 μ m sterile filter membrane. The particle size was 106.4 nm detected by a particle size analyzer, PDI was 0.08.

Example 28

[0151]

Table 13

Serial number	Prescription (g)			Soybean/API (g/g)	HSA/API (g/g)	Protectant (g/mL)
	API	Soybean oil	HSA			
1	2	2	10	1	5	10% Trehalose
2	2	3	10	1.5	5	10% Trehalose
3	2	4	10	2	5	10% Trehalose
4	2	5	10	2.5	5	10% Trehalose
5	3	4.5	9	1.5	3	10% Trehalose

[0152] β -elemene and soybean oil were weighed as shown in Table 13 and mixed to obtain an oil phase. 20% human serum albumin (50 mL, 10 g) was measured, namely an aqueous phase. The aqueous phase solution was poured into the oil phase, and sheared with a mixer (IKA, T25) at 5,000 rpm for 1 min and at 10,000 rpm for 5 min to form a primary

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emulsion. The obtained solution was transferred to a high-pressure homogenizer (ATS, AH-1500) for high-pressure homogenization at a homogenization pressure of 250-300 bar for 2 min, and at 1550-1550 bar for 6 min. 10 g of trehalose was weighed and dissolved to 50 mL by adding pure water (in which sample No. 5 was added with 15 g of trehalose and dissolved to 105 mL by adding pure water). The pipeline of the homogenizer was flushed with the aqueous solution of trehalose to replace the homogeneous solution remaining in the homogenizer, and the residual homogeneous solution was combined with the homogeneous solution, mixed, and filtered through a 0.45 μ m sterile filter membrane. Each sample was tested for hemolysis value. And the sample was divided into EP tubes, stored in a thermotank at 30°C, and the samples were taken at 0h, 24h, and 48h to determine particle size, PDI and β -elemene content. The results are shown in Table 14.

Table 14

Serial number	0h		24h			48h			Hemolysis value
	Particle size	PDI	Particle size	PDI	Content change %	Particle size	PDI	Content change %	
1	102.9	0.124	99.7	0.090	-4.22%	99.8	0.085	-5.42%	6.0
2	113.7	0.112	108.2	0.095	-1.81%	107.9	0.082	-4.82%	9.3
3	120.9	0.034	128.9	0.052	-2.33%	128.3	0.106	-4.07%	17.4
4	126.4	0.070	126.4	0.168	-2.33%	132.4	0.138	-2.91%	16.5
5	111.8	0.089	111.0	0.108	-5.52%	109.1	0.066	-6.08%	10.2

Example 29

[0153] 2g of β -elemene and 1.5g of soybean oil were weighed and mixed to obtain an oil phase. 50 mL (10 g) of 20% human serum albumin was measured, namely an aqueous phase. The aqueous phase solution was poured into the oil phase, and sheared with a mixer (IKA, T25) at 5,000 rpm for 1 min and at 10,000 rpm for 5 min to form a primary emulsion. And 5 mL of water for injection was used for cleaning the shearing head, and combined with the primary emulsion. The obtained solution was transferred to a high-pressure homogenizer (ATS, AH-1500) for high-pressure homogenization at a homogenization pressure of 250-300 bar for 2 min, and at 1500-1550 bar for 6 min, and the homogeneous solution was collected. The pipeline of the homogenizer was flushed with 22.5 mL of an aqueous solution of trehalose to replace the homogeneous solution remaining in the homogenizer, and then the pipeline of the homogenizer was flushed with 22.5 mL of ultrapure water to replace the residual aqueous solution of trehalose. The trehalose solution was combined with the homogeneous solution, mixed, and filtered via a 0.22 μ m sterile filter membrane. The sample was divided into EP tubes, stored in refrigerator at 2-8°C, and the particle size and PDI were detected by sampling on the 0th day and second month, respectively. The results are shown in Table 12.

Table 12

Time	Particle size	PDI
Day 0	87.93	0.107
2nd month	89.39	0.119

Example 30 Drug Efficacy Study

Preparation of sample

[0154] 4.5 g of β -elemene and 10.8 g of soybean oil were weighed and mixed to obtain an oil phase. 4 mL of ethanol and 16 mL of dichloromethane were measured, and mixed evenly to obtain organic phase A. The oil phase was added to the organic phase and the metered volume was adjusted to 36 mL to obtain organic phase B.

[0155] 13.5 g of human serum albumin was weighed and added to 405 mL of purified water, stirred to be dissolved. The organic phase B was added to the aqueous phase, and stirred with a high-speed shearing machine at gear A for 2 min, and then at gear B for 1 min to obtain primary emulsion A. The primary emulsion A was divided into 3 parts, and treated by a cell disrupter at 250w for 8 min to obtain primary emulsion B. The primary emulsion B was added into a high-pressure homogenizer, firstly at 200 bar for 3 cycles, and then at 1300 bar for 5 cycles to obtain albumin nanoparticles.

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The above nanoparticles were concentrated under reduced pressure at 37°C for 1 h, added with 12.30 g of 3% sucrose, filtered at 0.22 μm, and lyophilized. The particle size was 81.03 nm, PDI was 0.174.

Experimental Methods:

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[0156] Establishment of a human-derived brain tumor xenograft mouse model: 50 nude mice were anesthetized with 2.5% isoflurane, a small hole (<0.5 mm) was drilled in the skull of the nude mice, and cells expressing the luminescent reporter gene (5×10^4 U87MGR1 Human glioma cells) were injected into the left forehead (2.5 mm lateral and 0.5 mm anterior bregma, 2.5 mm deep).

10

[0157] In vivo bioluminescence imaging assay method: nude mice received a single subcutaneous injection of D-luciferin solution (120 mg/kg). 10 min after injection of fluorescein, light images of animals were taken with dark multicolor illumination during an exposure time of 1-5 min (for bioluminescence imaging of Gluc, a single intravenous injection of coelenterazine (20 mg/kg, at total volume of 150 μL) was given). Intratumoral luciferase activity was obtained by recording photon counts with a CCD camera over 5 min without lighting. Image intensity was quantitatively analyzed using Perkin Elmer's Living image software 4.3.1.

15

[0158] One week after inoculation, in vivo bioluminescence imaging was performed to evaluate the tumor volume in nude mice. 32 nude mice with similar tumor volume were selected and randomly divided into 8 mice (half male and half female)/group, and the dose were administered by tail vein injection for 3 weeks, 3 times/week:

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- 1) model group (control) (0.1% DMSO prepared by PBS), n=8;
- 2) 50 mg/kg temozolomide (TMZ), n=8;
- 3) 50 mg/kg Ailineng (ELE mic), n=8;
- 4) 50 mg/kg (Alb), n=8;

25

[0159] Tumor volume was measured weekly.

[0160] During the experiment, the animals were weighed weekly to monitor their healthy conditions.

[0161] Dosing was discontinued after two weeks of administration, followed by an observation for three weeks. The survival and tumor volume of mice in each group were recorded using Graphpad software to generate Kaplan-Meier plots. The tumor growth curve of the mouse orthotopic tumor model is shown in Figure 8 (the tumor-bearing nude mice were treated with Alb formulation, 3 times/week 3 2 weeks (n=8, half male and half female); compared with temozolomide (TMZ), $p < 0.01$), the survival curve of the mouse orthotopic tumor model is shown in Figure 9 (the tumor-bearing nude mice were treated with Alb, 3 times/week 3 2 weeks (n=8, half male and half female); compared with temozolomide (TMZ), $p < 0.01$).

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Example 31 Safety Study

Preparation of sample

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[0162] Elemene injection (trade name: Ailineng): CSPC Yuanda (Dalian) Pharmaceutical Co., Ltd., batch number: 17050302)

[0163] β-elemene micelle formulation:

17.08 g of RH40 was weighed and heated at 60°C to be dissolved. 2 mL of β-elemene was added, heated and stirred at 60°C for 5 min. 14.40 g of propylene glycol and 38.72 g of purified water were weighed and mixed, then heated at 60°C for 10 min. The aqueous solution of propylene glycol was added to the RH40 solution containing β-elemene, and stirred in a water bath at 60°C for 30 min to obtain β-elemene micelles. The above micelles were filtered at 0.22 μm, and divided into 5 mL/per formulation.

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[0164] β-elemene albumin formulation:

2.5001 g of β-elemene and 6.001 g of soybean oil were weighed and mixed for later use. 2 mL of ethanol and 8 mL of dichloromethane were measured, and mixed evenly for later use. The volume of the oil phase was adjusted to 12 mL by the organic phase for later use.

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[0165] 4.5015 g of human serum albumin was weighed and added to 135 mL of purified water, stirred to be dissolved. The organic phase was added to the aqueous phase, and stirred with a high-speed shearing machine at gear A for 2 min, and then at gear B for 0.5 min to obtain primary emulsion A. The primary emulsion A was divided into 3 parts, and treated by a cell disrupter at 250W for 8 min to obtain primary emulsion B. The primary emulsion B was added into a high-pressure homogenizer, firstly at 200 bar for 3 cycles, and then at 1300-1400 bar for 5 cycles to obtain albumin nanoparticles. The above nanoparticles were concentrated under a reduced pressure at 37°C for 1 h, added with 1.20 g of sucrose, filtered at 0.22 μm, and lyophilized.

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Solution Preparation:

[0166] The three formulations were prepared into 6 mg/mL solution using normal saline.

[0167] Ailineng: 7.5 mL of 20 mg/mL commercially available Ailineng was taken and diluted into 25 mL of 6 mg/mL sample solution with 17.5 mL of normal saline.

[0168] β -elemene micelle formulation: 5.91 mL of 25.41 mg/mL β -elemene micelle formulation was taken and diluted into 25 mL of 6 mg/mL sample solution with 19.09 mL of normal saline.

[0169] β -elemene albumin formulation: 12 β -elemene micelle formulations were taken, 2.01 mL of normal saline was added to each one, then mixed to obtain 24 mL of 6 mg/mL sample solution.

Test Methods:

[0170] Male KM mice, 20-25 g/mouse, were randomly divided into 13 groups according to body weight, with 7 mice in each group. The mice were grouped according to the prescribed dose by weight. The dosage of each sample was set as 100 mg/kg, 130 mg/kg, 170 mg/kg and 220 mg/kg, and another blank group was set. The mice were injected through tail vein, and the site of administration, reaction after administration and death conditions were observed after administration. The results of the study are shown in Table 15.

Table 15 Safety studies of different samples

Dosage Sample	100 mg/kg	130 mg/kg	170 mg/kg	220 mg/kg
Ailineng	reacted intensely; respiratory distress; motionless	reacted intensely; respiratory distress; lethargic, motionless; started to recover in about 5 min	3 died; reacted intensely; respiratory distress; lethargic; motionless, occasionally ataxia	6 died; respiratory distress, extremely low physical activity.
β -elemene micelle formulation	much less stresses than Ailineng group; a few with respiratory distress	much less stresses than Ailineng group; respiratory distress, activity decreasing, and started to recover in about 2 min	reacted intensely; respiratory distress; lethargic, motionless	3 died; respiratory distress; motionless
β -elemene albumin formulation	no obvious discomfort	no obvious discomfort, occasional mild respiratory distress	no obvious discomfort, occasional mild respiratory distress	no obvious discomfort, occasional respiratory distress

Claims

1. A pharmaceutical composition comprising elemene, an oil for injection and a protein carrier.

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2. The pharmaceutical composition according to claim 1, wherein the pharmaceutical composition is in the form of particles, and the particle size of the particles is less than 180 nm, preferably 50-180 nm.
3. The pharmaceutical composition according to claim 2, wherein the particle size of the particles is 70-170 nm.
4. The pharmaceutical composition according to claim 3, wherein the particle size of the particles is 70-150 nm.
5. The pharmaceutical composition according to claim 1, wherein the elemene is selected from one or more of α -elemene, β -elemene, γ -elemene and δ -elemene.
6. The pharmaceutical composition according to claim 5, wherein the elemene is β -elemene.
7. The pharmaceutical composition according to claim 1, wherein the protein carrier is albumin, and the albumin is selected from one or both of human serum albumin and bovine serum albumin.
8. The pharmaceutical composition according to claim 7, wherein the protein carrier is human serum albumin.
9. The pharmaceutical composition according to claim 1, wherein the weight ratio of the albumin carrier to the elemene is 0.5-20.
10. The pharmaceutical composition according to claim 9, wherein the weight ratio of the albumin carrier to the elemene is 0.5-10.
11. The pharmaceutical composition according to claim 1, wherein the weight ratio of the oil for injection and the elemene is 0.35-10.
12. The pharmaceutical composition according to claim 11, wherein the weight ratio of the oil for injection and the elemene is 0.5-10.
13. The pharmaceutical composition according to any one of claims 1-12, wherein the oil for injection is one or more of soybean oil, safflower oil, cottonseed oil, corn oil, sunflower oil, peanut oil, olive oil, sesame oil, camellia oil, medium and long chain fatty glyceride or medium chain triglyceride.
14. The pharmaceutical composition according to any one of claims 1-13, further comprising a lyoprotectant.
15. The pharmaceutical composition according to claim 14, wherein the lyoprotectant is selected from one or more of glucose, sucrose, maltose, lactose, mannose, trehalose, glycine and dextran.
16. The pharmaceutical composition according to claim 15, wherein the lyoprotectant is trehalose or sucrose.
17. The pharmaceutical composition according to claims 14-16, wherein the ratio of the weight of the lyoprotectant to the volume of the solution of the pharmaceutical composition is 3:100-20:100 g/mL.
18. The pharmaceutical composition according to any one of claims 1-16, further comprising one or more of isoosmotic adjusting agent, antioxidant, preservative, and pH regulator.
19. The pharmaceutical composition according to claim 18, wherein the isoosmotic adjusting agent is one or more of glycerin, sorbitol, mannitol or glucose.
20. The pharmaceutical composition according to claim 18, wherein the pH regulator is one or more of sodium hydroxide, sodium citrate, citric acid, phosphoric acid, acetic acid or hydrochloric acid.
21. The pharmaceutical composition according to claim 18, wherein the preservative is selected from one or more of hydroxybenzene alkyl esters, benzoic acid, sodium benzoate, sorbic acid, chlorhexidine acetate, benzalkonium bromide.
22. The pharmaceutical composition according to claim 18, the antioxidant is selected from one or more of sodium sulfite, sodium bisulfite, sodium metabisulfite, sodium thiosulfate, ascorbic acid, butylated hydroxyanisole, 2,6-di-

tert-butylated hydroxytoluene, and vitamin E.

23. The pharmaceutical composition according to any one of claims 1-22, wherein no organic solvent is included during preparation process thereof.
24. The pharmaceutical composition according to claim 23, wherein the pharmaceutical composition is prepared in the form of particles, and the particle size of the particles is less than 180 nm.
25. The pharmaceutical composition according to claim 24, wherein the particle size of the particles is 70-150 nm.
26. The pharmaceutical composition according to any one of claims 23-25, wherein in the pharmaceutical composition, the weight ratio of the soybean oil to the elemene is about 0.35-3; the weight ratio of the human serum albumin to the elemene is about 0.5-20.
27. The pharmaceutical composition according to claim 26, wherein in the pharmaceutical composition, the weight ratio of the soybean oil to the elemene is about 0.5-3; the weight ratio of the human serum albumin to the elemene is about 0.5-2.5.
28. The pharmaceutical composition according to claim 26, wherein in the pharmaceutical composition, the weight ratio of the soybean oil to the elemene is about 0.35-1.5, preferably 0.35-0.75; the weight ratio of the human serum albumin to the elemene is about 1.5-10.
29. The pharmaceutical composition according to claim 28, wherein in the pharmaceutical composition, the weight ratio of the soybean oil to the elemene is about 0.5; the weight ratio of the human serum albumin to the elemene is about 1.5.
30. The pharmaceutical composition according to claim 28, wherein in the pharmaceutical composition, the weight ratio of the soybean oil to the elemene is about 0.75; the weight ratio of the human serum albumin to the elemene is about 5.
31. The pharmaceutical composition according to claim 28, wherein in the pharmaceutical composition, the weight ratio of the soybean oil to the elemene is about 1.5; the weight ratio of the human serum albumin to the elemene is about 3-5.
32. The pharmaceutical composition according to any one of claims 1-22, wherein organic solvent is included during preparation process thereof.
33. The pharmaceutical composition according to claim 32, wherein the organic solvent is selected from one or more of chloroform, dichloromethane, tertiary butanol, isopropanol, ethyl acetate, ethanol, tetrahydrofuran, dioxane, acetonitrile, acetone, dimethyl sulfoxide, dimethylformamide, and methylpyrrolidone.
34. The pharmaceutical composition according to claim 33, wherein the organic solvent is a mixed solvent of dichloromethane and ethanol.
35. The pharmaceutical composition according to claim 34, wherein the volume ratio of dichloromethane to ethanol is 1:1-8.
36. The pharmaceutical composition according to claim 35, wherein the volume ratio of dichloromethane to ethanol is 1:4.
37. The pharmaceutical composition according to any one of claims 33-36, wherein the particle size of the particles is about 70-150 nm.
38. The pharmaceutical composition according to claim 37, wherein in the pharmaceutical composition, the weight ratio of the soybean oil to the elemene is about 2.4-10; the weight ratio of the human serum albumin to the elemene is about 0.5-10.
39. The pharmaceutical composition according to claim 38, wherein in the pharmaceutical composition, the weight ratio of the soybean oil to the elemene is about 2.4; the weight ratio of the human serum albumin to the elemene is about 3.
40. A method for preparing the pharmaceutical composition of any one of claims 1-39, comprising the steps of:

- (1) mixing elemene and an oil for injection evenly, optionally adding an organic solvent, to obtain a solution of the elemene and the oil for injection; dissolving a protein carrier in water to obtain a protein carrier solution;
 (2) mixing the two solutions obtained in step (1) to form an emulsion;
 (3) homogenizing the emulsion in step (2) under a high pressure, and optionally evaporating the emulsion under a reduced pressure to remove the organic solvent, to obtain a nanoparticle solution.

41. The method according to claim 40, wherein the organic solvent is used in step (1), preferably dissolving the elemene in the organic solvent and then mixing with the oil for injection.
42. The method according to claim 41, wherein the oil for injection is soybean oil, the albumin carrier is human serum albumin; the weight ratio of the soybean oil to the elemene is about 2.4-10; the weight ratio of the human serum albumin to the elemene is about 0.5-10.
43. The method according to claim 40, further comprising a step of lyophilizing the obtained solution of protein carrier nanoparticles.
44. The method according to claim 40, wherein, in step (2), a shearing or ultrasonic method is used for mixing; and in step (3), a high-pressure homogenization or micro-jet homogenization method is used for homogenization.
45. Use of the pharmaceutical composition of any one of claims 1-39 in the preparation of a medicament for the prevention or treatment of cancer.
46. The use according to claim 45, the cancer is adrenocortical carcinoma, cryptogenic myeloid metaplasia, AIDS-related cancer, anal cancer, appendiceal cancer, astrocytoma, basal cell carcinoma, cholangiocarcinoma, bladder cancer, bone cancer, glioma, ependymoma, oligodendroglioma, meningioma, craniopharyngioma, hemangioblastoma, medulloblastoma, neuroectodermal tumor, visual pathway and hypothalamic glioma and glioblastoma, breast cancer, bronchial adenoma, carcinoid tumor, central nervous system lymphoma, cervical cancer, colon cancer, colorectal cancer, chronic myeloproliferative disorder, endometrial cancer, ependymoma, esophagus cancer, Ewing's tumor family, eye cancer, gallbladder cancer, gastrointestinal carcinoid tumor, gastrointestinal stromal tumor, germ cell carcinoma, gestational trophoblastic tumor, head and neck cancer, liver cancer, laryngeal cancer, leukemia, lip and oral cancer, lung cancer, lymphoma, medulloblastoma, melanoma, mesothelioma, metastatic neck squamous cell carcinoma, multiple endocrine neoplasia syndrome, myelodysplastic syndrome, myelodysplastic/myeloproliferative disease, carcinoma of nasal cavity and sinuses, nasopharyngeal cancer, neuroblastoma, neuroendocrine cancer, oropharyngeal cancer, brain tumor, bone metastasis cancer, gastric cancer, intestinal cancer, esophageal cancer, ovarian cancer, pancreatic cancer, breast cancer, skin cancer, parathyroid carcinoma, penile carcinoma, peritoneal cancer, pharyngeal carcinoma, pheochromocytoma, pineoblastoma and supratentorial primitive neuroectodermal tumor, pituitary tumor, pleuropulmonary blastoma, lymphoma, primary central nervous system lymphoma, pulmonary lymphangiomyomatosis, rectal cancer, kidney cancer, renal pelvis and ureter cancer, rhabdomyosarcoma, salivary gland cancer, skin cancer, small intestine cancer, squamous epithelial cell carcinoma, testicular cancer, throat cancer, thymoma and thymic carcinoma, thyroid cancer, urethral cancer, or vaginal cancer.
47. The use according to claim 46, wherein the cancer is lung cancer, liver cancer, esophageal cancer, nasopharyngeal cancer, brain tumor, bone metastasis cancer, gastric cancer, intestinal cancer, uterine cancer, cervical cancer, germ cell carcinoma, endometrial cancer, gestational trophoblastic tumor, breast cancer, skin cancer, lymphoma, leukemia, or malignant melanoma.
48. The use according to claim 47, wherein the cancer is brain tumor, preferably glioma, brain stem glioma, cerebellar or cerebral astrocytoma, malignant glioma, ependymoma, oligodendroglioma, meningioma, craniopharyngioma, hemangioblastoma, medulloblastoma, visual pathway and hypothalamic glioma or glioblastoma.
49. The use according to claim 48, wherein the cerebellar or cerebral astrocytoma is fibrous cell astrocytoma or diffuse astrocytoma or anaplastic (malignant) astrocytoma.
50. A method of treating a human disease comprising administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition of any one of claims 1-37.
51. The method according to claim 50, wherein the pharmaceutical composition is administered by parenteral, by inhalation, intraperitoneal, intravesical, intramuscular, intravenous, intratracheal, subcutaneous, intraocular, intrathecal,

transdermal, rectal or vaginal.

52. The method according to claim 51, wherein the administration is intravenous.
- 5 53. A pharmaceutical formulation, comprising the pharmaceutical composition of any one of claims 1-39.
54. The pharmaceutical formulation according to claim 53, wherein the pharmaceutical formulation is a pharmaceutical formulation for intravenous administration, a pharmaceutical formulation for parenteral administration, a pharmaceutical formulation for gastrointestinal administration, a pharmaceutical formulation for respiratory administration, a pharmaceutical formulation for vaginal administration or other suitable pharmaceutical formulation; the pharmaceutical formulation for respiratory administration is aerosol.
- 10 55. The pharmaceutical formulation according to claim 53, wherein the pharmaceutical formulation is a solid formulation, a liquid formulation or a gas formulation.
- 15 56. The pharmaceutical formulation according to claim 53, wherein the liquid formulation is a liquid formulation for injection, comprising elemene and a pharmaceutically acceptable carrier, wherein the pharmaceutically acceptable carrier comprises albumin and soybean oil.
- 20 57. The pharmaceutical formulation according to claim 55, the pharmaceutical formulation is a granule formulation, the particle size of the granule formulation is 70-150 nm, wherein in the composition, the weight ratio of the albumin to the elemene is 0.5-20, and the weight ratio of the oil for injection to the elemene is 0.35-10.
- 25 58. The pharmaceutical formulation according to claim 55, wherein the liquid formulation is a stable aqueous suspension reconstituted from a sterile lyophilized powder.
59. The pharmaceutical formulation according to claim 53, further comprising other drugs, including anticancer drugs.
- 30 60. The pharmaceutical formulation according to claim 59, wherein the anticancer drug comprises temozolomide, paclitaxel, docetaxel, taxane, gemcitabine, an anti-VEGF drug, or a PD-1 antibody drug.
61. The pharmaceutical formulation according to claim 60, wherein the anti-VEGF drug comprises AVASTIN, ranibizumab, aflibercept, or conbercept.
- 35 62. The pharmaceutical formulation according to claim 56, wherein the PD-1 antibody drug comprises nivolumab, pembrolizumab, toripalimab, sintilimab, cemiplimab, atezolizumab, avelumab, or durvalumab.
63. A sealed container comprising the pharmaceutical composition of any one of claims 1-39 or the pharmaceutical formulation of any one of claims 53-62.
- 40 64. The sealed container according to claim 63, wherein the sealed container is a unit-dose container or a multi-dose container.
65. The sealed container according to claim 63, wherein the pharmaceutical composition is a liquid composition or a dry solid composition.
- 45 66. The sealed container according to claim 63, wherein the pharmaceutical composition is lyophilized.
67. The sealed container according to claim 63, wherein the pharmaceutical composition is sterile.
- 50 68. The sealed container according to claim 63, wherein the sealed container is a prefilled syringe.

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Figure 1

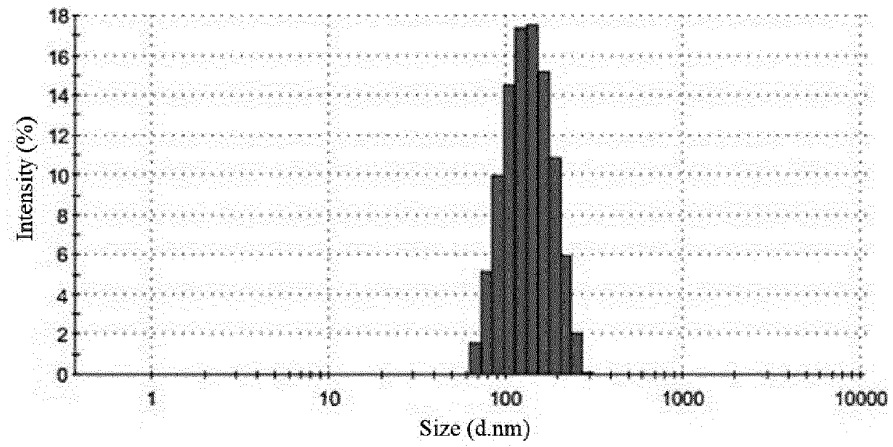


Figure 2

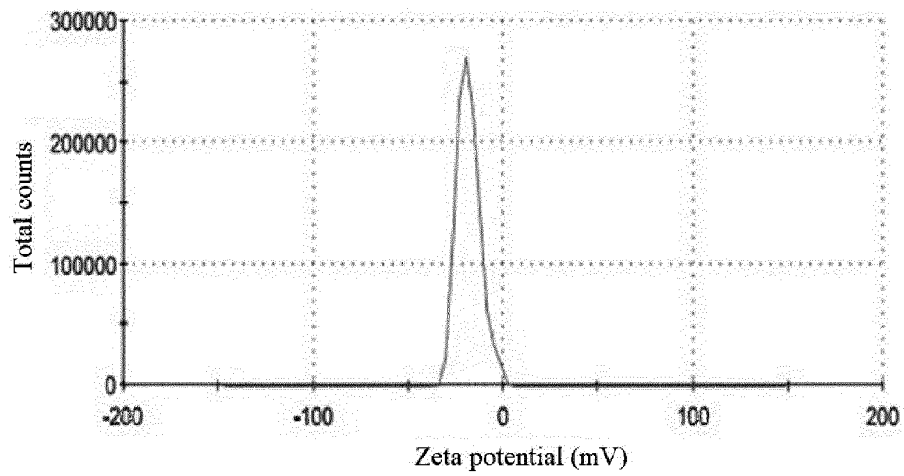


Figure 3

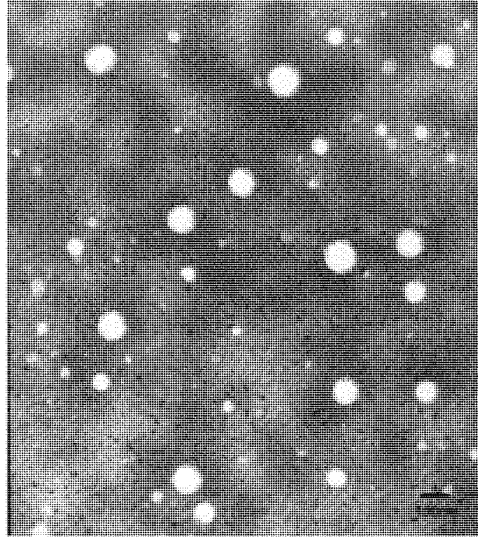


Figure 4

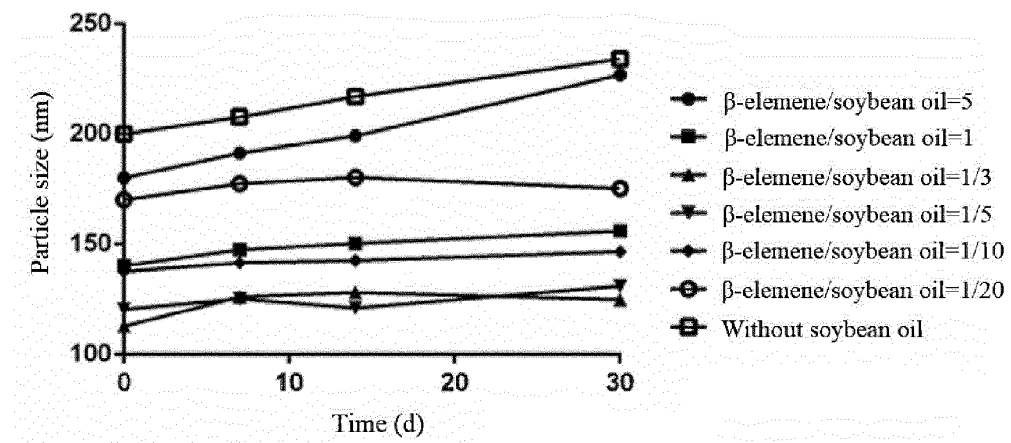


Figure 5

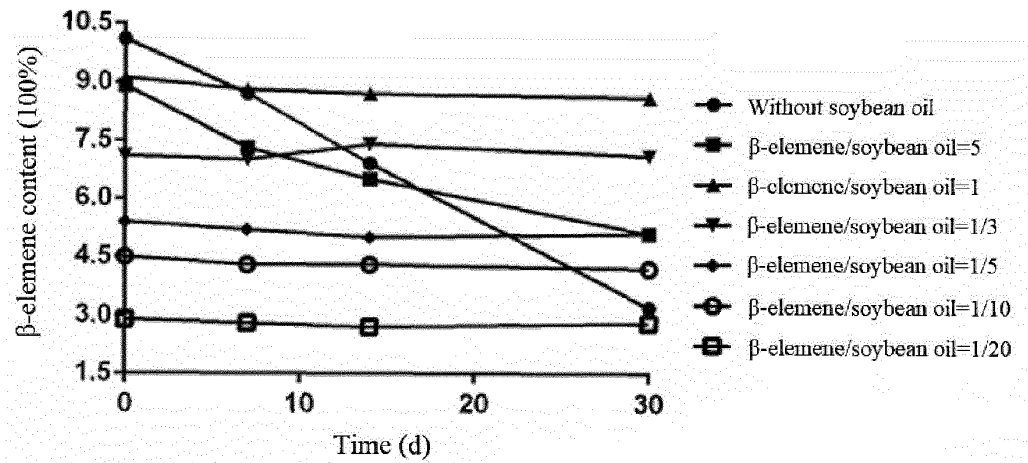


Figure 6

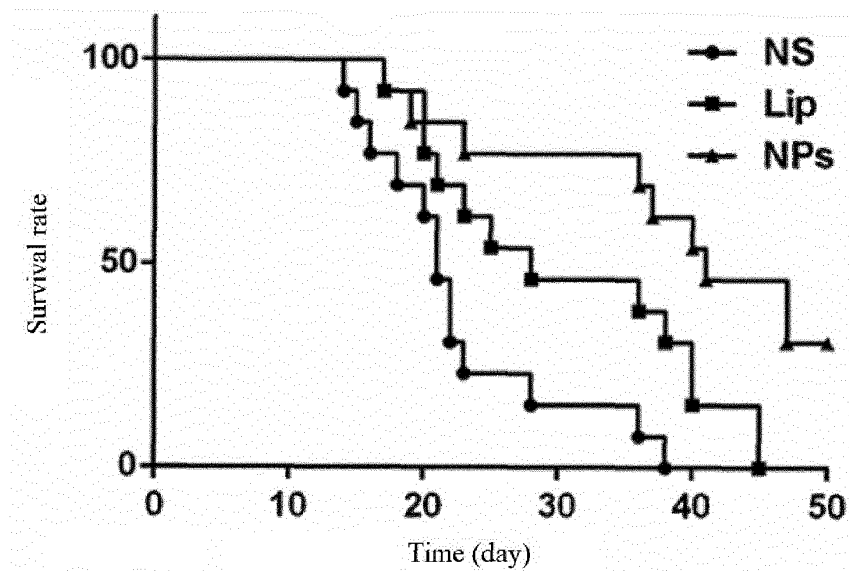


Figure 7

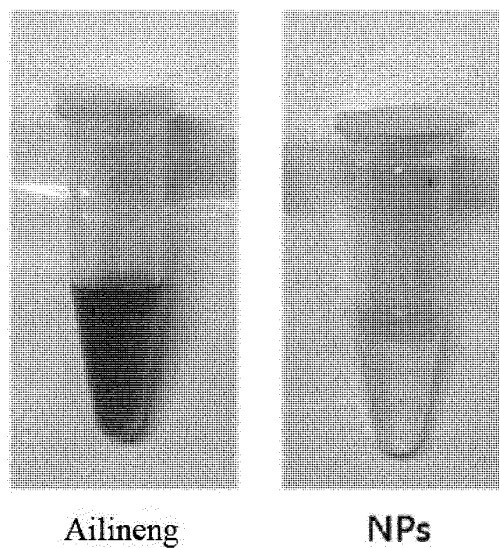


Figure 8

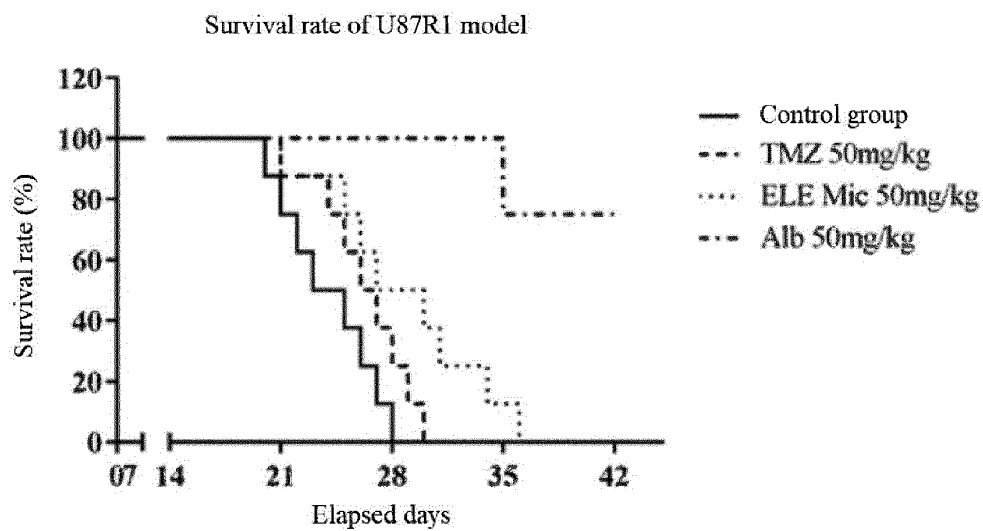
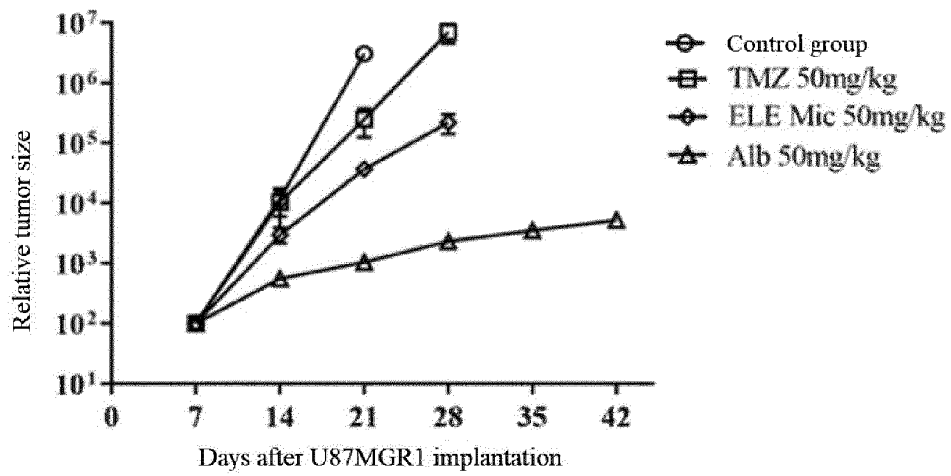


Figure 9



INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2020/133470

A. CLASSIFICATION OF SUBJECT MATTER

A61K 9/19(2006.01)i; A61K 47/44(2017.01)i; A61K 9/51(2006.01)i; A61K 47/42(2017.01)i; A61K 31/015(2006.01)i; A61P 35/00(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K:A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNABS, CNTXT, SIPOABS, DWPI, WOTXT, EPTXT, USTXT, CNKI, 万方, WANFANG, PUBMED, ISI-Web of Science, 成都康弘药业, 榄香烯, 白蛋白, 油, 注射, elemene+, albumin+, IgA, apolipoprotein, lipoprotein, glycoprotein, cancer, tumor?, thyroglobulin, thyroglobulin, transferrin?, 葵涛, 柯潇, 郑强, 叶亮

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2005186230 A1 (SD PHARMACEUTICALS, INC.) 25 August 2005 (2005-08-25) tables 1 and 2, and description, paragraphs [0177], [0043], [0143], [0145]-[0174], [0161]-[0165] and [0180]	1-68
Y	CN 101306181 A (XIE, Tian) 19 November 2008 (2008-11-19) claims 1-9	1-68
Y	刘艳菊 (LIU, Yanju). "荧光光谱及分子模拟研究 β -榄香烯与牛血清白蛋白的相互作用 (Study on Interaction between β -elemene and Bovine Serum Albumin by Fluorescence Spectroscopy and Molecular Modeling)" <i>中国测试 (China Measurement & Test)</i> , Vol. 41, No. 9, 30 September 2015 (2015-09-30), page 52, left column, lines 1-23, page 54, section 2.5, and page 55, section 3	1-68
Y	牛晓颖 (NIU, Xiaoying). "双光谱法研究 β -榄香烯甘氨酸与牛血清白蛋白的相互作用 (Study on the Interaction Between β -elemene Glycine and Bovine Serum Albumin by Double Spectroscopy)" <i>中医学报 (China Journal of Chinese Medicine)</i> , Vol. 34, No. 3, 31 March 2019 (2019-03-31), page 581, the last paragraph to page 582, paragraph 1, and page 585, the last paragraph	1-68

☒ Further documents are listed in the continuation of Box C.
 ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

19 February 2021

Date of mailing of the international search report

03 March 2021

Name and mailing address of the ISA/CN

China National Intellectual Property Administration (ISA/CN)
No. 6, Xitucheng Road, Jimenqiao, Haidian District, Beijing
100088
China

Authorized officer

Facsimile No. (86-10)62019451

Telephone No.

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2020/133470

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	沈展 (SHEN, Zhan). "转铁蛋白功能化的 β -榄香烯-雷公藤红素共传递微乳协同靶向抗结直肠癌研究 (Transferrin-functionalized Co-Delivery Microemulsion of β -elemene and Celastrol for Synergistic Anti-Colorectal Cancer Treatment)" <i>中草药 (Chinese Traditional and Herbal Drugs)</i> , Vol. 50, No. 2, 31 January 2019 (2019-01-31), page 472, left column, paragraph 3, and page 479, the last 2 paragraphs	1-68
A	CN 110404067 A (JINZHOU MEDICAL UNIVERSITY) 05 November 2019 (2019-11-05) entire document	1-68
A	CN 1200266 A (MEDICINAL SCIENCE & TECHNOLOGY DEVELOPMENT INSTITUTE, CHINA ACADEMY OF SCIENCE & TECHNOLOGY DEVELOPMENT) 02 December 1998 (1998-12-02) entire document	1-68
A	CN 1408347 A (CHINA GRAND ENTERPRISES, INC.) 09 April 2003 (2003-04-09) entire document	1-68
A	CN 101921164 A (LONG RANGE INTERNATIONAL USA, LTD.) 22 December 2010 (2010-12-22) entire document	1-68
A	CN 105998019 A (QINGDAO YUNTIAN BIOTECHNOLOGY CO., LTD.) 12 October 2016 (2016-10-12) entire document	1-68
A	CN 109562080 A (MONKAM NITCHEU GUY FAUSTIN) 02 April 2019 (2019-04-02) entire document	1-68
A	CN 108853513 A (JINGCHU UNIVERSITY OF TECHNOLOGY) 23 November 2018 (2018-11-23) entire document	1-68
A	CN 109330994 A (HANGZHOU PUSHKANG BIOTECHNOLOGY CO., LTD.) 15 February 2019 (2019-02-15) entire document	1-68
A	US 2004192641 A1 (None) 30 September 2004 (2004-09-30) entire document	1-68
A	US 2014045927 A1 (TIANJIN TASLY GROUP CO., LTD.) 13 February 2014 (2014-02-13) entire document	1-68

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2020/133470

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Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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1. ☒ Claims Nos.: **50-52**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] Claims 50-52 set forth a method for treating human diseases, which includes a treatment solution for human body, and does not comply with PCT rule 39.1(iv). The search on claims 50-52 is made on the basis of the following modification solution: the use of the pharmaceutical composition described in any one of claims 1-37 in the preparation of drugs for treating diseases.

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2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

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3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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Form PCT/ISA/210 (continuation of first sheet) (January 2015)

EP 4 070 786 A1

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2020/133470

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Patent document cited in search report			Publication date (day/month/year)		Patent family member(s)		Publication date (day/month/year)	
US	2005186230	A1	25 August 2005		None			
CN	101306181	A	19 November 2008		CN	100423714	C	08 October 2008
					CN	1451377	A	29 October 2003
					CN	101306181	B	29 June 2011
					CN	101306182	B	23 November 2011
					CN	101306182	A	19 November 2008
CN	110404067	A	05 November 2019		None			
CN	1200266	A	02 December 1998		CN	1084187	C	08 May 2002
CN	1408347	A	09 April 2003		CN	100374106	C	12 March 2008
CN	101921164	A	22 December 2010		EP	1773741	A2	18 April 2007
					US	8507562	B2	13 August 2013
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					RU	2007104176	A	20 August 2008
					JP	2008505893	A	28 February 2008
					EP	1773741	A4	09 July 2008
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CN	109562080	A	02 April 2019		MX	2019003685	A	26 September 2019
					FR	3058059	A1	04 May 2018
					ZA	201902910	B	24 June 2020
					CA	3043456	A1	03 May 2018
					WO	2018078539	A1	03 May 2018
					KR	20190077449	A	03 July 2019
					FR	3058059	B1	10 July 2020
					EP	3429568	A1	23 January 2019
					IL	266623	D0	31 July 2019
					FR	3058058	A1	04 May 2018
CN	108853513	A	23 November 2018		None			
CN	109330994	A	15 February 2019		None			
US	2004192641	A1	30 September 2004		None			
US	2014045927	A1	13 February 2014		US	2017007570	A1	12 January 2017
					US	2019216768	A1	18 July 2019
					US	2011077291	A1	31 March 2011

Form PCT/ISA/210 (patent family annex) (January 2015)

Electronic Acknowledgement Receipt	
EFS ID:	47038682
Application Number:	17384070
International Application Number:	
Confirmation Number:	1061
Title of Invention:	High Concentration VEGF Receptor Fusion Protein Containing Formulations
First Named Inventor/Applicant Name:	Kenneth S. Graham
Customer Number:	147803
Filer:	Lance A. Termes/Heather Kulin
Filer Authorized By:	Lance A. Termes
Attorney Docket Number:	125984-266215
Receipt Date:	16-NOV-2022
Filing Date:	23-JUL-2021
Time Stamp:	16:53:50
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	10430US02_IDS_Transmittal_11622.pdf	23712 ac99ee983e02224b3368568d8f13860c4c26b758a	no	3

Warnings:

Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	10430US02_IDS_SB08_111622.pdf	62159	no	3
			d0f612d55b43b3735e15bd6233c0f84ecbcaa86		
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Foreign Reference	459_WO2022089440A1.pdf	7295019	no	98
			3c9ef0b8e7f8d3ab3d52c71830401f29529898f1		
Warnings:					
Information:					
4	Foreign Reference	460_WO2022094340A1.pdf	14399173	no	140
			cb0349f8d3708b806a96e41e6b3b81c4f801ceb7		
Warnings:					
Information:					
5	Foreign Reference	445_WO2022101826A1.pdf	8690116	no	92
			54fa46f1736b552a85fa381dc23e38d00fe22fec		
Warnings:					
Information:					
6	Foreign Reference	449_WO2022175601A1.pdf	4971390	no	58
			0aa32bda3d64ff4fa15e602d2fcfef264e1d354a		
Warnings:					
Information:					
7	Foreign Reference	444_WO2022195505A1.pdf	3293287	no	40
			ccb4b85728f202c5700a67593943df8aa941dab5		
Warnings:					
Information:					
8	Foreign Reference	462_WO2022201084A1.pdf	7242159	no	73
			6c8a1f6883dd5f8706d854db6736d7ad6f21666a		
Warnings:					
Information:					

9	Foreign Reference	463_WO2022217110A1.pdf	20521777	no	202
			0a8c5363e4661a29a8d058551b0690c92352e59f		
Warnings:					
Information:					
10	Foreign Reference	454_EP3503883_2.pdf	1020404	no	36
			2f7130b6c32165bfc9eb581316cfacf130a8cb94		
Warnings:					
Information:					
11	Foreign Reference	455_EP3716992B1_2.pdf	2495950	no	64
			cd8497ce5b708b6fdbf8f1725139bc11884f5ea4		
Warnings:					
Information:					
12	Foreign Reference	450_EP4070786A1_2.pdf	1291174	no	39
			79a3bb0cf71a24dc87f7707a3bfe3f80a0808460		
Warnings:					
Information:					
13	Non Patent Literature	442_NPL-Naoto_2013_2.pdf	422625	no	23
			c17d648fd87347a4572c6eea741c4b62619b81		
Warnings:					
Information:					
Total Files Size (in bytes):			71728945		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Kenneth S. Graham, et al.

Application No.: 17/384,070

Filed: Jul-23-2021

For: HIGH CONCENTRATION VEGF
RECEPTOR FUSION PROTEIN
CONTAINING FORMULATIONS

Customer No.: 147803

Confirmation No.: 1061

Examiner: Not Yet Assigned

Art Unit: Not Yet Assigned

SUPPLEMENTAL INFORMATION
DISCLOSURE STATEMENT UNDER
37 CFR §1.97 and §1.98

Date of Transmission: November 16, 2022

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

The references cited on the attached IDS are being called to the attention of the Examiner. Copies of the references in compliance with the requirements of 37 CFR §1.98(a)(2) are submitted herewith.

It is respectfully requested that the cited references be expressly considered during the prosecution of this application, and the references be made of record therein and appear among the “references cited” on any patent to issue therefrom.

The cited references may include office actions from related cases. The Examiner is encouraged to review the full prosecution history from related cases. Applicant understands

that the prosecution history can be viewed electronically on PALM. Nevertheless, if the Examiner wishes Applicant to provide copies of any documents from related applications to the extent not already provided, he or she is requested to contact the undersigned.

Applicant cites the following applications, which are directed to related subject matter and are commonly owned or under an assignment obligation to be commonly owned:

Attorney Docket No.	Application No.	Filing Date	Publication No.	Status
125984-248591	62/669,506	05-10-2018	N/A	Expired
125984-248592	62/752,127	10-29-2018	N/A	Expired
125984-248593	62/769,876	11-20-2018	N/A	Expired
125984-248594	62/813,882	03-05-2019	N/A	Expired
125984-248595	16/409,631	05-10-2019	US 11,103,552	Issued
125984-248596	PCT/US2019/031879	05-10-2019	WO 2019/217927	Expired

As provided for by 37 CFR §1.97(g) and (h), no inference should be made that the information and references cited are prior art merely because they are in this statement and no representation is being made that a search has been conducted or that this statement encompasses all the possible relevant information.

Applicant believes that no fee is required for submission of this statement.

However, if a fee is required in connection with this paper, the Commissioner is authorized to deduct such fee from the undersigned's Deposit Account No. 500393.

Respectfully submitted,

/Lance A. Termes/

Lance A. Termes
Reg. No. 43184

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1211 SW 5th Avenue, Suite 1900
Portland, OR 97204
Tel: 503-222-9981

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (not for submission under 37 CFR 1.99)	Application Number	17/384,070
	Filing Date	Jul-23-2021
	First Named Inventor	Kenneth S. Graham
	Art Unit	Not Yet Assigned
	Confirmation Number	1061
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	125984-266215 / 10430US02

U.S. PATENTS						
Examiner Initial*	Cite No	Patent Number	Kind Code ¹	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear
	415	11,160,881		11-02-2021	Rangaramanujam et al.	
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	417	11,185,635		11-30-2021	Bryant et al.	
	418	11,209,444		12-28-2021	Baldwin et al.	
	419	11,241,498		02-08-2022	Tang et al.	
	420	11,253,572		02-22-2022	Yancopoulos et al.	

U.S. PATENT APPLICATION PUBLICATIONS						
Examiner Initial*	Cite No	Publication Number	Kind Code ¹	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear
	421	2021-0340605		11-04-2021	Perlee et al.	
	422	2021-0353713		11-18-2021	WEXLER-COHEN et al.	
	423	2021-0361769		11-25-2021	KAUVAR et al.	
	424	2021-0369616		12-02-2021	TALLEY	
	425	2021-0371457		12-02-2021	Casteels et al.	
	426	2021-0379012		12-09-2021	Corson et al.	
	427	2022-0025032		01-27-2022	BIGELOW et al.	
	428	2022-0031952		02-03-2022	BRYANT et al.	
	429	2022-0054586		02-24-2022	KIM et al.	
	430	2022-0079876		03-17-2022	Blizzard et al.	
	431	2022-0087939		03-27-2022	PATEL et al.	
	432	2022-0111015		04-14-2022	CONSTABLE et al.	
	433	2022-0112278		04-14-2022	LI et al.	

FOREIGN PATENT DOCUMENTS							
Examiner Initial*	Cite No	Foreign Document Number ³	Country Code ²	Kind Code ⁴	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear
	434	3640643	EP	A1	12-22-2021	DREYMAN et al.	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (not for submission under 37 CFR 1.99)	Application Number	17/384,070
	Filing Date	Jul-23-2021
	First Named Inventor	Kenneth S. Graham
	Art Unit	Not Yet Assigned
	Confirmation Number	1061
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	125984-266215 / 10430US02

FOREIGN PATENT DOCUMENTS								
Examiner Initial*	Cite No	Foreign Document Number ³	Country Code ²	Kind Code ⁴	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear	T ⁵
	435	21/252647	WO	A1	12-16-2021	ALKAHEST INC.		
	436	22/008699	WO	A1	01-13-2022	F. HOFFMANN-LA ROCHE AG		
	437	22/011323	WO	A1	01-13-2022	ICONIC THERAPEUTICS, INC.		
	438	22/013171	WO	A1	01-20-2022	MERCK PATENT GMBH		
	439	22/026510	WO	A1	02-03-2022	SEAGEN INC.		
	440	22/038038	WO	A2	02-24-2022	EPO EXPERIMENTELLE PHARMAKOLOGIE & ONKOLOGIE BERLIN-BUCH GMBH		
	441	22/056326	WO	A1	03-17-2022	ELIXIR MEDICAL CORPORATION		

NON-PATENT LITERATURE DOCUMENTS			
Examiner Initial*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, pages(s), volume-issue number(s), publisher, city and/or country where published	T ₅

EXAMINER SIGNATURE	
Examiner Signature	Date Considered
<p>*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.</p>	
<p>¹ See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. ² Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). ³ For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. ⁴ Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. ⁵ Applicant is to place a check mark here if English language translation is attached.</p>	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (not for submission under 37 CFR 1.99)	Application Number	17/384,070
	Filing Date	Jul-23-2021
	First Named Inventor	Kenneth S. Graham
	Art Unit	Not Yet Assigned
	Confirmation Number	1061
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	125984-266215 / 10430US02

CERTIFICATION STATEMENT			
Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):			
☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).			
OR			
☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).			
☐ See attached certification statement.			
☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.			
☒ None			
SIGNATURE			
A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.			
Signature	/Lance A. Termes/	Date (YYYY-MM-DD)	2022-05-16
Name/Print	Lance A. Termes	Registration Number	43184

(19)



(11)

EP 3 640 643 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
22.12.2021 Bulletin 2021/51

(51) Int Cl.:
G01N 33/574 (2006.01)

(21) Application number: **19201633.5**

(22) Date of filing: **24.01.2017**

(54) **METHOD FOR PREDICTING THE OUTCOME OF A TREATMENT WITH AFLIBERCEPT OF A PATIENT SUSPECTED TO SUFFER FROM A CANCER BY MEASURING THE LEVEL OF A PLASMA BIOMARKER**

VERFAHREN ZUR VORHERSAGE DES AUSGANGS EINER BEHANDLUNG MIT AFLIBERCEPT EINES PATIENTEN MIT VERDACHT AUF KREBSLEIDEN DURCH MESSUNG DES PLASMABIOMARKERSPIEGELS

PROCÉDÉ PERMETTANT DE PRÉDIRE LE RÉSULTAT D'UN TRAITEMENT AVEC AFLIBERCEPT D'UN PATIENT SUSPECTÉ DE SOUFFRIR D'UN CANCER PAR MESURE DU NIVEAU D'UN BIOMARQUEUR DE PLASMA

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR

(30) Priority: **25.01.2016 EP 16305065**

(43) Date of publication of application:
22.04.2020 Bulletin 2020/17

(62) Document number(s) of the earlier application(s) in
accordance with Art. 76 EPC:
17701686.2 / 3 408 673

(73) Proprietor: **Sanofi**
75008 Paris (FR)

(72) Inventors:
• **DREYMANN, Jennifer**
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• **CHIRON-BLONDEL, Marielle**
75008 Paris (FR)

(74) Representative: **Sanofi**
54, rue La Boétie
75008 Paris (FR)

(56) References cited:
WO-A1-2016/008975 US-B1- 6 749 853

- **DIETHER LAMBRECHTS ET AL:** "Evaluation of efficacy and safety markers in a phase II study of metastatic colorectal cancer treated with aflibercept in the first-line setting", **BRITISH JOURNAL OF CANCER**, vol. 113, no. 7, 10 September 2015 (2015-09-10), pages 1027-1034, XP055283970, GB ISSN: 0007-0920, DOI: 10.1038/bjc.2015.329
- **J. BAAR ET AL:** "A Vasculature-Targeting Regimen of Preoperative Docetaxel with or without Bevacizumab for Locally Advanced Breast Cancer: Impact on Angiogenic Biomarkers", **CLINICAL CANCER RESEARCH**, vol. 15, no. 10, 5 May 2009 (2009-05-05), pages 3583-3590, XP055284319, US ISSN: 1078-0432, DOI: 10.1158/1078-0432.CCR-08-2917
- **MARKUS WEHLAND ET AL:** "Biomarkers for Anti-Angiogenic Therapy in Cancer", **INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES** 2012 MDPI AG CHE, vol. 14, no. 5, 1 January 2013 (2013-01-01), pages 9338-9364, XP055283946, ISSN: 1661-6596, DOI: 10.3390/ijms14059338
- **J. F. DE GROOT ET AL:** "Myeloid Biomarkers Associated with Glioblastoma Response to Anti-VEGF Therapy with Aflibercept", **CLINICAL CANCER RESEARCH**, vol. 17, no. 14, 1 June 2011 (2011-06-01), pages 4872-4881, XP055367887, US ISSN: 1078-0432, DOI: 10.1158/1078-0432.CCR-11-0271

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

EP 3 640 643 B1

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| <ul style="list-style-type: none">• M. CHIRON ET AL: "Differential Antitumor Activity of Aflibercept and Bevacizumab in Patient-Derived Xenograft Models of Colorectal Cancer", MOLECULAR CANCER THERAPEUTICS, vol. 13, no. 6, 31 March 2014 (2014-03-31) , pages 1636-1644, XP055367889, US ISSN: 1535-7163, DOI: 10.1158/1535-7163.MCT-13-0753 | <ul style="list-style-type: none">• CLAUS ZEHETNER ET AL: "Systemic Counterregulatory Response of Placental Growth Factor Levels to Intravitreal Aflibercept Therapy", INVESTIGATIVE OPHTHALMOLOGY & VISUAL SCIENCE, vol. 56, no. 5, 21 May 2015 (2015-05-21), pages 3279-3286, XP055367860, US ISSN: 1552-5783, DOI: 10.1167/iovs.15-16686 |
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Description

[0001] The present invention concerns the use of PIGF as a biomarker for predicting the outcome of the treatment with aflibercept, or ziv-aflibercept, of a patient suspected to suffer from a colon cancer, a colorectal cancer or a rectal cancer.

[0002] Aflibercept, or ziv-aflibercept, also referred to as VEGFR1R2-Fc.DELTA.C1 Flt1D2.Flk1D3.Fc.DELTA.C1 or AVE0005, is a homo dimer protein, with each dimer comprising two identical monomers, each of which is a fusion protein comprising the signal sequence of VEGFR1 fused to the D2 Ig domain of the VEGFR1 receptor, itself fused to the D3 Ig domain of the VEGFR2 receptor, in turn fused to the Fc domain of IgG1.

[0003] The protein chain is glycosylated, with N-acetyl-glucosamine, fucose, galactose, mannose and sialic acids contributing to the carbohydrate structures. The N-linked oligosaccharides consist of mainly bi-antennary structures with zero, one or two terminal sialic acids. The monomer has the amino acid sequence SEQ ID N°1.

[0004] The U.S. Food and Drug Administration (FDA) already approved aflibercept under the trade name EYLEA® for the treatment of patients with neovascular (wet) age-related macular degeneration (AMD). In particular, EYLEA® is the trade name for aflibercept as generated, processed and formulated for intravitreal injection.

[0005] At the time of registration of aflibercept (ZALTRAP®) for cancer indication, and in light of aflibercept's approved use in treating AMD, the FDA requested that a different name (ziv-aflibercept) be given for the compound's use in the treatment of cancer. Thus, ziv-aflibercept is the United States Adopted Name (USAN) accepted by FDA to designate a pharmaceutical composition comprising aflibercept as generated, processed and formulated for injection via intravenous infusion. Ziv-aflibercept has been approved by the FDA for sale under the tradename ZALTRAP® for the treatment of metastatic colorectal cancer (mCRC).

[0006] The European Medicines Agency (EMA) approved ZALTRAP® as well however did not request separate names for the compound. Thus, in the European Union the name "aflibercept" is used regardless of the indication.

[0007] ZALTRAP® and EYLEA® are obtained by slightly different processes. They both contain aflibercept or ziv-aflibercept, but the ratio of aggregates of aflibercept or ziv-aflibercept is slightly different in ZALTRAP® and EYLEA®.

[0008] ZALTRAP® approval was based on data obtained from the VELOUR trial - a multicenter, randomized, placebo-controlled phase III trial, which compared the efficacy of aflibercept *versus* placebo in combination with the FOLFIRI regimen for patients with mCRC previously treated with an oxaliplatin containing regimen.

[0009] Despite the efficacy and the safety of the treatment of cancer by aflibercept it remains a goal to better identify patients who should benefit more from the treatment. Indeed the ability to identify Metastatic Colorectal Cancer (mCRC) patients who will benefit from aflibercept would further improve clinical utility of this drug.

[0010] To date, no validated predictive serum or plasma biomarkers have been identified that correlate with treatment outcomes to aflibercept.

[0011] The profiling of tumor and plasma samples derived from patients involved in clinical trials and subsequent analysis of their genomic / proteomic and clinical data could allow the discovery and potential validation of predictive biomarkers.

[0012] It has now been discovered that high levels of VCAM-1, ICAM-1 and/or PIGF at baseline correlated with shorter survival times.

[0013] Vascular cell adhesion molecule-1 (VCAM-1) also known as CD106 has the sequence SEQ ID N°1 (NCBI Reference Sequence: NP_001069.1). The term "VCAM-1" encompasses its homologues and isoforms and variants thereof, as well as fragments of the sequences, provided that the variant proteins (including isoforms), homologous proteins and/or fragments are recognized by one or more VCAM-1 specific antibodies.

[0014] Intercellular Adhesion Molecule 1 (ICAM-1) also known as CD54 has the sequence SEQ ID N°2 (NCBI Reference Sequence: NP_000192.2). The term "ICAM-1" encompasses its homologues and isoforms and variants thereof, as well as fragments of the sequences, provided that the variant proteins (including isoforms), homologous proteins and/or fragments are recognized by one or more ICAM-1 specific antibodies.

[0015] "Placental Growth Factor" or "PIGF" comprises the 2 isoforms PIGF1 et PIGF2 which have respectively the sequences SEQ ID N°3 (NCBI Reference Sequence: NP_002623.2) and SEQ ID N°4 (NCBI Reference Sequence: NP_001193941.1). The term "PIGF" encompasses PIGF1 et PIGF2 their homologues and isoforms and variants thereof, as well as fragments of the sequences, provided that the variant proteins (including isoforms), homologous proteins and/or fragments are recognized by one or more PIGF specific antibodies.

[0016] The correlation of ICAM-1 with patient outcome was tested in clinical trials where patients were treated with bevacizumab.

[0017] The correlation of ICAM-1 with patient outcome was found in a trial wherein lung cancer patients were treated with bevacizumab, cisplatin and etoposide (Horn et al, J Clin Oncol 2009; 27:6006-6011). The authors found that "patients who had high ICAM levels had a non significant trend towards improved OS compared with patients who had low levels", whereas in another study (Dowlati et al, 2008, Clin Cancer Res 14(5), 1407) "Patients with low baseline ICAM had a higher response than those with high ICAM".

[0018] But the correlation of ICAM-1 with patient outcome was not found in colorectal cancer patients treated with bevacizumab, capecitabine and oxaliplatin (Liu et al; Cancer Medicine 2013; 2(2): 234-242).

[0019] These articles show the level of unpredictability between various biomarkers studies with the same biologics i.e. bevacizumab.

[0020] Furthermore bevacizumab is an anti-VEGF-A antibody. Aflibercept is not an antibody but a chimeric protein. It consists of portions of extracellular domains of human VEGF receptors 1 and 2 fused to human IgG1 Fc portion. Aflibercept binds not only to VEGF-A but also to VEGF-B and placental growth factor (PlGF).

[0021] Thus bevacizumab and aflibercept have structure and functions that are very different and the man skilled in the art would not transpose directly the results obtained with bevacizumab to aflibercept.

[0022] The correlation of VCAM-1 with patient outcome was found in the two clinical trials mentioned above (Liu et al; Cancer Medicine 2013; 2(2): 234-242 and Horn et al, J Clin Oncol 2009; 27:6006-6011). However in these two studies there is not a placebo arm to let us know if the effect is predictive or prognostic.

[0023] Wehland et al. (Int J Mol Sci. 2013 May; 14(5): 9338-9364) is a review on biomarkers for anti-angiogenic therapy in Cancer, concluding that no general or cancer-specific biomarker has yet emerged, which could help select patients with a positive prognosis for anti-angiogenic therapy.

[0024] De Groot et al. (Clin Cancer Res. 2011 July 15; 17(14): 4872—4881) reports compelling evidence that elevated levels of myeloid-associated chemokines such as MIF, IP-10, MCP3/CCL7, and CTACK/ CCL27 are predictive of radiographic response, and that a decrease in VEGFR1+ monocytes from baseline to 24 hours was associated with response, both in in patients with glioblastoma.

[0025] Chiron et al. (Mol Cancer Ther. 2014 Jun;13(6):1636-44) indicates that aflibercept was more active in a greater number of colorectal cancer Patient-Derived Xenograft (PDX) models than bevacizumab.

[0026] Zehetner et al. (Invest Ophthalmol Vis Sci. 2015 May;56(5):3279-86) reports a significant systemic upregulation of the proangiogenic cytokine PlGF after intravitreal administration of aflibercept, and that might represent a counter-regulatory response to antiangiogenic therapy.

[0027] Lambrechts et al. (British Journal of Cancer (2015) 113, 1027-1034) describes that high IL8 plasma levels at baseline is associated with worse PFS in patients having metastatic colorectal cancer and treated with aflibercept, suggesting that IL8 may act as a potentially predictive biomarker of aflibercept treatment outcome. WO2016008975 discloses the use of interleukin-8 (IL-8) as a biomarker for predicting the outcome of the treatment with aflibercept, or ziv-aflibercept of a patient suspected to suffer from a cancer.

[0028] The invention relates to the use of PlGF as a biomarker for predicting the outcome of the treatment with aflibercept, or ziv-aflibercept of a patient suspected to suffer from a colon cancer, a colorectal cancer or a rectal cancer.

[0029] In one aspect, the present invention provides a method of determining whether a patient suspected to suffer from a colon cancer, a colorectal cancer or a rectal cancer is a candidate for aflibercept, or ziv-aflibercept therapy for the said cancer comprising the step of subjecting a patient's biological sample to at least one assay to measure at baseline the level of PlGF, wherein when the biological sample of the biomarker level is low relative to a reference level of expression of PlGF, the patient is identified as a candidate for therapy for cancer.

[0030] In another aspect, the present invention provides a method of determining whether a patient suspected to suffer from a colon cancer, a colorectal cancer or a rectal cancer is a candidate for aflibercept, or ziv-aflibercept therapy for the said cancer comprising the step of subjecting a patient's biological sample to at least one assay to measure at baseline the level of PlGF, wherein when the biological sample level of the biomarker is high relative to a reference level of expression of PlGF, the patient is identified as not being a candidate for therapy for cancer. The threshold or reference level of expression of PlGF allows to define sensitive and non-sensitive populations.

[0031] In an embodiment the reference level of expression of PlGF is comprised between around 12 ng/mL and around 19 ng/mL. Yet in another embodiment the reference level of expression of PlGF is around 17 ng/mL.

[0032] The invention relates also to a method for treating a patient with a colon cancer, a colorectal cancer or a rectal cancer with aflibercept, or ziv-aflibercept, comprising administering a therapeutically effective amount of aflibercept, or ziv-aflibercept to the patient, wherein the level of PlGF in the patient's biological sample is low relative to a reference level of expression of PlGF.

[0033] Yet in another aspect the invention relates to a method for improving the progression- free survival (PFS) and/or the overall survival (OS) of a patient with a colon cancer, a colorectal cancer or a rectal cancer, comprising administering a therapeutically effective amount of aflibercept, or ziv-aflibercept to the patient, wherein the level of PlGF in the patient's biological sample is low relative to a reference level of expression of PlGF.

[0034] In an embodiment of one of the methods described above the biological sample is chosen from the group consisting of blood, serum and plasma.

[0035] In an embodiment the colorectal cancer is a metastatic colorectal cancer.

[0036] In another embodiment of the invention, the subject is treated with aflibercept and further undergoes a chemotherapeutic treatment with oxaliplatin, 5-fluorouracil (5-FU) and folinic acid (i.e. the FOLFOX treatment), folinic acid, 5-fluorouracil and irinotecan (i.e. the FOLFIRI treatment), or 5-fluorouracil and folinic acid (i.e. the FUFOL or LV5FU2

treatment).

[0037] The chemotherapeutic treatment may combine at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or at most 10, 9, 8, 7, 6, 5, 4, 3, 2 agents, such as e.g. a combination of oxaliplatin, 5-fluorouracil (5-FU) and folinic acid (i.e. the FOLFOX treatment or the modified FOLFOX6 treatment as described in the example below), a combination of folinic acid, 5-fluorouracil and irinocetan (i.e. the FOLFIRI treatment), or a combination of 5-fluorouracil and folinic acid (i.e. the FUFOL or LV5FU2 treatment).

[0038] In this regard the application WO2012146610 relates to a method of treatment of the mCRC by aflibercept, or ziv-aflibercept in combination with FOLFIRI.

[0039] In an embodiment of one of the methods described above therapeutically effective amounts of aflibercept, or ziv-aflibercept, oxaliplatin, 5-fluorouracil (5-FU) and folinic acid are administered to said patient.

[0040] In an embodiment of one of the methods described above therapeutically effective amounts of aflibercept, or ziv-aflibercept, folinic acid, 5-fluorouracil (5-FU) and irinocetan are administered to said patient.

[0041] In a further embodiment of one of the methods described above folinic acid at a dosage comprised between about 200 mg/m² and about 600 mg/m², 5-fluorouracil (5-FU) at a dosage comprised between about 2000 mg/m² and about 4000 mg/m², irinocetan at a dosage comprised between about 100 mg/m² and about 300 mg/m² and aflibercept at a dosage comprised between about 1 mg/kg and about 10 mg/kg are administered to patient.

[0042] In a further embodiment of one of the methods described above folinic acid at a dosage of about 400 mg/m², 5-fluorouracil (5-FU) at a dosage of about 2800 mg/m², irinocetan at a dosage of about 180 mg/m² and aflibercept at a dosage of about 4 mg/kg are administered to patient.

[0043] In a further embodiment of one of the methods described above folinic acid is administered intravenously at a dosage of about 400 mg/m², 5-fluorouracil (5-FU) is administered intravenously at a dosage of about 2800 mg/m², irinocetan is administered intravenously at a dosage of about 180 mg/m² and aflibercept is administered intravenously at a dosage of about 4 mg/kg and wherein the combination is administered every two weeks.

[0044] In a further embodiment of one of the methods described above folinic acid, 5-fluorouracil (5-FU), irinocetan and aflibercept are administered intravenously every two weeks for a period comprised between 9 and 18 weeks.

[0045] In a further embodiment of one of the methods described above folinic acid is administered intravenously immediately after aflibercept administration. It can be also administered intravenously immediately after aflibercept administration over a period of about 2 hours.

[0046] In a further embodiment of one of the methods described above irinocetan is administered intravenously immediately after aflibercept administration. It can be also administered intravenously immediately after aflibercept administration over a period of about 90 minutes.

[0047] In a further embodiment of one of the methods described above 5-fluorouracil (5-FU) is administered immediately after aflibercept administration.

[0048] In a further embodiment of one of the methods described above a first quantity of 5-fluorouracil (5-FU) is administered intravenously immediately after aflibercept administration and a second quantity of 5-FU is administered intravenously after the first quantity in continuous infusion.

[0049] In a further embodiment of one of the methods described above about 400 mg/m² of 5-fluorouracil (5-FU) is administered intravenously over a period of 2 to 4 minutes after aflibercept administration and wherein 2400 mg/m² of 5-FU is administered intravenously over around 46 hours after the administration of the 400 mg/m² in continuous infusion.

[0050] In an embodiment said patient has previously been treated with therapy based on oxaliplatin or on bevacizumab.

[0051] In another embodiment said patient has failed with chemotherapy, radiotherapy or surgery.

[0052] The invention relates also to aflibercept, or ziv-aflibercept for use in treating a patient suspected to suffer from cancer, wherein the patient has been identified as having lower level of a PIGF in biological sample as compared to the reference level of expression of PIGF.

[0053] The above methods and use of the invention may be, for instance, in vitro or ex vivo methods and use.

[0054] Means for measuring the expression level of VCAM-1, ICAM-1 and PIGF protein are well-known in the art and include immunoassay such as ELISA assay. For instance the means for measuring VCAM-1 include antibodies specifically binding to VCAM-1.

[0055] The level of VCAM-1 protein may be, for instance, determined using immunological detection methods such as an ELISA assay. The methods involve an antibody which binds to VCAM-1 protein, for example a monoclonal or polyclonal antibody, an antibody variant or fragments such as a single chain antibody, a diabody, a minibody, a single chain Fv fragment (sc(Fv)), a Sc(Fv)2 antibody, a Fab fragment or a F(ab')₂ fragment, or a single domain antibody. Such antibodies are well known in the art and are commercially available. They may also notably be obtained by immunization of animals (for example rabbits, rats or mice) with VCAM-1 protein. Antibodies may be used to determine protein expression in a range of immunological assays including competitive and non-competitive assay systems using techniques such as western blotting, immunohistochemistry/ immunofluorescence (i.e. protein detection on fixed cells or tissues), radioimmunoassay such as RIA (radio-linked immunoassay), ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, immunodiffusion assays, agglutination assays, complement-fixation as-

says, immunoradiometric assays, fluorescent immunoassays, e.g. FIA (fluorescence-linked immunoassay), chemiluminescence immunoassays, ECLIA (electrochemiluminescence immunoassay) and protein A immunoassays. Such assays are routine and well known to the person skilled in the art (Ausubel et al (1994) Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York).

[0056] Protein expression of VCAM-1 may be determined by proteomic method such as mass spectrometry assays (LC-MS or LC-MS/MS). Qualitative and quantitative mass spectrometric techniques are known and used in the art. To this aim, target peptides specific for marker proteins are selected and quantified based on calibration curves established with synthetic peptides labeled with stable isotopes. Enzymatic digests, spiked with a defined amount of isotope labeled target peptides, are analyzed by liquid chromatography coupled with mass spectrometry. The ratio between labeled and non-labeled target peptides is measured to assess target peptide concentrations and therefore protein marker concentration.

[0057] The means for measuring the expression level of VCAM-1 may also include reagents such as e.g. reaction and/or washing buffers. The means may be present, e.g., in vials or microtiter plates, or be attached to a solid support such as a microarray as can be the case for primers and probes.

[0058] Similar means are at the disposal of the man skilled in the art for detecting ICAM-1 and PIGF.

[0059] In an embodiment VCAM-1, ICAM-1 and PIGF proteins can be measured with a bead-based multiplex assay, the Luminex™ technology.

[0060] Aflibercept, or ziv-aflibercept is provided in a formulation which is not prejudicial to the patient to be treated.

[0061] In an embodiment aflibercept, or ziv-aflibercept is provided in a formulation with sucrose and polysorbate 20 (stabilisers), sodium chloride, citrate buffer, and sodium phosphate buffer, adjusted to final pH.

[0062] In another embodiment aflibercept, or ziv-aflibercept, is supplied in two drug product presentations:

- a presentation at 100 mg aflibercept, or ziv-aflibercept / 4.0 mL (nominal concentration).
- a second presentation at 200 mg aflibercept, or ziv-aflibercept / 8.0 mL (nominal concentration).

[0063] Both presentations are manufactured from the same bulk sterile solution at 25 mg/mL of aflibercept, or ziv-aflibercept.

[0064] Prior to infusion to the patient, the concentrate solution is diluted with 0.9% sodium chloride solution or 5% dextrose.

[0065] The anti-cancer agents used in the above recited method or use are provided in a pharmaceutically acceptable carrier, excipient or diluent which is not prejudicial to the patient to be treated.

[0066] Pharmaceutically acceptable carriers and excipient that may be used in the compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminium stearate, lecithin, self-emulsifying drug delivery systems (SEDDS) such as d-a-tocopherol polyethyleneglycol 1000 succinate, surfactants used in pharmaceutical dosage forms such as Tweens or other similar polymeric delivery matrices, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

[0067] As appreciated by skilled artisans, compositions are suitably formulated to be compatible with the intended route of administration. Examples of suitable routes of administration include parenteral route, including for instance intramuscular, subcutaneous, intravenous, intraperitoneal or local intratumoral injections. The oral route can also be used, provided that the composition is in a form suitable for oral administration, able to protect the active principle from the gastric and intestinal enzymes.

[0068] The term "effective amount" refers to an amount of a drug alone or in combination with other drug or treatment regimen effective to treat a disease or disorder in a mammal. In the case of cancer, the therapeutically effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the disorder. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy in vivo can, for example, be measured by assessing the duration of survival, duration of progression free survival (PFS), the response rates (RR), duration of response, and/or quality of life.

[0069] The terms "Therapy", "therapeutic", "treatment" and "treating" are used herein to characterize a therapeutic method or process that is aimed at (1) slowing down or stopping the progression, aggravation, or deterioration of the symptoms of the disease state or condition to which such term applies; (2) alleviating or bringing about ameliorations of the symptoms of the disease state or condition to which such term applies; and/or (3) reversing or curing the disease state or condition to which such term applies.

[0070] The term "overall survival (OS)" refers to the length of time during and after treatment the patient survives. As the skilled person will appreciate, a patient's overall survival is improved or enhanced, if the patient belongs to a subgroup of patients that has a statistically significant longer mean survival time as compared to another subgroup of patients.

[0071] The term "progression-free survival (PFS)" refers to the length of time during and after treatment during which, according to the assessment of the treating physician or investigator, the patient's disease does not become worse, i.e., does not progress. As the skilled person will appreciate, a patient's progression-free survival is improved or enhanced if the patient belongs to a subgroup of patients that has a longer length of time during which the disease does not progress as compared to the average or mean progression free survival time of a control group of similarly situated patients.

[0072] A "subject" or a "patient" may be a human or a non-human mammal, such as monkeys, dogs, cats, guinea pigs, hamsters, rabbits, cows, horses, goats and sheep.

[0073] The term "reference level" herein refers to a predetermined value. As the skilled artisan will appreciate the reference level is predetermined and set to meet the requirements in terms of e.g. specificity and/or sensitivity. These requirements can vary, e.g. from regulatory body to regulatory body. It may for example be that assay sensitivity or specificity, respectively, has to be set to certain limits, e.g. 80%, 90% or 95%. These requirements may also be defined in terms of positive or negative predictive values. Nonetheless, based on the teaching given in the present invention it will always be possible to arrive at the reference level meeting those requirements. In one embodiment the reference level is determined in healthy individuals. The reference value in one embodiment has been predetermined in the disease entity to which the patient belongs. In certain embodiments the reference level can e.g. be set to any percentage between 25% and 75% of the overall distribution of the values in a disease entity investigated. In other embodiments the reference level can e.g. be set to the median, tertiles or quartiles as determined from the overall distribution of the values in a disease entity investigated. In one embodiment the reference level is set to the median value as determined from the overall distribution of the values in a disease entity investigated.

BRIEF DESCRIPTION OF THE DRAWINGS

[0074]

Figure 1 represents Kaplan-Meier curves for PFS endpoint for sensitive and non-sensitive populations defined with VCAM-1.

Figure 2 represents Kaplan-Meier curves for OS endpoint for sensitive and non-sensitive populations defined with VCAM-1.

Figure 3 represents Kaplan-Meier curves for PFS endpoint for sensitive and non-sensitive populations defined with ICAM-1.

Figure 4 represents Kaplan-Meier curves for OS endpoint for sensitive and non-sensitive populations defined with ICAM-1.

Figure 5 represents Kaplan-Meier curves for PFS endpoint for sensitive and non-sensitive populations defined with VCAM-1 and ICAM-1.

Figure 6 represents Kaplan-Meier curves for OS endpoint for sensitive and non-sensitive populations defined with VCAM-1 and ICAM-1.

Figure 7 represents Kaplan-Meier curves for PFS endpoint for sensitive and non-sensitive populations defined with PIGF.

EXAMPLE: Predictive effect of PIGF on PFS in the AFLAME study

[0075] Aspects on VCAM-1 and ICAM-1 (including Figures 1 to 6) are embodiments of the study EFC11338 (AFLAME) described below, but are embodiments not covered by the claimed invention.

STUDY EFC11338 (AFLAME)

[0076] EFC11338 was designed as a Multinational, Randomized, Double-Blind Study of Aflibercept Versus Placebo with Irinotecan/5-FU Combination (FOLFIRI) in Patients with Metastatic Colorectal Cancer (MCR) After Failure of an Oxaliplatin Based Regimen.

Objectives:

[0077]

To identify potential predictive biomarkers for response to treatment on efficacy endpoints (Progression Free Survival

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(PFS), Overall Survival (OS) and Overall Response Rate (ORR))

To identify potential prognostic biomarkers for efficacy endpoints (PFS, OS and ORR)

To identify potential correlation between biomarkers and other baseline characteristics

To identify potential correlation of longitudinal plasma measurements with clinical endpoints

5 To identify potential groups of homogeneous individuals based on their molecular profiles in an unsupervised way and to estimate correlation with clinical outcomes (PFS, OS and ORR)

To assess the safety profile of the population identified by prognostic/predictive biomarkers

Dosage and schedule of administration:

10

[0078] Patients were administered either aflibercept or placebo, depending on arm assigned. Immediately after, patients received irinotecan, 5-FU and leucovorin (FOLFIRI regimen). This treatment was repeated every 2 weeks.

Aflibercept/placebo

15

[0079]

Arm A, aflibercept: 4 mg/kg was administered IV over 1 hour on Day 1, every 2 weeks,

OR

20

Arm B, placebo: 4 mg/kg was administered IV over 1 hour on Day 1, every 2 weeks.

FOLFIRI regimen

[0080] Immediately after aflibercept/placebo administration, all the patients received:

25

- Irinotecan 180 mg/m² IV infusion in 500 mL D5W over 90 minutes and dl leucovorin* 400 mg/m² IV infusion over 2 hours, at the same time, in bags using a Y-line, followed by:
- 5-FU 400 mg/m² IV bolus given over 2-4 minutes, followed by:
- 5-FU 2400 mg/m² continuous IV infusion in 500 mL D5W (recommended)

30

over 46-hours.

Duration of treatment:

35

[0081] Patient was treated until disease progression, unacceptable toxicity or patient's refusal

Duration of observation:

[0082] Patients were followed when on study treatment and during follow up period until death or the study cut-off date for OS, whichever comes first. The cut-off date for OS is one year after the last patient enrolled.

40

Number of subjects:

[0083]

45

Intent-to-Treat (ITT) population: 332 (109 in the placebo group + 223 in the aflibercept group)

Evaluable population for response rate: 295 (96 in the placebo group + 199 in the aflibercept group)

Evaluable for Luminex biomarkers - Group 1 biomarkers: 295 (99 in the placebo group + 196 in the aflibercept group)

Evaluable for Luminex biomarkers - Group 2 biomarkers: 108 (37 in the placebo group + 71 in the aflibercept group)

50

Evaluable for ELISA biomarkers: 329 (107 in the placebo group + 222 in the aflibercept group)

[0084] Due to an operational error, some patients randomized to the aflibercept arm have received placebo for one or several treatment cycles; or vice versa. A total of 198 patients received at least one misallocated treatment kit.

55

Criteria for evaluation:

[0085] Biomarkers:

Different types of biomarker data were investigated in the current translational research proposal in the AFLAME study:

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- 107 plasma angiogenic factors and inflammatory cytokines measured at baseline, during and after treatment (end of cycle 1 infusion, 48h post aflibercept/placebo on cycle 2 or 3, 30 days after last aflibercept/placebo administration) with Luminex® technology
- Free VEGF-A and PIGF measured in plasma samples at baseline with ELISA (Enzyme-Linked Immunosorbent Assay) technology

PREPARATION OF SAMPLES AND ANALYSIS

[0086] Plasma biomarkers were measured either with Luminex™ technology (bead-based multiplex assay) or with ELISA.

Plasma biomarkers measured with Luminex™ technology

[0087] Plasma angiogenic factors and inflammatory cytokines have been measured at baseline pre cycle 1.

[0088] Thirty proteins measured on all the samples were defined as Group 1 biomarkers and 77 proteins measured only for some samples were defined as Group 2 biomarkers. The biomarkers from Group 1 were angiogenic/inflammatory molecules and have been selected based on aflibercept mechanism of action (inhibiting 3 angiogenic factors and their receptors), key candidate biomarkers identified on independent aflibercept studies or literature/experts.

Plasma biomarkers measured with ELISA

[0089] In addition to plasma biomarkers measured with Luminex™ technology, free VEGF-A and free PIGF concentrations of baseline plasma samples were measured with ELISA technology.

RESULTS

Univariate analysis

[0090] Biomarkers have been tested for predictive and prognostic effects for PFS.

[0091] VCAM-1 and ICAM-1 have been identified as potentially predictive with corrected Benjamini-Hochberg (BH) p-value ≤ 0.2 (295 subjects).

[0092] Then sensitive and non-sensitive populations have been defined using VCAM-1 and ICAM-1 (BH p-value ≤ 0.2).

[0093] PIGF has been identified as potentially predictive by ELISA (unadjusted p-value = 0.075).

Identification of sensitive and non-sensitive populations with VCAM-1

[0094] The threshold of 6.32 corresponding to 553 ng/mL has been determined to define sensitive (197 individuals) and non-sensitive populations (98 individuals) respectively corresponding to individuals with low values for VCAM-1 and high values for VCAM-1.

[0095] Figure 1 and Figure 2 represent Kaplan-Meier curves for PFS and OS endpoints for sensitive and non-sensitive populations illustrating the better treatment effect for low VCAM-1 group compared to high VCAM-1 group. For OS, in addition to a better treatment effect compared to placebo, sensitive population showed globally a better prognostic (increased OS for low VCAM-1 group in placebo and aflibercept arms).

[0096] Table 1 shows the response rate for sensitive and non-sensitive populations by treatment group. There was an increased response rate (26%) for the aflibercept/folfiri treatment in sensitive population compared to the non-sensitive population (10%).

Table 1 - Response Rate for non-sensitive/sensitive populations defined with VCAM-1 for PFS - Preprocessed data - VCAM-1 and RR evaluable population

Population	Placebo/Folfiri	Aflibercept/Folfiri
Sensitive VCAM-1 ≤ 6.32 (N = 189)	3/68 (4%)	31/121 (26%)
Non-sensitive VCAM-1 > 6.32 (N = 94)	1/26 (4%)	7/68 (10%)
Total	4/94 (4%)	38/189 (20%)

Identification of sensitive and non-sensitive populations with ICAM-1

[0097] The threshold of 5.04 corresponding to 144 ng/mL has been determined to define sensitive (205 individuals) and non-sensitive populations (90 individuals) respectively corresponding to individuals with low values for ICAM-1 and high values for ICAM-1.

[0098] Figure 3 and Figure 4 represent Kaplan-Meier curves for PFS and OS endpoints for sensitive and non-sensitive populations illustrating the better treatment effect for low ICAM-1 group compared to high ICAM-1 group. For OS, in addition to a better treatment effect compared to placebo, sensitive population showed globally a better prognostic (increased OS for low ICAM-1 group in placebo and aflibercept arms).

[0099] Table 2 shows the response rate for sensitive and non-sensitive populations by treatment group. There has been an increased response rate (25%) for the aflibercept/fofiri treatment in sensitive population compared to the non-sensitive population (9%).

Table 2 - Response Rate for non-sensitive/sensitive populations defined with ICAM-1 for PFS - Preprocessed data
- ICAM-1 and RR evaluable population

Population	Placebo/Folfiri	Aflibercept/Folfiri
Sensitive ICAM-1 ≤ 5.04 (N = 197)	3/63 (5%)	33/134 (25%)
Non-sensitive ICAM-1 > 5.04 (N = 86)	1/31 (3%)	5/55 (9%)
Total	4/94 (4%)	38/189 (20%)

Multivariate analysis

[0100] The multivariate predictive score $0.089 \times \text{ICAM} - 1 + 0.17 \times \text{VCAM} - 1$ has been dichotomized using quantile 10% to 90% as threshold.

[0101] Sensitive population showed a significant difference in PFS and in OS in favor of aflibercept over placebo (HR= 0.47 for PFS and HR = 0.66 for OS), which was increased compared to the non-sensitive population (HR= 0.98 for PFS and HR = 1.04 for OS).

[0102] Median PFS difference between aflibercept and placebo was equal to 2.59 months in sensitive population, showing a greater but moderate difference compared to non-sensitive population (0.5 month).

[0103] Median OS difference between aflibercept and placebo was equal to 3.75 months in sensitive population, showing a greater difference compared to non-sensitive population (-0.39 month). For non-sensitive population there was a decrease in median OS for placebo arm and treated arm (8.90 months and 8.51 months) compared to the other populations illustrating a potential prognostic effect of multivariate score in addition to the predictive effect.

[0104] In conclusion sensitive population showed a decreased HR compared to the non-sensitive population for PFS and OS with moderate gain in term of median.

[0105] Figure 5 and Figure 6 represent Kaplan-Meier curves for PFS and OS endpoints for sensitive and non-sensitive populations illustrating the better treatment effect for low score group compared to high score group. For OS, in addition to a better treatment effect compared to placebo, sensitive population showed globally a better prognostic (increased OS for low score group in placebo and aflibercept arms).

Identification of sensitive and non-sensitive populations with PIGF

[0106] The threshold of 2.82 corresponding to 17 pg/ml has been determined to define sensitive (230 individuals) and non-sensitive populations (99 individuals) respectively corresponding to individuals with low values for PIGF and high values for PIGF.

[0107] Figure 7 which represents the Kaplan-Meier curves for PFS endpoint for sensitive and non-sensitive populations defined with PIGF illustrates the determination of the PIGF cut off for sensitive and non-sensitive populations.

CONCLUSIONS**[0108]**

- VCAM-1 and ICAM-1 have been identified as potentially predictive biomarkers for PFS in a univariate framework (unadjusted p-value equal to 0.00017 for VCAM-1 and 0.0043 for ICAM-1).
- The third biomarker that showed up to be potentially predictive was PIGF measured by ELISA (unadjusted p-value

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= 0.075).

- Linear combination of VCAM-1 and ICAM-1 has been identified as potentially predictive for PFS.

5 SEQUENCE LISTING

[0109]

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<120> METHOD FOR PREDICTING THE OUTCOME OF A TREATMENT WITH AFLIBERCEPT OF A PATIENT SUSPECTED TO SUFFER FROM A CANCER BY MEASURING THE LEVEL OF A PLASMA BIOMARKER

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Claims

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1. Use of Placenta Growth Factor (PIGF) for predicting the outcome of the treatment with aflibercept of a patient suspected to suffer from a colon cancer, a colorectal cancer or a rectal cancer.

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2. A method of determining whether a patient suspected to suffer from a colon cancer, a colorectal cancer or a rectal cancer is a candidate for aflibercept therapy for the said cancer comprising the step of subjecting a patient's biological sample to at least one assay to measure at baseline the level of PIGF, wherein when the PIGF level in the biological sample is low relative to a reference level of expression of PIGF, the patient is identified as a candidate for therapy for cancer.

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3. A method of determining whether a patient suspected to suffer from a colon cancer, a colorectal cancer or a rectal cancer is a candidate for aflibercept therapy for the said cancer comprising the step of subjecting a patient's biological sample to at least one assay to measure at baseline the level of PIGF, wherein when the PIGF level in the biological sample is high relative to a reference level of expression of PIGF, the patient is identified as not being a candidate for therapy for cancer.

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4. A method according to any of claims 2 and 3 wherein the reference level of expression of PIGF is between 12 and 19 pg/ml.

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5. A method according to any of claims 2 to 4, wherein the reference level of expression of PIGF is 17 pg/ml.

6. A method according to any of claims 2 to 5, wherein the biological sample is chosen from the group consisting of blood, serum and plasma.

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7. A method according to claim 6 wherein the colorectal cancer is a metastatic colorectal cancer.

8. A method according to any of claims 3 to 7, wherein the PIGF level which is determined is the circulating level.

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9. Aflibercept for use in treating a patient suspected to suffer from a colon cancer, a colorectal cancer or a rectal cancer, wherein the patient has been identified as having lower level of PIGF in biological sample as compared to the reference level of expression of PIGF.

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10. Combination comprising aflibercept, folinic acid, 5-fluorouracil (5-FU) and irinotecan for use in treating a patient suspected to suffer from a colon cancer, a colorectal cancer or a rectal cancer, wherein the patient has been identified as having a low level of PIGF in a biological sample compared to the reference level of expression of PIGF.

11. Combination comprising aflibercept, folinic acid, 5-fluorouracil (5-FU) and irinotecan for use according to claim 10 wherein the subject is treated with aflibercept and further undergoes a chemotherapeutic treatment with folinic acid, 5-fluorouracil and irinotecan.

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12. Aflibercept or combination comprising aflibercept, folinic acid, 5-fluorouracil (5-FU) and irinotecan for use according to any of claims 9 to 11 wherein the reference level of expression of PIGF is between 12 and 19 pg/ml.

13. Aflibercept or combination comprising aflibercept, folinic acid, 5-fluorouracil (5-FU) and irinotecan for use according to claim 12 wherein the reference level of expression of PIGF is 17 pg/ml

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14. Aflibercept or combination comprising aflibercept, folinic acid, 5-fluorouracil (5-FU) and irinotecan for use according to any one of claims 9 to 13 wherein the colorectal cancer is a metastatic colorectal cancer.

- 5 15. Combination comprising aflibercept, folinic acid, 5-fluorouracil (5-FU) and irinotecan for use according to any one of claims 9 to 14, wherein folinic acid at a dosage comprised between 200 mg/m² and 600 mg/m², 5-fluorouracil (5-FU) at a dosage comprised between 2000 mg/m² and 4000 mg/m², irinotecan at a dosage comprised between 100 mg/m² and 300 mg/m² and aflibercept at a dosage comprised between 1 mg/kg and 10 mg/kg are administered to patient.

Patentansprüche

- 10 1. Verwendung von PIGF (Placenta Growth Factor) zur Vorhersage des Ergebnisses der Behandlung eines Patienten, bei dem ein Leiden an einem Kolonkarzinom, einem Kolorektalkarzinom oder einem Rektumkarzinom vermutet wird, mit Aflibercept.
- 15 2. Verfahren zur Bestimmung, ob ein Patient, bei dem ein Leiden an einem Kolonkarzinom, einem Kolorektalkarzinom oder einem Rektumkarzinom vermutet wird, ein Kandidat für eine Aflibercept-Therapie gegen die Krebserkrankung ist, umfassend den Schritt, bei dem eine biologische Probe des Patienten wenigstens einem Testverfahren zur Messung des Grundniveaus von PIGF unterzogen wird, wobei der Patient bei einem niedrigen PIGF-Niveau in der biologischen Probe relativ zu einem Referenzexpressionsniveau von PIGF als Kandidat für eine Krebstherapie identifiziert wird.
- 20 3. Verfahren zur Bestimmung, ob ein Patient, bei dem ein Leiden an einem Kolonkarzinom, einem Kolorektalkarzinom oder einem Rektumkarzinom vermutet wird, ein Kandidat für eine Aflibercept-Therapie gegen die Krebserkrankung ist, umfassend den Schritt, bei dem eine biologische Probe des Patienten wenigstens einem Testverfahren zur Messung des Grundniveaus von PIGF unterzogen wird, wobei der Patient bei einem hohen PIGF-Niveau in der biologischen Probe relativ zu einem Referenzexpressionsniveau von PIGF als kein Kandidat für eine Krebstherapie identifiziert wird.
- 25 4. Verfahren nach einem der Ansprüche 2 und 3, wobei das Referenzexpressionsniveau von PIGF zwischen 12 und 19 pg/ml liegt.
- 30 5. Verfahren nach einem der Ansprüche 2 bis 4, wobei das Referenzexpressionsniveau von PIGF bei 17 pg/ml liegt.
6. Verfahren nach einem der Ansprüche 2 bis 5, wobei die biologische Probe aus der Gruppe bestehend aus Blut, Serum und Plasma gewählt ist.
- 35 7. Verfahren nach Anspruch 6, wobei es sich bei dem Kolorektalkarzinom um ein metastatisches Kolorektalkarzinom handelt.
8. Verfahren nach einem der Ansprüche 3 bis 7, wobei es sich bei dem PIGF-Niveau, das bestimmt wird, um den Blutkreislaufspiegel handelt.
- 40 9. Aflibercept zur Verwendung bei der Behandlung eines Patienten, bei dem ein Leiden an einem Kolonkarzinom, einem Kolorektalkarzinom oder einem Rektumkarzinom vermutet wird, wobei bei dem Patienten das Vorliegen eines niedrigeren Niveaus von PIGF in einer biologischen Probe im Vergleich zu dem Referenzexpressionsniveau von PIGF identifiziert wurde.
- 45 10. Kombination, umfassend Aflibercept, Folinsäure, 5-Fluoruracil (5-FU) und Irinotecan, zur Verwendung bei der Behandlung eines Patienten, bei dem ein Leiden an einem Kolonkarzinom, einem Kolorektalkarzinom oder einem Rektumkarzinom vermutet wird, wobei bei dem Patienten das Vorliegen eines niedrigen Niveaus von PIGF in einer biologischen Probe verglichen mit dem Referenzexpressionsniveau von PIGF identifiziert wurde.
- 50 11. Kombination, umfassend Aflibercept, Folinsäure, 5-Fluoruracil (5-FU) und Irinotecan, zur Verwendung nach Anspruch 10, wobei das Individuum mit Aflibercept behandelt wird und sich ferner einer chemotherapeutischen Behandlung mit Folinsäure, 5-Fluoruracil und Irinotecan unterzieht.
- 55 12. Aflibercept oder Kombination, die Aflibercept, Folinsäure, 5-Fluoruracil (5-FU) und Irinotecan umfasst, zur Verwendung nach einem der Ansprüche 9 bis 11, wobei das Referenzexpressionsniveau von PIGF zwischen 12 und 19 pg/ml liegt.

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13. Aflibercept oder Kombination, die Aflibercept, Folinsäure, 5-Fluoruracil (5-FU) und Irinotecan umfasst, zur Verwendung nach Anspruch 12, wobei das Referenzexpressionsniveau von PIGF bei 17 pg/ml liegt.
- 5 14. Aflibercept oder Kombination, die Aflibercept, Folinsäure, 5-Fluoruracil (5-FU) und Irinotecan umfasst, zur Verwendung nach einem der Ansprüche 9 bis 13, wobei es sich bei dem Kolorektalkarzinom um ein metastatisches Kolorektalkarzinom handelt.
- 10 15. Kombination, die Aflibercept, Folinsäure, 5-Fluoruracil (5-FU) und Irinotecan umfasst, zur Verwendung nach einem der Ansprüche 9 bis 14, wobei Folinsäure in einer Dosis, die zwischen 200 mg/m² und 600 mg/m² liegt, 5-Fluoruracil (5-FU) in einer Dosis, die zwischen 2000 mg/m² und 4000 mg/m² liegt, Irinotecan in einer Dosis, die zwischen 100 mg/m² und 300 mg/m² liegt, und Aflibercept in einer Dosis, die zwischen 1 mg/kg und 10 mg/kg liegt, einem Patienten verabreicht werden.

15 Revendications

1. Utilisation du facteur de croissance placentaire (PIGF) pour la prédiction de l'issue du traitement avec de l'aflibercept chez un patient suspecté de souffrir d'un cancer du côlon, d'un cancer colorectal ou d'un cancer rectal.
- 20 2. Procédé pour déterminer si un patient suspecté de souffrir d'un cancer du côlon, d'un cancer colorectal ou d'un cancer rectal est un candidat pour une thérapie à l'aflibercept pour ledit cancer comprenant l'étape de soumission d'un échantillon biologique du patient à au moins une analyse pour mesurer avant le traitement le taux de PIGF, dans lequel, lorsque le taux de PIGF dans l'échantillon biologique est bas par rapport à un taux de référence d'expression de PIGF, le patient est identifié comme un candidat pour une thérapie pour le cancer.
- 25 3. Procédé pour déterminer si un patient suspecté de souffrir d'un cancer du côlon, d'un cancer colorectal ou d'un cancer rectal est un candidat pour une thérapie à l'aflibercept pour ledit cancer comprenant l'étape de soumission d'un échantillon biologique du patient à au moins une analyse pour mesurer avant le traitement le taux de PIGF, dans lequel, lorsque le taux de PIGF dans l'échantillon biologique est élevé par rapport à un taux de référence d'expression de PIGF, le patient est identifié comme n'étant pas un candidat pour une thérapie pour le cancer.
- 30 4. Procédé selon l'une quelconque des revendications 2 et 3, le taux de référence d'expression de PIGF étant compris entre 12 et 19 pg/ml.
- 35 5. Procédé selon l'une quelconque des revendications 2 à 4, le taux de référence d'expression de PIGF étant de 17 pg/ml.
6. Procédé selon l'une quelconque des revendications 2 à 5, l'échantillon biologique étant choisi dans le groupe constitué par le sang, le sérum et le plasma.
- 40 7. Procédé selon la revendication 6, le cancer colorectal étant un cancer colorectal métastatique.
8. Procédé selon l'une quelconque des revendications 3 à 7, le taux de PIGF qui est déterminé étant le taux circulant.
- 45 9. Aflibercept pour une utilisation dans le traitement d'un patient suspecté de souffrir d'un cancer du côlon, d'un cancer colorectal ou d'un cancer rectal, le patient ayant été identifié comme ayant un taux plus bas de PIGF dans l'échantillon biologique par rapport au taux de référence d'expression de PIGF.
- 50 10. Combinaison comprenant de l'aflibercept, de l'acide folinique, du 5-fluorouracile (5-FU) et de l'irinotécan pour une utilisation dans le traitement d'un patient suspecté de souffrir d'un cancer du côlon, d'un cancer colorectal ou d'un cancer rectal, le patient ayant été identifié comme ayant un taux bas de PIGF dans un échantillon biologique par rapport au taux de référence d'expression de PIGF.
- 55 11. Combinaison comprenant de l'aflibercept, de l'acide folinique, du 5-fluorouracile (5-FU) et de l'irinotécan pour une utilisation selon la revendication 10, le sujet étant traité avec de l'aflibercept et subissant en outre un traitement chimiothérapeutique avec de l'acide folinique, du 5-fluorouracile et de l'irinotécan.
12. Aflibercept ou combinaison comprenant de l'aflibercept, de l'acide folinique, du 5-fluorouracile (5-FU) et de l'irinotécan

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pour une utilisation selon l'une quelconque des revendications 9 à 11, le taux de référence d'expression de PIGF étant compris entre 12 et 19 pg/ml.

- 5 **13.** Aflibercept ou combinaison comprenant de l'aflibercept, de l'acide folinique, du 5-fluorouracile (5-FU) et de l'irinotécan pour une utilisation selon la revendication 12, le taux de référence d'expression de PIGF étant de 17 pg/ml.
- 10 **14.** Aflibercept ou combinaison comprenant de l'aflibercept, de l'acide folinique, du 5-fluorouracile (5-FU) et de l'irinotécan pour une utilisation selon l'une quelconque des revendications 9 à 13, le cancer colorectal étant un cancer colorectal métastatique.
- 15 **15.** Combinaison comprenant de l'aflibercept, de l'acide folinique, du 5-fluorouracile (5-FU) et de l'irinotécan pour une utilisation selon l'une quelconque des revendications 9 à 14, l'acide folinique, le 5-fluorouracile (5-FU), l'irinotécan et l'aflibercept étant administrés au patient respectivement à un dosage compris entre 200 mg/m² et 600 mg/m², un dosage compris entre 2 000 mg/m² et 4 000 mg/m², un dosage compris entre 100 mg/m² et 300 mg/m², et un dosage compris entre 1 mg/kg et 10 mg/kg.

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Figure 1

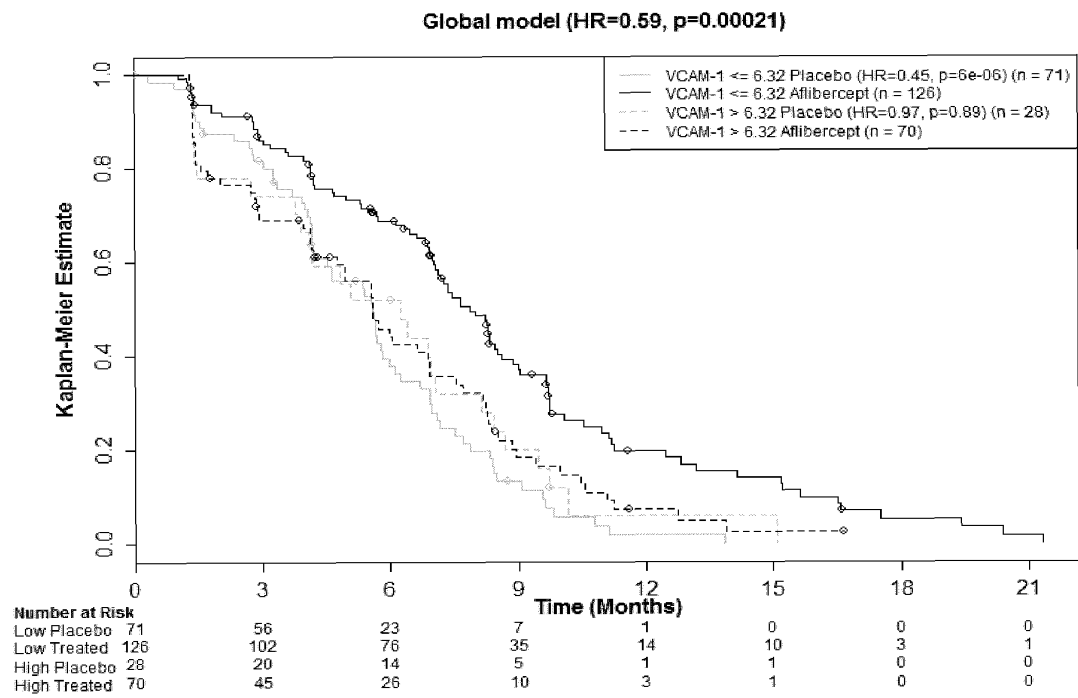


Figure 2

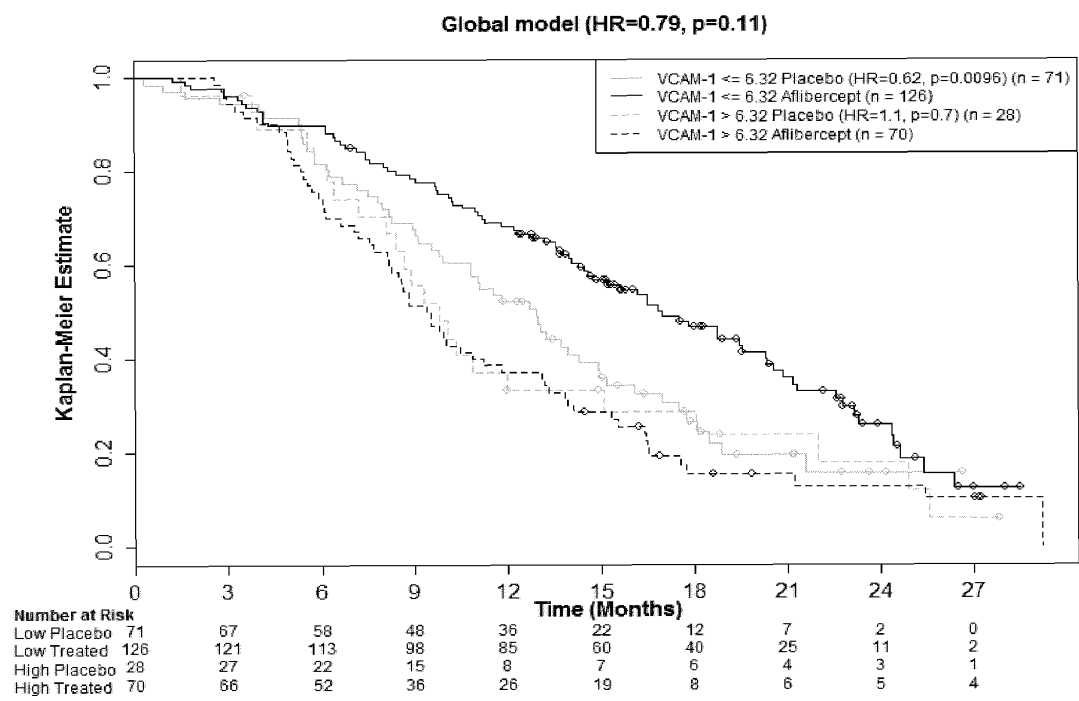


Figure 3

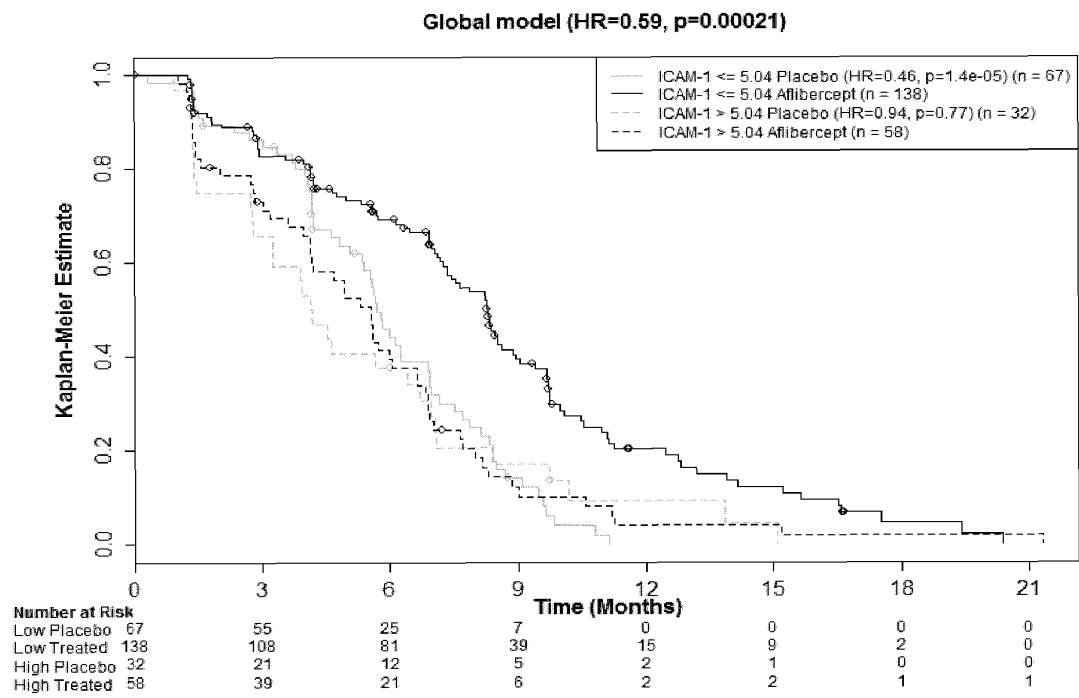


Figure 4

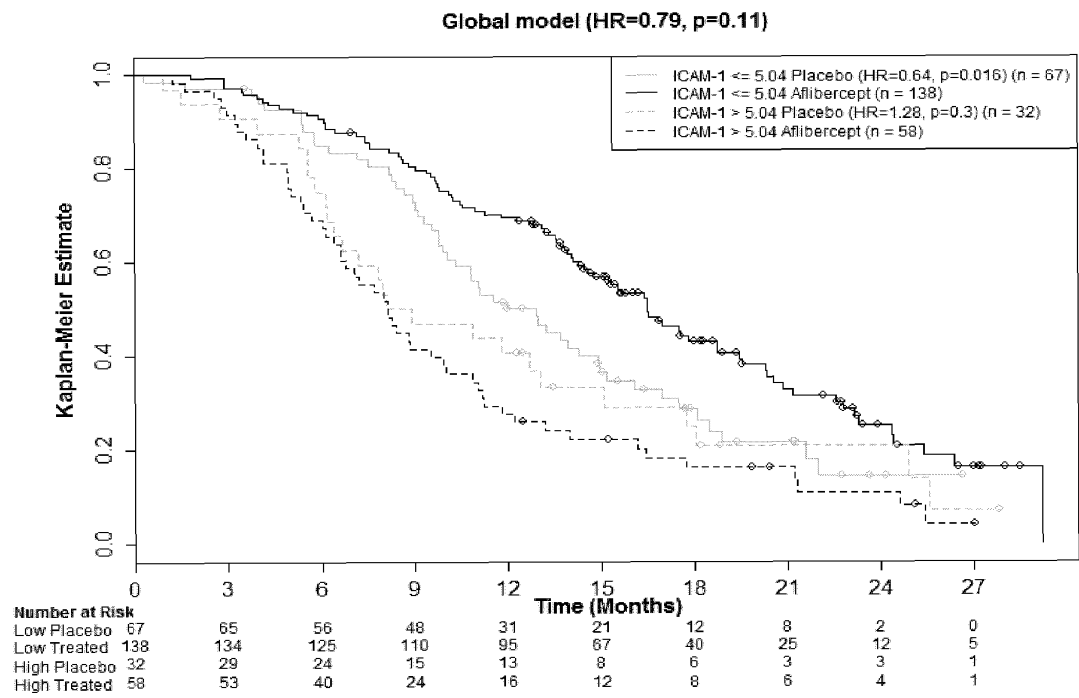


Figure 5

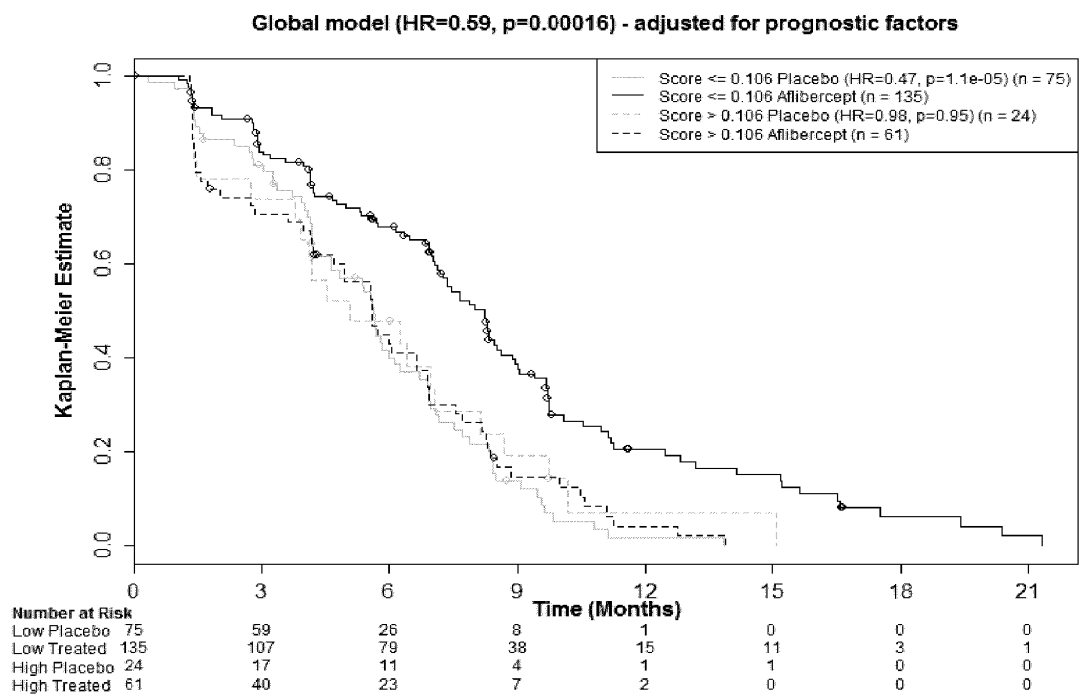


Figure 6

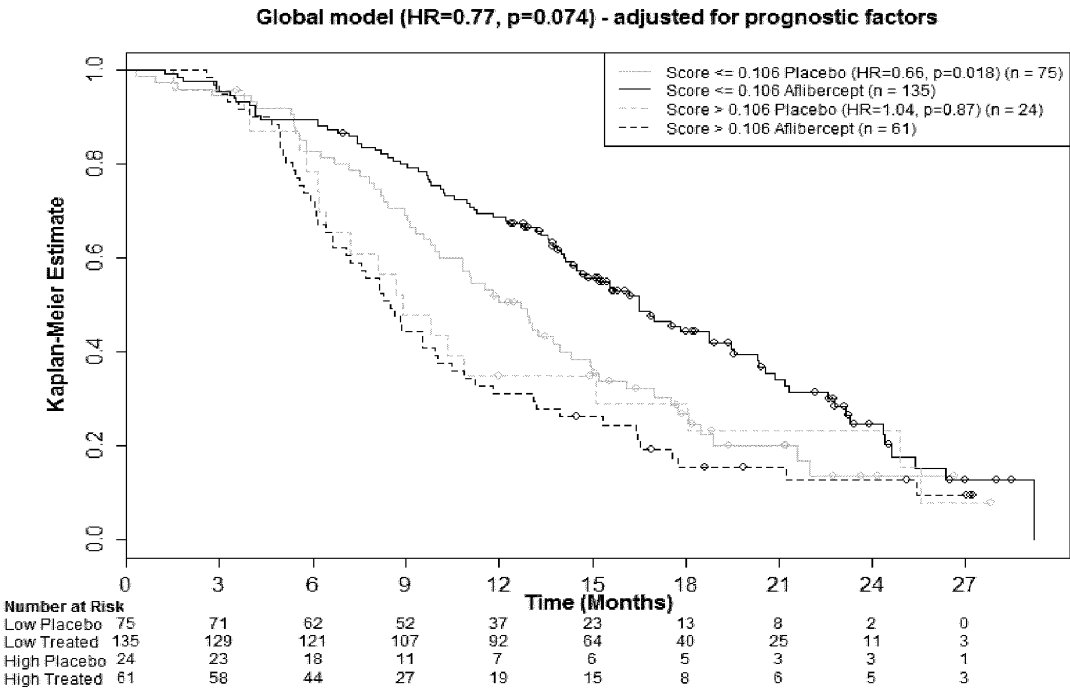
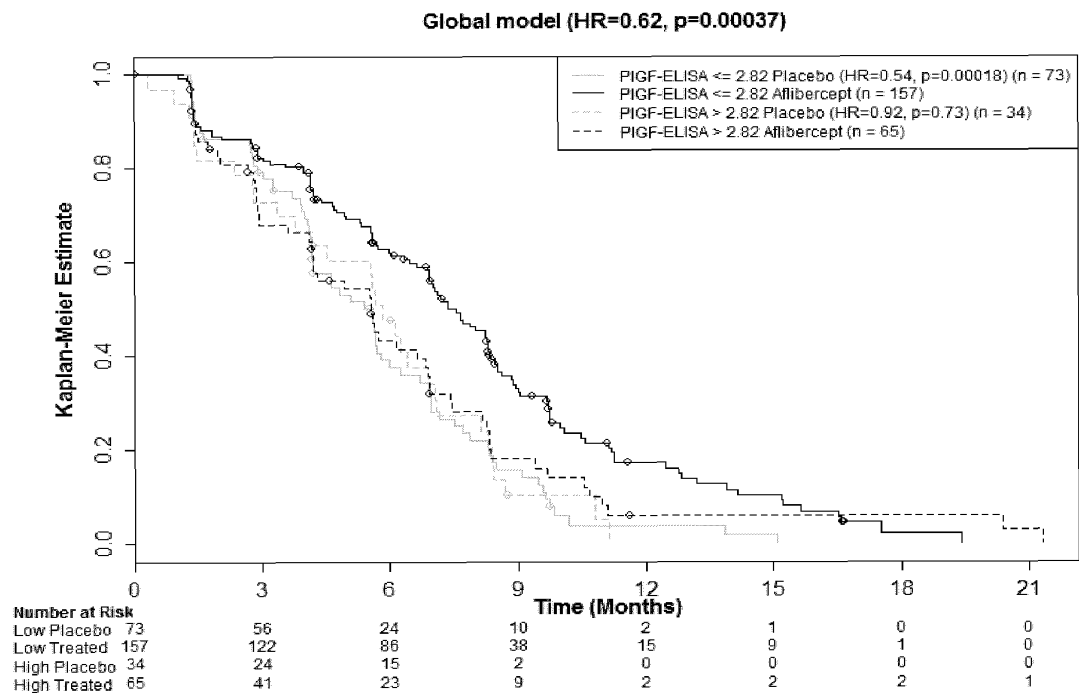


Figure 7



REFERENCES CITED IN THE DESCRIPTION

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(54) Title: METHODS OF IMPROVING RETINA-ASSOCIATED DISEASE OUTCOME USING CCR3-INHIBITORS

(57) Abstract: The disclosure provides a method of reducing the frequency of administration of an anti-VEGF agent to a subject diagnosed with wet age-related macular degeneration. The method comprises administering a CCR3 inhibitory agent to the subject; administering the anti-VEGF agent to the subject after the administering of the CCR3 inhibitory agent; and administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency. The disclosure further provides a method of treating a subject suffering from a retina-associated disease, the method comprising administering an anti-VEGF agent to a subject suffering from a retina-associated disease and previously treated with a CCR3 inhibitory agent, wherein the anti-VEGF agent is administered at a frequency less than the recommended frequency for the anti-VEGF agent.

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Methods of Improving Retina-Associated Disease Outcome Using CCR3-Inhibitors

Cross Reference to Related Applications

[0001] Pursuant to 35 U.S.C. § 119(e), this application claims priority to the filing date of United States Provisional Application No. 63/037,970, filed June 11, 2020; and United States Provisional Patent Application No. 63/040,345, filed June 17, 2020; the disclosures of which applications are herein incorporated by reference.

Background

[0002] Among the variety of retinal-associated diseases, there are those that manifest themselves early in life as well as those that manifest themselves in connection with aging. An example of the former type of disease is retinopathy of prematurity (ROP) and Stargardt's disease. Examples of age-related retina-associated diseases include: age-related macular degeneration (AMD) which is the most common degenerative disease of the macula; retinal vein occlusion (RVO), myopic choroidal neovascularization (mCNV) and diabetic retinopathy (with and without macular edema). Untreated, retina-associated disease can lead to legal blindness.

[0003] AMD is the leading cause of irreversible blindness in people 50 years of age or older in the developed world. (Jager, R. et al., The New England Journal of Medicine, 358(2606-17), 2008). AMD is a term that is used to describe a family of diseases that are all characterized by a progressive loss of central vision associated with abnormalities of Bruch's membrane, the choroid, the neural retina and/or the retinal pigment epithelium. In the early stages of AMD, which is often referred to as age-related maculopathy (ARM) or non-exudative or "dry" AMD (early and intermediate disease), accumulation of drusen (biochemical byproducts of the photoreceptor cells which accumulate in Bruch's membrane which are categorized by their appearance) and disturbances of the retinal pigment epithelium (RPE) are often observed.

[0004] AMD that becomes clinically advanced is classified into two forms – "dry," nonexudative or atrophic AMD called "Geographic Atrophy" and exudative "wet" (wAMD) or neovascular AMD (nAMD). Advanced dry AMD or Geographic Atrophy occurs in approximately 15% and wet AMD 10% of AMD patients. Wet AMD is considered the more immediate debilitating form of AMD and is thought to be caused by the growth of abnormal

choroidal neovascular membranes (CNVM). These new blood vessels grow from the choriocapillaris, growing under the RPE or retina, and leak serum and blood. This fluid accumulates in the sub-RPE and subretinal and retinal (e.g., intraretinal fluid) spaces along with the neurosensory retina, and in turn causes measurable thickening of the macula. If the fovea becomes involved with the CNVM, the resulting edema and hemorrhage can significantly impair visual acuity (VA), leading to dramatic vision loss.

[0005] Estimates suggest that about 10% of those aged 65-74 years old and 30% of those aged 75-85+ years old, exhibit signs of AMD. The current standard of care for wet AMD is anti-angiogenic therapies such as ranibizumab (Lucentis®) and aflibercept (Eylea®) by intravitreal (IVT) administration (i.e. injection directly into the eye). Another standard of care utilized off-label is bevacizumab (Avastin®). Such therapies target vascular endothelial growth factors (VEGF, VEGF-A) and their angiogenic-promoting properties. Additional treatments targeting VEGF include brolucizumab, KSI301 (Kodiak Sciences), abicipar, GB-102, (Graybug Vision), and anti-VEGF genetic therapies such as RGX-314 (RegenxBio) and ADVM-022 (Adverum). A concentrated bevacizumab eye port delivery system and conbercept (KH902) fusion protein have also been explored. Modifications to standard of care have mostly concentrated not on identifying new targets or polypharmacy like the instant application, but ways to extend the durability or pharmacology of anti-VEGF therapies. However even these attempts (e.g., brolucizumab) at increasing the duration of doses in order to lower the per year dosing rate have been met with safety concerns (Helzner J, *Retinal Physician*, June 2020, P. E5).

[0006] Glucocorticoids delivered IVT have also been explored in patients with retina related disease. However, anti-VEGF IVT treatments have been generally associated with fewer side effects and ocular complications. (Kuppermann BD *et al.*, *Ophthalmologica*, 234:40-54 (2015)). For example, glucocorticoids delivered IVT can have a high rate of cataract formation and steroid-induced glaucoma. (Hadayer A *et al.*, *Expert Opin Drug Delivery*, 13(8):1083-91 (2016) and Corpus K *et al.*, *Philipp J Ophthalmol*, 40:52-56 (2015)). Further, almost all cells in the body are sensitive to glucocorticoids, producing pleiotropic effects that are undesirable to human health. (Quatrini L *et al.*, *Cell Mol Immunol*, 18:269-78 (2021)).

[0007] Unfortunately, monthly IVT anti-VEGF injections have been associated with the adverse effects of geographic atrophy. (Desai, SJ, *et al.*, *Curr Ophthalmol. Rep.* (Feb. 01, 2017)).

In nAMD there have been serious concerns raised of increased intraocular pressure and increased risk of glaucoma. (Fowler SC and Schneider EW, More frequent dosing for refractory nAMD?, Retina Specialist, May 13, 2020). Further, IVT injections can be associated with serious adverse events such as endophthalmitis, retinal detachment, and traumatic cataract. Currently, there exist no effective, less-invasive therapies, underscoring an unmet need for a non-IVT delivered anti-VEGF based therapy for treating AMD, such as in orally administered form. In addition to alleviating and reversing the symptoms and dramatic detrimental effects on the vision of patients, such a therapy would have the added benefit of increased compliance. IVT injections bear increased cost and risk to patients and are burdensome to both patients and caregivers. Requirements for a cold chain and prefilled vials and syringes involve substantial financial investment. And even when injections are given in a bi-monthly fashion, patients often need to visit the office of the retinal specialist more frequently for diagnostic tests to ensure the stability of the retina. Due to the state of their vision, these visits frequently involve a caregiver. Recent estimates have suggested that between drive time, wait time, treatment, recovery, and booking time, each visit can take more than 10 hours of combined time. Because of the burden on patients and care staff, physicians and patients alike have attempted to create “treat and extend regimens” which decrease the frequency of injections and visits. However, these decreased injection frequencies lead to decreased treatment outcomes over time.

[0008] In the instances where glucocorticoid therapy adjunctive to anti-VEGF treatment have been explored, both practical and safety concerns remain. Glucocorticoid (dexamethasone) implants in the eye in conjunction with anti-VEGF agents delivered IVT have exhibited modest efficacy in reducing the frequency of anti-VEGF injections in neovascular AMD; at best, the adjunct therapy allowed the interval between anti-VEGF injections to be extended by less than 30 days. Further, the eye implants release glucocorticoids continuously over the life of the implant (e.g., 3-4 months), thereby requiring constant exposure to the subject. (Kuppermann, *supra.*). Further, implants expire, requiring invasive reinsertion of replacement implants into patients’ eyes. (Corpus, *supra.*)

[0009] The mechanistic basis of anti-VEGF therapies also bears risk. VEGF, particularly VEGF-A has a physiologic cytoprotective role in the retina. Modulating VEGF expression and activity can be toxic to multiple cell types. (Ambati, J., et al., Neuron 75(1):26-39, Jul 2012). Evidence shows that anti-VEGF-A therapy can also contribute to physiologic alterations in the

retinal vasculature in the short-term as well as RPE toxicity in the long-term. (Papadopoulou DN, et al., Ophthalmology 116(9):1755-61 (2009); Sacu S, et al., Invest. Ophthalmol. Vis. Sci. 52(6):3046-50 (2011); and Rofagha S, et al. Am. J. Ophthalmol. 159(5):915-24 (2015)).

[0010] An additional drawback to the use of anti-VEGF agents such as aflibercept, ranibizumab, and bevacizumab is the need for frequent dosing such as once per month (q4wk) or bimonthly (q8wk) or even as frequent as twice per month. (Peden MC et al., Retina Specialist, Jun:28-32 (2016)). Not only does such frequent dosing result in higher cost and repeated, invasive injections into affected eyes, but a significant number of patients have proven to have a poor or non-response to anti-VEGF agents upon standard treatment. Further, many patients have experienced a loss of efficacy of anti-VEGF agents after repeated administration over time, becoming refractory to treatment. (Id.). Persistent presence of fluid at the retina as observed by optical coherence tomography (OCT) or angiography has been observed in 51.5% of patients receiving IVT ranibizumab and in 67.4% of patients receiving IVT bevacizumab after two years of monthly injections. (Id.) Similarly, 19.7% to 36.6% of patients receiving IVT aflibercept either monthly or bimonthly have active exudation via OCT or angiography after 1 year. (Id.)

[0011] Despite the promise of anti-VEGF therapy for the treatment of retinal disorders including nAMD, there is a need for new agents and methods to overcome the drawbacks of IVT anti-VEGF agents.

Summary

[0012] The methods of the disclosure are surprisingly effective at improving the outcome of IVT anti-VEGF dosing regimens by decreasing the necessary frequency of dosing of such agents in both treatment naïve and refractory patients through the use of C-C motif chemokine receptor 3 (CCR3) pathway antagonists. The methods provide a response that is unexpectedly greater in robustness and durability compared to prior methods using anti-VEGF agents, steroids or a combination of the two. Naïve patients who undergo treatment with a small molecule CCR3 antagonist in conjunction with anti-VEGF agents require significantly less frequent doses of anti-VEGF agents, while those with refractory disease surprisingly experience renewed sensitivity.

[0013] The disclosure provides methods of treating patients for retinal-associated disease, including, e.g., dry and wet age-associated macular degeneration, retinal vein occlusion, retinopathy of prematurity, diabetic retinopathy (with and without macular edema), myopic

choroidal neovascularization and geographic atrophy. Aspects of the methods include treating the patient with a dosing regimen of a CCL11/CCR3 pathway antagonist and anti-VEGF agents so as to decrease the recommended frequency of anti-VEGF agents. The methods also include administering of a CCL11/CCR3 pathway antagonist to re-sensitize patients with refractory disease to anti-VEGF agents.

[0014] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Brief Description of the Figures

[0015] **Figure 1, Figure 2 and Figure 3** are graphs reporting the Snellen-based visual acuity ratio in three subjects with naïve unilateral wAMD (i.e. having wAMD in one eye). The subjects are referred to as Patient Case 1, Patient Case 2, and Patient Case 3, respectively. The x-axis depicts time after the last dose of Compound 1 CCR3 antagonist had been administered. Arrows report when an anti-VEGF agent was administered intravitreally (IVT). Notes at the top of the graph annotate changes to morphological features of the retina, particularly the fovea, leading to decision to retreat with an anti-VEGF agent. The Snellen eye chart is a commonly employed and well-recognized test of visual acuity in clinical practice. *See* Aflibercept (Eylea): Treatment of Neovascular (Wet) Age-Related Macular Degeneration (wAMD) [Internet]. Ottawa (ON): Canadian Agency for Drugs and Technologies in Health; 2015 Aug. APPENDIX 5, VALIDITY OF OUTCOME MEASURES.

[0016] **Figure 4 and Figure 5** report the Snellen-based visual acuity ratio in each of the eyes of a subject with refractory, bilateral wAMD (i.e., having wAMD in both eyes). The subject is referred to as Patient Case 4. The x-axis depicts time after the last dose of Compound 1 had been administered. Arrows report when an anti-VEGF agent was administered intravitreally (IVT).

[0017] **Figure 6, Figure 7 and Figure 8** report the number of ETDRS letters read in the three subjects with naïve unilateral wAMD. The x-axis depicts time after the last dose of Compound 1 had been administered. Arrows report when an anti-VEGF agent was administered intravitreally (IVT). Notes at the top of the graph annotate changes to morphological features of the retina, particularly the fovea, leading to decision to retreat with an anti-VEGF agent. The number of

ETDRS letters read was converted from the Snellen-based visual acuity ratios in Figures 1 through 3. *See Beck RW et al., Am J Ophthalmol. 135. 194-205 (2003).*

[0018] **Figure 9** and **Figure 10** report the number of ETDRS letters read in the two eyes of the subject with refractory, bilateral wAMD. The x-axis depicts time after the last dose of Compound 1 had been administered. Arrows report when an anti-VEGF agent was administered intravitreally (IVT). Notes at the top of the graph annotate changes to morphological features of the retina, particularly the fovea, leading to decision to retreat with an anti-VEGF agent.

[0019] **Figure 11**, **Figure 12** and **Figure 13** report central retinal thickness (CRT) in the three subjects with naïve unilateral wAMD. The x-axis depicts time after the last dose of Compound 1 CCR3 antagonist had been administered. Arrows report when an anti-VEGF agent was administered intravitreally (IVT). As with the preceding figures, status of subretinal fluid, intraretinal fluid and choroidal neovascularization (CNV) are also noted over time.

[0020] **Figure 14** and **Figure 15** report central retinal thickness in the subject with refractory, bilateral wAMD. The x-axis depicts time after the last dose of Compound 1 had been administered. Arrows report when an anti-VEGF agent was administered intravitreally (IVT). The status of subretinal fluid, intraretinal fluid and choroidal neovascularization (CNV) are also noted over time.

[0021] **Figure 16**, **Figure 17** and **Figure 18** report central retinal pigmental epithelium (RPE) detachment height in the three subjects with naïve unilateral wAMD. The x-axis depicts time after the last dose of Compound 1 CCR3 antagonist had been administered. Arrows report when an anti-VEGF agent was administered intravitreally (IVT). As with the preceding figures, status of subretinal fluid, intraretinal fluid and choroidal neovascularization (CNV) are also noted over time.

[0022] **Figure 19** and **Figure 20** report central retinal pigmental epithelium (RPE) detachment height in the subject with refractory, bilateral wAMD. The x-axis depicts time after the last dose of Compound 1 CCR3 antagonist had been administered. Arrows report when an anti-VEGF agent was administered intravitreally (IVT). As with the preceding figures, status of subretinal fluid, intraretinal fluid and choroidal neovascularization (CNV) are also noted over time.

[0023] **Figure 21** is a chart depicting the sequencing of treatment for each subject. Patient Cases 1 through 3 (naïve unilateral subjects) received oral Compound 1 twice per day (BID) for 6 weeks prior to anti-VEGF IVT injections. The time between doses of anti-VEGF agents is shown in months (“mo” or “mos”). The mean months between doses of anti-VEGF agents is reported in the box labeled “Naïve Mean” with standard deviations (SD) shown in parentheses.

[0024] For the subject with refractory, bilateral wAMD (Patient Case 4), 11 anti-VEGF IVT treatments had been received in the left eye and 18 in the right eye prior to treatment with Compound 1. The mean months between doses (after Compound 1 treatment) is reported in the box labeled “Refract Mean.”

[0025] **Figure 22** is a table reporting the days between doses, months between doses (or since last dose), mean months without anti-VEGF injection with standard deviation, the range of months between doses, doses per month, and doses per year for each treated anti-VEGF IVT eye for Subjects 1-3 (naïve) and Subject 4 (refractory).

[0026] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Detailed Description

[0027] The methods and compositions disclosed in PCT Patent Publication Number WO2018/187473, U.S. Patent Application No. 12/727,318, U.S. Patent Application No. 13/596,225, U.S. Patent Application No. 14/175,082, U.S. Patent Application No. 13/267,417, U.S. Patent Application No. 14/160,646, U.S. Patent Application No. 14/572,889, U.S. Patent Application No. 13/851,547, U.S. Patent Application No. 13/851,564, and U.S. Patent Application No. 13/851538 are hereby incorporated by reference in their entirety as if set forth herein.

[0028] Methods of treating symptoms of retina-related disease are provided, the method comprising administering compounds from the compounds and formulae discussed below as well as anti-VEGF agents. An aspect of the disclosure comprises a method of reducing the frequency of administering an anti-VEGF agent to a subject diagnosed with a retina related disorder or disease, such as wet age-related macular degeneration, the method comprising:

administering a CCR3 inhibitory agent to the subject; administering the anti-VEGF agent to the subject after the administering of the CCR3 inhibitory agent; and administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency or frequency given in standard practice. In other aspects of the disclosure, the administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency may comprise substituting various different anti-VEGF agents for one or more subsequent doses. For example, the first subsequent dose may be ranibizumab and one or more additional subsequent doses may be bevacizumab, aflibercept, or brolucizumab. Thus mixing anti-VEGF agents is contemplated by the disclosure. In aspects of the disclosure when a first anti-VEGF agent is provided in the initial administration(s) and a second anti-VEGF agent is provided in subsequent administrations, and the first and second anti-VEGF agents differ, the frequency of the doses in the subsequent administrations is less than (i.e., reduced compared to) the recommended frequency of the second anti-VEGF agent or less than the frequency of the second anti-VEGF agent given in standard practice.

[0029] Additional aspects of the disclosure include using CCR3 antagonists as the CCR3 inhibitory agent. Further aspects of the disclosure include using Eotaxin-1 antagonists as the CCR3 inhibitory agent. An additional aspect of the disclosure includes using small molecule agents as the CCR3 inhibitory agent or CCR3 antagonist. Yet an additional aspect of the disclosure utilizes Compound 1 as the small molecule agent. A further aspect of the disclosure utilizes CCR3 inhibitory agents that are not delivered through intravitreal or other type of injection into the eye. A further aspect of the disclosure utilizes CCR3 inhibitory agents that are administered orally to the subject diagnosed with the retina related disorder or disease.

[0030] Further aspects of the disclosure comprise utilization of antibodies or antibody fragments to vascular endothelial growth factor (VEGF). Additional aspects comprise utilization of said antibodies that selectively bind to or inhibit the activity of VEGF. Examples of such antibodies may include, by way of example and not limitation, bevacizumab, ranibizumab, and brolucizumab. Additional aspects of the disclosure comprise use of recombinant fusion proteins as the anti-VEGF agent. Such fusion proteins may be, by way of example and not limitation, aflibercept.

[0031] Another aspect of the disclosure comprises administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency wherein said recommended frequency is greater than or equal to twelve doses every twelve months (i.e., greater than or equal to twelve doses administered within a twelve-month window). Further aspects comprise administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency wherein the recommended frequency is less than one dose every six months. Further aspects comprise administering additional subsequent doses of the anti-VEGF agent to the subject wherein the recommended frequency is less than one dose every five months. Further aspects comprise administering additional subsequent doses of the anti-VEGF agent to the subject wherein the recommended frequency is less than one dose every four months. Further aspects comprise administering additional subsequent doses of the anti-VEGF agent to the subject wherein the recommended frequency is less than one dose every three months. An additional aspect comprises administering additional subsequent doses of the anti-VEGF agent to the subject wherein the recommended frequency is less than one dose every two months.

[0032] Further aspects comprise administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency wherein the recommended frequency is greater than or equal to eleven doses every twelve months. Further aspects comprise administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency wherein the recommended frequency is greater than or equal to ten doses every twelve months. Further aspects comprise administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency wherein the recommended frequency is greater than or equal to nine doses every twelve months. Further aspects comprise administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency wherein the recommended frequency is greater than or equal to eight doses every twelve months. An additional aspect comprises administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency wherein the recommended frequency is greater than or equal to seven doses every twelve months.

[0033] An additional aspect comprises administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency wherein the recommended frequency is greater than or equal to six doses every twelve months. An additional aspect comprises administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency wherein the recommended frequency is greater than or equal to five doses every twelve months. An additional aspect comprises administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency wherein the recommended frequency is greater than or equal to four doses every twelve months. An additional aspect comprises administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency wherein the recommended frequency is greater than or equal to three doses every twelve months. An additional aspect comprises administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency wherein the recommended frequency is greater than or equal to two doses every twelve months. Another aspect of the disclosure includes equivalents to the aforementioned frequencies, (e.g., a frequency of twelve doses every twelve months can also be understood to include one dose per month and a frequency of six doses per twelve months can also be understood to include one dose every two months, etc.). Another aspect of the disclosure includes irregular dosing frequencies, (e.g., a frequency of twelve doses every twelve months can also be understood to include twelve doses in twelve months that may not follow an absolute regular schedule such as two of the twelve doses administered over one and a half months so long as twelve doses were administered within a twelve-month period overall). Another aspect of the disclosure comprises administering an anti-VEGF agent to a subject intravitreally or otherwise by injection into the eye.

[0034] Another aspect of the disclosure further comprises observing or diagnosing a morphological or functional change in an eye of the subject diagnosed with wet age-related macular degeneration (or other retina-associated disease) and who has received one or more additional subsequent doses of the anti-VEGF agent after having been administered a dosing regimen of CCR3/eotaxin-1 pathway antagonist, such as a CCR3 antagonist. Put another way, in any of the methods described herein, the method optionally further comprises examining an eye of the subject to detect a morphological or functional change in the eye. An additional aspect

further comprises determining whether to administer additional subsequent doses of the anti-VEGF agent based on the outcome of observing or diagnosing the morphological or functional change in the eye of the subject diagnosed with wet age-related macular degeneration or other retinal disorder or disease. Optionally, any of the methods described herein may further comprise administering one or more doses of anti-VEGF agent following examination of an eye of the subject, or may comprise delaying one or more further administrations of an anti-VEGF agent to the subject. Further aspects of the disclosure comprise the preceding methods wherein the morphological change is selected from increased intraretinal fluid, increased intraretinal fluid, increased choroidal neovascularization, and increased central retinal pigmental epithelium detachment height. Additional aspects of the disclosure comprise the preceding methods wherein the functional change is selected from decreased visual acuity. The types and degree of change in morphological or functional characteristics would be readily determined by physicians or others having ordinary skill in the art. Additional aspects of the disclosure comprise the preceding methods wherein the functional change is selected from a clinically relevant change in central retinal thickness (CRT), central macular thickness (CMT), or CSF thickness, which refers to the retinal thickness in the central 1 mm subfield of the retina. Another aspect comprises a functional change in clinically relevant CRT, CMT, or CSF as determined from optical coherence tomography (OCT).

[0035] An additional aspect of the disclosure comprises a method of reducing the frequency of administering an anti-VEGF agent to a subject diagnosed with a retina related disorder or disease, the method comprising concurrently administering the anti-VEGF agent with a CCR3 inhibitory agent wherein the anti-VEGF agent is administered at a frequency less than the recommended frequency or frequency given in standard practice. Another aspect comprises the aforementioned concurrent administration of the anti-VEGF agent with a CCR3 inhibitory agent wherein the frequency of administration of the anti-VEGF agent is adjusted based on a morphological or functional change in the eye of the subject. An additional aspect comprises administering a loading dose of the anti-VEGF agent and either concurrently administering a CCR3 inhibitory agent from the start of the loading dose period onward or beginning the concurrent CCR3 inhibitor administration after the anti-VEGF loading dose period is completed.

[0036] Yet an additional aspect of the disclosure comprises first administering a CCR3 inhibitory agent to a treatment-naïve subject with respect to treatment of the retina related disorder and subsequently administering the anti-VEGF agent.

[0037] In any of the methods described herein, the CCR3 inhibitory agent may be administered within about 360 days of administration of the anti-VEGF agent (e.g., within about 330 days, within about 300 days, within about 270 days, within about 240 days, within about 210 days, within about 180 days, within about 150 days, within about 120 days, within about 90 days, within about 75 days, within about 60 days, within about 45 days, within about 30 days, within about 14 days, within about 7 days, within about 3 days, or within about 1 day of administering the anti-VEGF agent). For example, the CCR3 inhibitory agent may be administered between about 1-180 days prior to the initial anti-VEGF agent administration (i.e., within six months, within three months, within two months, within one month, within three weeks, within two weeks, or within one week of the initial administration of the anti-VEGF agent to the subject in the context of the method of the disclosure). In various aspects of the method described herein, the CCR3 inhibitory agent may be administered until a morphological or functional change in the eye of the subject warrants administration of an anti-VEGF agent. Administration of the CCR3 inhibitory agent may be continued after commencing the anti-VEGF agent, but this is not required. Alternatively, the method comprises ceasing the administration of the CCR3 inhibitory agent.

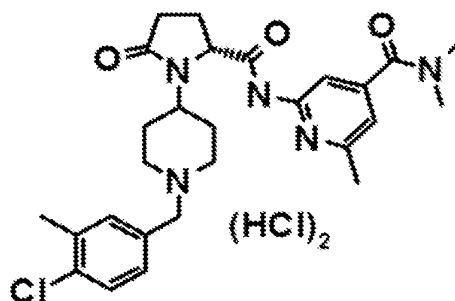
[0038] The disclosure further provides a method of treating a subject suffering from a retina-associated disease, the method comprising administering an anti-VEGF agent to a subject suffering from a retina-associated disease and previously treated with a CCR3 inhibitory agent, wherein the anti-VEGF agent is administered at a frequency less than the recommended frequency for the anti-VEGF agent. Optionally, the subject was treated with the CCR3 inhibitory agent within six months of administration of the anti-VEGF agent.

[0039] Additional aspects of the disclosure comprise the preceding methods wherein the subject has been diagnosed with, by way of example and not limitation, dry macular degeneration (dry AMD), retinal vein occlusion either branched (BRVO) or central (CRVO), retinopathy of prematurity, diabetic retinopathy (with or without macular edema), diabetic macular edema (DME) and geographic atrophy.

a. CCR3 Antagonists

i. Compound 1 & Its Analogs

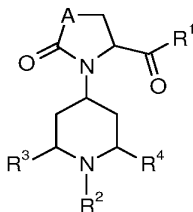
[0040] The methods of the disclosure further comprise administration to a subject of **Compound 1**. Compound 1 is a small molecule antagonist of CCR3 which specifically and selectively binds to CCR3.



Compound 1

[0041] Structural and chemical analogs to Compound 1 also are contemplated for use in the methods the disclosure. Examples of such analogs and their structural and chemical formulae may be found in PCT patent publication number WO2018/187473, US patent application no. 12/727,318, US patent application no. 13/596,225, US patent application no. 14/175,082, US patent application no. 13/267,417, US patent application no. 14/160,646, US patent application no. 14/572,889, US patent application no. 13/851,547, US patent application no. 13/851,564, and US patent application no. 13/851,538 which are incorporated by reference in their entirety as if set forth herein.

[0042] In any of the methods described herein, the CCR3 inhibitory agent may comprise a compound of Formula 1:

**Formula 1**

wherein

A is CH₂, O or N-C₁₋₆-alkyl;

R¹ is selected from

- NHR^{1.1}, NMeR^{1.1};
- NHR^{1.2}, NMeR^{1.2};
- NHCH₂-R^{1.3};
- NH-C₃₋₆-cycloalkyl, whereas optionally one carbon atom is replaced by a nitrogen atom, whereas the ring is optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, O-C₁₋₆-alkyl, NHSO₂-phenyl, NHCONH-phenyl, halogen, CN, SO₂-C₁₋₆-alkyl, COO-C₁₋₆-alkyl;
- a C₉ or C₁₀-bicyclic-ring, whereas one or two carbon atoms are replaced by nitrogen atoms and the ring system is bound via a nitrogen atom to the basic structure of formula **1** and whereas the ring system is optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, COO-C₁₋₆-alkyl, C₁₋₆-haloalkyl, O-C₁₋₆-alkyl, NO₂, halogen, CN, NHSO₂-C₁₋₆-alkyl, methoxy-phenyl;
- a group selected from NHCH(pyridinyl)CH₂COO-C₁₋₆-alkyl, NHCH(CH₂O-C₁₋₆-alkyl)-benzimidazolyl, optionally substituted with halogen or CN;
- or 1-aminocyclopentyl, optionally substituted with methyl-oxadiazole;

R^{1.1} is phenyl, optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, C₂₋₆-alkenyl, C₂₋₆-alkynyl, C₁₋₆-haloalkyl, C₁₋₆-alkylene-OH, C₂₋₆-alkenylene-OH, C₂₋₆-alkynylene-OH, CH₂CON(C₁₋₆-alkyl)₂, CH₂NHCONH-C₃₋₆-cycloalkyl, CN, CO-pyridinyl, CONR^{1.1.1}R^{1.1.2}, COO-C₁₋₆-alkyl, N(SO₂-C₁₋₆-alkyl)(CH₂CON(C₁₋₄-alkyl)₂) O-C₁₋₆-alkyl, O-pyridinyl, SO₂-C₁₋₆-alkyl, SO₂-C₁₋₆-alkylene-OH, SO₂-C₃₋₆-cycloalkyl, SO₂-piperidinyl, SO₂NH-C₁₋₆-alkyl, SO₂N(C₁₋₆-alkyl)₂, halogen, CN, CO-morpholinyl, CH₂-pyridinyl or a heterocyclic ring optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, NHC₁₋₆-alkyl and =O;

R^{1.1.1} is H, C₁₋₆-alkyl, C₃₋₆-cycloalkyl, C₁₋₆-haloalkyl, CH₂CON(C₁₋₆-alkyl)₂, CH₂CO-azetindinyl, C₁₋₆-alkylen-C₃₋₆-cycloalkyl, CH₂-pyranyl, CH₂-tetrahydrofuranyl, CH₂-furanyl, C₁₋₆-alkylen-OH or thiadiazolyl, optionally substituted with C₁₋₆-alkyl;

R^{1.1.2} is H, C₁₋₆-alkyl, SO₂C₁₋₆-alkyl;

or R^{1.1.1} and R^{1.1.2} together are forming a four-, five- or six-membered carbocyclic ring, optionally containing one N or O, replacing a carbon atom of the ring, optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, C₁₋₄-alkylene-OH, OH, =O;

or

R^{1.1} is phenyl, wherein two adjacent residues are together forming a five- or six-membered carbocyclic aromatic or non-aromatic ring, optionally containing independently from each other one or two N, S, or SO₂, replacing a carbon atom of the ring, wherein the ring is optionally substituted with C₁₋₄-alkyl or =O;

R^{1.2} is selected from

- heteroaryl, optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, C₂₋₆-alkenyl, C₂₋₆-alkynyl, C₃₋₆-cycloalkyl, CH₂COO-C₁₋₆-alkyl, CONR^{1.2.1}R^{1.2.2}, COR^{1.2.3}, COO-C₁₋₆-alkyl, CONH₂, O-C₁₋₆-alkyl, halogen, CN, SO₂N(C₁₋₆-alkyl)₂ or heteroaryl optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl;
- heteroaryl, optionally substituted with a five- or six-membered carbocyclic non-aromatic ring containing independently from each other two N, O, S, or SO₂, replacing a carbon atom of the ring;
- a aromatic or non-aromatic C₉ or 10-bicyclic-ring, whereas one or two carbon atoms are replaced by N, O or S each optionally substituted with one or two residues selected from the group consisting of N(C₁₋₆-alkyl)₂, CONH-C₁₋₆-alkyl, =O;
- a heterocyclic non-aromatic ring, optionally substituted with pyridinyl;

- 4,5-dihydro-naphtho[2,1-d]thiazole, optionally substituted with NHCO-C₁₋₆-alkyl,

R^{1.2.1} is H, C₁₋₆-alkyl, C₁₋₆-alkylene-C₃₋₆-cycloalkyl, C₁₋₄-alkylene-phenyl, C₁₋₄-alkylene-furanyl, C₃₋₆-cycloalkyl, C₁₋₄-alkylene-O-C₁₋₄-alkyl, C₁₋₆-haloalkyl or a five- or six-membered carbocyclic non-aromatic ring, optionally containing independently from each other one or two N, O, S, or SO₂, replacing a carbon atom of the ring, optionally substituted with 4-cyclopropylmethyl-piperazinyl

R^{1.2.2} is H, C₁₋₆-alkyl;

R^{1.2.3} is a five- or six-membered carbocyclic non-aromatic ring, optionally containing independently from each other one or two N, O, S, or SO₂, replacing a carbon atom of the ring;

R^{1.3} is selected from phenyl, heteroaryl or indolyl, each optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, C₃₋₆-cycloalkyl, O-C₁₋₆-alkyl, O-C₁₋₆-haloalkyl, phenyl, heteroaryl;

R² is selected from the group consisting of C₁₋₆-alkylene-phenyl, C₁₋₆-alkylene-naphthyl, and C₁₋₆-alkylene-heteroaryl; each optionally substituted with one, two or three residues selected from the group consisting of C₁₋₆-alkyl, C₁₋₆-haloalkyl, O-C₁₋₆-alkyl, O-C₁₋₆-haloalkyl, halogen;

R³ is H, C₁₋₆-alkyl;

R⁴ is H, C₁₋₆-alkyl;

or **R³** and **R⁴** together are forming a CH₂-CH₂ group.

[0043] The antagonistic potency of Compound 1 was determined in several human CCR3-dependent assays (see WO2018/187473). Compound 1's potency was determined via a receptor binding assay, with the IC₅₀ measured at 4.0 ± 1.8 nM and the K_i at 3.2 ± 0.6 nM. IC₅₀ for a calcium influx assay using human CCR3-transfected CHEM1-Gα15 cells was determined to be 0.9 ± 0.2 nM. Antagonism by Compound 1 of human eotaxin-1 induced eosinophil shape change in human whole blood was achieved with an IC₅₀ of 42.5 ± 43.5 nM.

[0044] Potencies for several other mammalian species were also determined in different assays. Species included cynomolgus (macaque) monkeys, mouse, rat, and canines. With

respect to receptor binding assays, the K_i for Compound 1 on mouse CCR3 was 124.3 ± 0.9 nM, and the IC_{50} 87.3 ± 5.6 nM. For rat CCR3, the K_i for the investigational drug of the invention was 1488.6 ± 127.6 nM and the IC_{50} 1719.0 ± 129.9 nM.

ii. Additional CCR3 Antagonists

1. Small Molecules

[0045] An aspect of the disclosure is CCR3 small molecule antagonists which are both potent and selective antagonists of human CCR3. An additional aspect of the disclosure is CCR3 small molecule antagonists which exhibit an inhibitory concentration 50 (IC_{50}) of 500 nM or less in one or more of various assays. Yet another aspect of the disclosure is CCR3 small molecule antagonists which exhibit an inhibitory concentration 50 (IC_{50}) of 200 nM or less in one or more of various assays. A further aspect of the disclosure is CCR3 small molecule antagonists which exhibit an inhibitory concentration 50 (IC_{50}) of 100 nM or less in one or more of various assays. Another aspect of the disclosure is CCR3 small molecule antagonists which exhibit an inhibitory concentration 50 (IC_{50}) of 50 nM or less in one or more various assays. Yet another aspect of the disclosure is CCR3 small molecule antagonists which exhibit an inhibitor concentration 50 (IC_{50}) of 10 nM or less in one or more various assays. An “ IC_{50} ” is the concentration of an inhibitor where the response induced by the agonist ligand is reduced by half. An aspect of the disclosure is that the response can be the activity of the CCR3 receptor protein which binds CCR3 antagonists (including small molecule and antibody antagonists), calcium influx in CCR3-transfected cells, eosinophil shape change, CCR3 internalization, or chemotaxis (migration) assays.

[0046] Various assays to determine an IC_{50} value for a CCR3 small molecule antagonist are well-known to those having ordinary skill in the art.

Eosinophil Shape Change Assay (ESC)

[0047] Eosinophils undergo dramatic shape changes in response to immunological and chemotactic factors, which can be measured in an eosinophil shape change (ESC) assay. It can be determined, for example, using whole blood samples which are incubated with 30 nmol/L of coxtaxin-1 at 37°C for 7 minutes. Samples are then fixed on ice for 20 minutes followed by erythrocyte lysis at room temperature for 15 minutes. ESC is determined by flow cytometry using forward scatter (FSC) as a measure of cell size and side scatter (SSC) as a measure of

granularity. The percentage inhibition of ESC is then calculated and plotted in order to determine the IC₅₀.

CCR3 Internalization

[0048] CCR3 ligand-induced internalization is a critical step in eosinophil functional response to stimulatory signals. Inhibition of CCR3 internalization by CCR3 antagonists can be determined using blood samples incubated with 30 nmol/L of eotaxin-1 at 37°C for 30 minutes. The samples are then incubated with allophycocyanin (APC)-conjugated antibody against CCR3 at room temperature in the dark for 30 minutes, followed by erythrocyte lysis and cell fixation. Samples are then washed and analyzed for FSC, SSC, and APC-fluorescence by flow cytometry. The percentage inhibition of CCR3 internalization is then calculated and plotted.

Calcium Influx (Calcium Mobilization) Assay

[0049] CCR3 Ca²⁺ mobilization studies can be carried out as described previously (White RJ, et al., J Biol Chem, 275(47):36626-31 (2000)). The assay can be performed using Fluo 3-loaded RBL-2H3-CCR3 cells and a microtiter plate-based assay using a fluorescent imaging plate reader (FLIPR). RBL-2H3-CCR3 cells are grown to confluence in RPMI 1640 medium containing 10% fetal calf serum in T-150 flasks with 5% CO₂ at 37°C. Cells are removed from the T-150 flask by removing the medium and then treating the cells with 5 mL of Versene for 5 min at room temperature. Cells are then washed once in RPMI 1640 medium, 10% fetal calf serum and subsequently plated into sterile 96-well black at 40,000 cells/well and incubated for 18–24 hours. On the day of assay, the medium is removed and replaced with 100 µl of Earle's minimal essential medium with Earle's salts containing L-glutamine, 0.1% bovine serum albumin, 4 µM Fluo-3 acetoxymethyl ester (Fluo-3/AM; Molecular Probes, Inc., Eugene, OR), and 1.5 mM sulfinpyrazone. Plates are then incubated for 60 min at 37°C, medium is removed and replaced with the same medium without Fluo-3/AM, and plates are incubated for 10 min at 37°C. Cells are washed three times and incubated at 37°C in 100 µL of assay buffer (120 mM NaCl, 4.6 mM KCl, 1.03 mM KH₂PO₄, 25 mM NaHCO₃, 1.0 mM CaCl₂, 11 mM glucose, 20 mM HEPES (pH 7.4) with 1.5 mM sulfinpyrazone. Plates are then placed into FLIPR for analysis. The maximal change in fluorescence after addition of agonist (eotaxin-1) is quantitated. The percentage of maximal agonist-induced Ca²⁺ mobilization is determined for each concentration of antagonist,

and the IC₅₀ is defined as the concentration of antagonist that inhibits 50% of the maximal response induced by 33 nM eotaxin-1.

Chemotaxis (Migration) Assay

[0050] Chemotactic responsiveness of eosinophils (which express CCR3) can be determined using ChemoTx™ plates (see Wise EL, J Biol Chem, 282(38):27935-43 (2007)). An assay buffer of RPMI supplemented with 0.1% BSA are loaded with duplicate concentrations of agonist in the presence or absence of a CCR3 antagonist. These solutions are applied in a final volume of 31 µL to the lower wells of the chemotaxis chamber. A filter is put into place and 2 × 10⁵ eosinophils in the same assay buffer are applied to the upper surface. Following a 5-hour incubation at (humid) 37°C in the presence of 5% CO₂, the number of migrating eosinophils traversing a 5 µm pore filter are counted, providing the number of migrated cells. The greater the inhibition by an antagonist, the less migration is observed.

[0051] Another aspect of the disclosure is CCR3 small molecule antagonists which have a Ki value of 200 nM or less in a receptor displacement binding assay (also referred to as a receptor binding assay). The Ki value is the dissociation constant which describes the binding affinity between the inhibitor and the receptor. An additional aspect of the disclosure is CCR3 small molecule antagonists which have a Ki value of 100 nM or less in a receptor displacement binding assay. Yet an additional aspect of the disclosure is CCR3 small molecule antagonists which have a Ki value of 50 nM or less in a receptor displacement binding assay. Yet another aspect of the disclosure is CCR3 small molecule antagonists which have a Ki value of 10 nM or less in a receptor displacement binding assay. IC₅₀ values for receptor displacement can also be determined for CCR3 receptor binding and are a measure of the concentration of antagonist needed to inhibit 50% of the agonist ligand (eotaxin-1) from binding CCR3 receptors.

Receptor Displacement Binding Assay

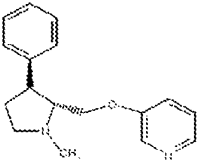
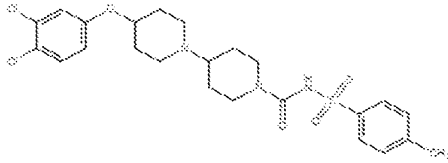
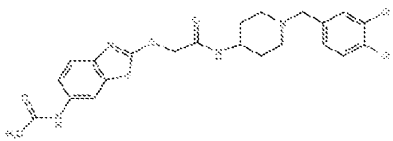
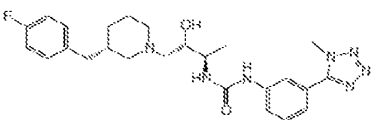
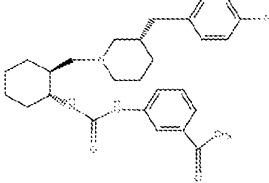
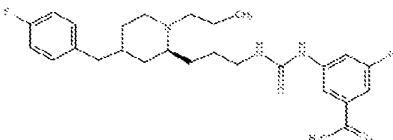
[0052] Dissociation constant (Ki) values for CCR3 small molecule antagonists can be determined using techniques known to those having ordinary skill in the art. Similarly, IC₅₀ values for CCR3 antagonists can be determined with such techniques. An example of a receptor displacement binding assay to determine dissociation constants for receptors such as CCR3 similarly uses radioligand displacement (see Warrior U, et al., J Biomol Screening, 8(3):324-31 (2003) and Becker OM et al., PNAS, 101(31):11304-309 (2004)). Unlabeled (cold) antagonist

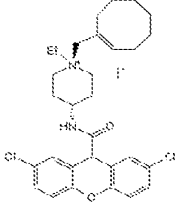
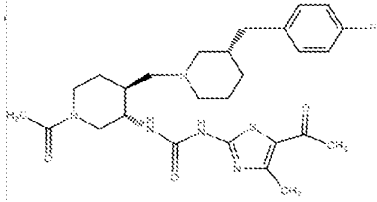
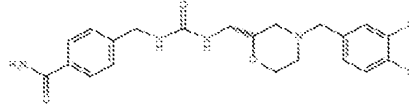
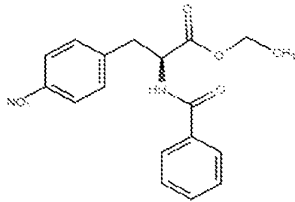
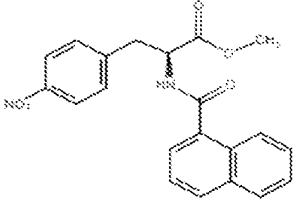
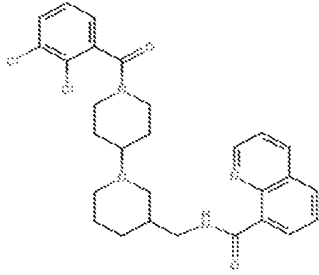
compounds are initially tested at a 10- μ M concentration in duplicate with K562 cell membranes (containing CCR3 receptors) and radioligand. Hits showing >50% inhibition at 10 μ M are validated by a full-concentration dose-response curve, measured between 10-10 M (.1 nM) and 10-4 M (.1 mM). The radioligand is [125I] eotaxin (0.1 nM, K_d = 0.7 nM).

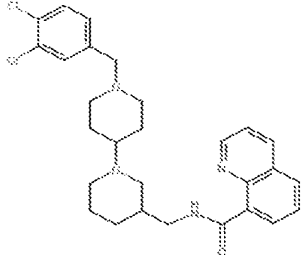
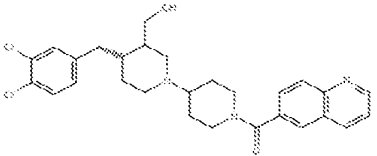
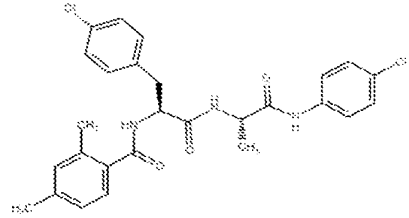
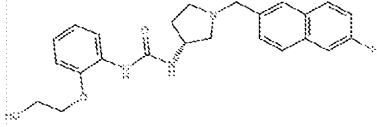
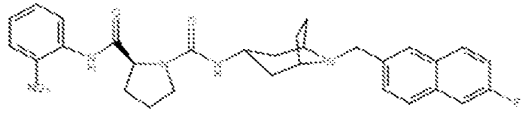
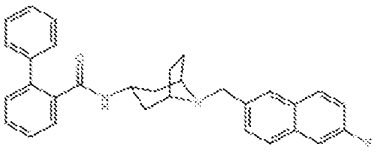
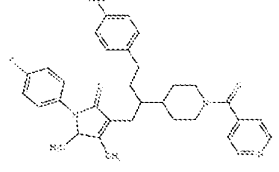
[0053] Thus, the disclosure contemplates use of other small molecule CCR3 antagonists (other than Compound 1 of the disclosure and its analogs) in the context of the method described herein. Many other small molecule CCR3 antagonists are known to those having ordinary skill in the art. These include CCR3 antagonists disclosed by Pease JE and Horuk R, *Expert Opin Drug Discov*, 9(5):467-83 (2014) and *Expert Opin Ther Patents* 19(1) (2009), such as (by way of example, and not limitation) ASM8, GSK766994, GW824575, DPC168, BMS-639623, QAP-642, AZD3778, SB-328437, YM-344031, Compound 80, Compound 27, Compound 32, YM 344484, Compound 3a (Astra Zeneca), Compound 3b (Astra Zeneca), AZ10565259, AZ12436092, 2-(4-fluorophenyl)-4-(2-(1-isonicotinoylpiperidin-4-yl)-4-(4-nitrophenyl)butyl)-1,5-dimethyl-1,2-dihydro-3H-pyrazol-3-one (Astra Zeneca), Compound 20 (Astra Zeneca), and Compound 60 (Astra Zeneca). Aspects of the disclosure may employ these molecules as small molecule CCR3 antagonists serving as CCR3 inhibitory agents.

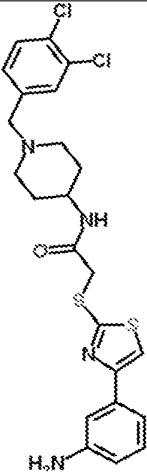
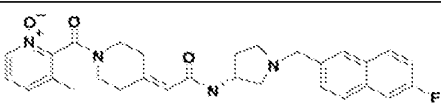
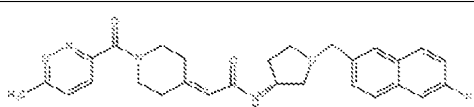
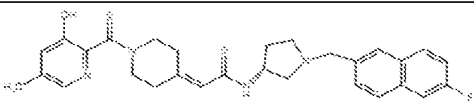
[0054] Further aspects of the disclosure include administration of small molecules disclosed in U.S. Patent Number 7,705,153, U.S. Patent Number 9,206,186, U.S. Patent Number 8,030,303, U.S. Patent Number 7,935,700, and International Patent Application Serial Number PCT/EP2004/002496, as small molecule CCR3 antagonists.

[0055] The disclosure contemplates aspects wherein the CCR3 inhibitory agent comprises a CCR3 antagonist compound from the following table:

Compound Structure	Reference
	<p>Pease & Horuk, <i>Expert Opin. Ther. Patents</i> 19(1) (2009).</p> <p>IC₅₀ = 195 nM</p>
	<p>U.S. Patent No. 7,307,090</p> <p>IC₅₀ = 8 nM</p>
	<p>Pease & Horuk, <i>Expert Opin. Ther. Patents</i> 19(1) (2009).</p> <p>IC₅₀ = 1.5 nM</p>
	<p>Pease JE and Horuk R, <i>Expert Opin Drug Discov</i>, 9(5):467-83 (2014)</p> <p>IC₅₀ (binding) = 300 pM (0.3 nM)</p> <p>IC₅₀ (chemotaxis) = 38 pM</p>
	<p>Pease & Horuk, <i>Expert Opin. Ther. Patents</i> 19(1) (2009).</p> <p>IC₅₀ = 2 nM</p>
	<p>Watson PS <i>et al.</i>, <i>Bioorg & Med Chem</i>, 282(38):27935-43 (2007).</p> <p>IC₅₀ (binding) = 2 nM</p> <p>IC₅₀ (Calcium Influx) = 26 nM</p> <p>IC₅₀ (Chemotaxis) = 19 nM</p>

	<p>Wise EL <i>et al.</i>, <i>J Biol Chem</i>, 282(38): 27935-43 (2007) and Sabroe I, <i>et al.</i>, <i>J Biol Chem</i>, 275(34): 25985-92 (2000).</p>
	<p>Pease & Horuk, <i>Expert Opin Ther Patents</i> 19(1) (2009). IC₅₀ = 1.9 nM</p>
	<p>Pease & Horuk, <i>Expert Opin Ther Patents</i> 19(1) (2009). IC₅₀ = 10 nM</p>
	<p>Mori A. <i>et al.</i>, <i>Int Immunol</i>, 19(8):913-21 (2007).</p>
	<p>Pease & Horuk, <i>Expert Opin Ther Patents</i> 19(1) (2009).</p>
	<p>Pease & Horuk, <i>Expert Opin Ther Patents</i> 19(1) (2009).</p>

	<p>Pease & Horuk, <i>Expert Opin Ther Patents</i> 19(1) (2009).</p> <p>IC₅₀ = 30 nM</p>
	<p>Pease & Horuk, <i>Expert Opin Ther Patents</i> 19(1) (2009).</p>
	<p>Pease & Horuk, <i>Expert Opin Ther Patents</i> 19(1) (2009).</p> <p>IC₅₀ = 3.5 nM</p>
	<p>Nitta A, <i>et al.</i>, <i>Bioorg & Med Chem Lett</i>, 22 6872-81 (2012).</p> <p>IC₅₀ = 1.7 nM</p>
	<p>Nitta A, <i>et al.</i>, <i>Bioorg & Med Chem Lett</i>, 22 6872-81 (2012).</p> <p>IC₅₀ = 4.9 nM</p>
	<p>Sato I, <i>et al.</i>, <i>Bioorg & Med Chem Lett</i>, 16 144-56 (2008).</p> <p>IC₅₀ = 20 nM</p>
	<p>Pease & Horuk, <i>Expert Opin Ther Patents</i> 19(1) (2009).</p> <p>IC₅₀ = 12 nM</p>

	U.S. Patent No. 7,115,635
	Suzuki K, <i>et al.</i> , <i>BBRC</i> , 339:1217-23 (2006). IC_{50} (binding) = 3 nM IC_{50} (Calcium influx) = 5.4 nM IC_{50} (chemotaxis) = 19.9 nM
	Suzuki K, <i>et al.</i> , <i>Eur J Pharm</i> , 563 224-32 (2007). IC_{50} (Calcium influx) = 6.2 nM
	Morokata T <i>et al.</i> , <i>J Pharm Exp Ther</i> 317(1):244-50 (2006).

[0056] The disclosure further contemplates methods wherein the CCR3 inhibitory agent is comprised of one of the following small molecule CCR3 antagonists (further described in U.S. Patent Nos. 10,130,634 (morpholine compounds) and 7,935,700 (morpholine compounds) which have IC_{50} values below 50 nM with many below 10 nM, incorporated herein by reference in their entireties and particular with respect to their description of the following compounds):

(2S)-[4-(carboxymethyl)thiazol-2-ylthio]-N-[[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl]acetamide

(2S)-[4-(carboxymethyl)thiazol-2-ylthio]-N-[[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl]acetamidehydrobromide

(2S)-[4-(3-aminophenyl)thiazol-2-ylthio]-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-[4-(3-carbamoyl-4-hydroxyphenyl)thiazol-2-ylthio]N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}[4-(pyridin-4-yl)thiazol-2-ylthio]acetamide,

(2S)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}[4-(3,4-dimethoxyphenyl)thiazol-2-ylthio]acetamide,

(2S)-(4-carbamoylthiazol-2-ylthio)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-{4-[(2-amino-2-oxoethyl)aminocarbonyl]thiazol-2-ylthio}-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}[4-(5-methyl-1,2,4-oxadiazol-3-yl)thiazol-2-ylthio]acetamidehydrochloride,

(2S)-(5-amino-8H-indeno[1,2-d]thiazol-2-ylthio)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-(4-carboxyphenylthio)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-{4-[(2-amino-2-oxoethyl)aminocarbonyl]phenylthio}N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-{4-[(2-carboxyethyl)aminocarbonyl]thiazol-2-ylthio}N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide hydrochloride,

(2S)-(5-acetamino-1,3,4-thiadiazol-2-ylthio)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-4-(4-carbamoylthiazol-2-ylthio)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}butylamide hydrochloride,

(2S)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}[4-(1H-tetrazol-5-yl)thiazol-2-ylthio]acetamide,

(2S)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}([1,3]thiazolo[5,4-b]pyridin-2-ylthio) acetamide,

(2S)-(E)-[4-(2-carbamoyl-1-yl)thiazol-2-ylthio]-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-[4-(carbamoylmethyl)thiazol-2-ylthio]-N-{[4-(3,4-fluorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-[4-(carbamoylmethyl)thiazol-2-ylthio]-N-{[4-(4-fluorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-(4-carboxy-5-methylthiazol-2-ylthio)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-(4-carbamoyl-5-methylthiazol-2-ylthio)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-(4-carbamoylthiazol-2-ylthio)-N-{[4-(3-chloro-4-fluorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-[4-(2-amino-2-oxoethyl)aminocarbonylthiazol-2-ylthio]-N-{[4-(3,4-difluorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-(5-amino-1,3,4-thiadiazol-2-ylthio)-N-{[4-(3-chlorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-(5-amino-1,3,4-thiadiazol-2-ylthio)-N-{[4-(3,4-difluorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}(pyrimidin-2-ylthio)acetamide,
(2S)-(3-acetyl-2-oxo-2H-chromen-6-ylthio)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-N-{[4-(3,4-difluorobenzyl)morpholin-2-yl]methyl}(6-oxo-1,6-dihydropyridazin-3-ylthio)acetamide,
(2S)-[6-(carbamoylmethyl)pyrazin-2-ylthio]-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-4-(cyclopentanesulfonyl)-N-{[4-(3-chloro-4-fluorobenzyl)morpholin-2-yl]methyl}butylamide,
(2S)-[4-(carboxymethyl)thiazol-2-ylthio]-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-[4-(carboxymethyl)thiazol-2-ylthio]-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide hydrobromide,

(2S)-[4-(2-carboxypropan-2-yl)thiazol-2-ylthio]-N-{{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-(5-amino-1,3,4-thiadiazol-2-ylthio)-N-{{[4-(3,4-difluorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-N-{{[4-(3,4-difluorobenzyl)morpholin-2-yl]methyl}-(6-oxo-1,6-dihydropyridazin-3-ylthio)acetamide,

(2S)-[4-(carboxymethyl)thiazol-2-ylthio]-N-{{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-[4-(carboxymethyl)thiazol-2-ylthio]-N-{{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)[4-(3-aminophenyl)thiazol-2-ylthio]-N-{{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-[4-(3-carbamoyl-4-hydroxyphenyl)thiazol-2-ylthio]-N-{{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-N-{{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}-[4-(pyridin-4-yl)thiazol-2-ylthio]acetamide,

(2S)-N-{{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}-[4-(3,4-dimethoxyphenyl)thiazol-2-ylthio]acetamide,

(2S)-(4-carbamoylthiazol-2-ylthio)-N-{{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-{4-[(2-amino-2-oxoethyl)aminocarbonyl]thiazol-2-ylthio}-N-{{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-N-{{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}-[4-(5-methyl-1,2,4-oxadiazol-3-yl)thiazol-2-ylthio]acetamide hydrochloride,

(2S)-(5-amino-SH-indeno[1,2-d]thiazol-2-ylthio)-N-{{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-(4-carboxyphenylthio)-N-{{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-{4-[(2-amino-2-oxoethyl)aminocarbonyl]phenylthio}-N-{{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-{4-[(2-carboxyethyl)aminocarbonyl]thiazol-2-ylthio}-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide hydrochloride,
(2S)-(5-acetamino-1,3,4-thiadiazol-2-ylthio)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-4-(4-carbamoylthiazol-2-ylthio)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}butyramide hydrochloride,
(2S)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}-[4-(1H-tetrazol-5-yl)thiazol-2-ylthio]acetamide,
(2S)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}-([1,3]thiazolo[5,4-b]pyridin-2-ylthio)acetamide,
(2S)-(E)-[4-(2-carbamoyl-1-yl)thiazol-2-ylthio]-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-[4-(carbamoylmethyl)thiazol-2-ylthio]-N-{[4-(3,4-difluorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-[4-(carbamoylmethyl)thiazol-2-ylthio]-N-{[4-(4-fluorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-(4-carboxy-5-methylthiazol-2-ylthio)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-(4-carbamoyl-5-methylthiazol-2-ylthio)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-(4-carbamoylthiazol-2-ylthio)-N-{5[4-(3-chloro-4-fluorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-[4-(2-amino-2-oxoethyl)aminocarbonylthiazol-2-ylthio]-N-{[4-(3,4-difluorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-(5-amino-1,3,4-thiadiazol-2-ylthio)-N-{[4-(3-chlorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-(5-amino-1,3,4-thiadiazol-2-ylthio)-N-{[4-(3,4-difluorobenzyl)morpholin-2-yl]methyl}acetamide,
(2S)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}-(pyrimidin-2-ylthio)acetamide,
(2S)-(3-acetyl-2-oxo-2H-chromen-6-ylthio)-N-{[4-(3,4-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,

(2S)-N-{{[4-(3,4-difluorobenzyl)morpholin-2-yl]methyl}-(6-oxo-1,6-dihydropyridazin-3-ylthio)acetamide,
 (2S)-[6-dichlorobenzyl)morpholin-2-yl]methyl}acetamide,
 (2S)-4-(cyclopentanesulfonyl)-N-{{[4-(3-chloro-4-fluorobenzyl)morpholin-2-yl]methyl}butyramide.

[0057] The disclosure further contemplates methods wherein the CCR3 inhibitory agent is comprised of one of the following phenoxy or phenylsulfonamide small molecule CCR3 antagonists (further described in U.S. Patent No. 9,206,186, incorporated herein by reference in its entirety and particular with respect to its description of the following compounds) all of which exhibit a binding IC_{50} less than 100 nM and a calcium influx assay IC_{50} less than 100 nM:

N-(R)-(+)-(1-aza-bicyclo[2.2.2]oct-3-yl)-5-cyano-2-(3,5-dichloro-phenoxy)-benzenesulfonamide,
 5-cyano-2-(3,5-dichloro-phenoxy)-N-(2-dimethylamino-ethyl)-N-[2-(2,5-dioxo-pyrrolidin-1-yl)ethyl]-benzenesulfonamide,
 4-(3,5-dichloro-phenoxy)-3-[(3S)-(1H-indol-3-ylmethyl)-piperazine-1-sulfonyl]-benzonitrile,
 4-(3,5-dichlorophenoxy)-3-{ [2-(1 H-1,2,4-triazol-1-ylmethyl)-1-piperazinyl] sulfonyl} benzonitrile hydrochloride.

[0058] The disclosure also further contemplates methods wherein the CCR3 inhibitory agent is comprised of the bipiperidine CCR3 antagonists described U.S. Patent No. 7,705,153, incorporated herein by reference in its entirety) all of which exhibit a K_i value of between 3 nM and 50 nM.

2. Antibodies

[0059] Optionally, the methods of the disclosure comprise administration to a subject antibodies specific for CCR3 or its ligand, Eotaxin-1/CCL11. Such antibodies are known to those having ordinary skill in the art. These include, by way of example and not limitation, MAB320-SP and MAB155-SP (R&D Systems, Minneapolis, MN), MA5-23831 (ThermoFisher Scientific, Waltham, MA), and Cat # 12-1939-42 (Invitrogen, Waltham, MA). Additional examples can be found in Senechal S *et al.*, Lab Invest 82:929-39 (2002) and Williams TJ,

Front. Immunol. 6(84) (2015) (CAT-213, iCo-008, Bertilimumab). The antibodies may be administered systemically (e.g., IV) or by IVT injection.

b. Co-Crystals and Salts, Formulations, Dosage Forms/Ingredients and Dosages

[0060] The disclosure further contemplates aspects wherein co-crystals and salts of Compound 1 or its analogs are administered as CCR3 inhibitory agents. The disclosure further contemplates aspects using formulations of Compound 1 and its analogs. Use of various dosage forms, ingredients, and dosages of Compound 1 and its analogs (or any of the CCR3 inhibitory agents described herein) are also contemplated by the disclosure and suitable for use in any of the methods described herein. Examples of these aspects may be found in PCT Patent Publication Number WO2018/187473, U.S. Patent Application No. 12/727,318, U.S. Patent Application No. 13/596,225, U.S. Patent Application No. 14/175,082, U.S. Patent Application No. 13/267,417, U.S. Patent Application No. 14/160,646, U.S. Patent Application No. 14/572,889, U.S. Patent Application No. 13/851,547, U.S. Patent Application No. 13/851,564, and U.S. Patent Application No. 13/851,538 which are incorporated by reference in their entirety as if set forth herein.

c. Definitions

[0061] Terms not specifically defined herein should be given the meanings that would be given to them by one of skill in the art in light of the disclosure and the context. As used in the specification, however, unless specified to the contrary, the following terms have the meaning indicated and the following conventions are adhered to.

[0062] The term “about” means 5% more or less of the specified value. Thus, about 100 minutes could also be read as from 95 to 105 minutes.

[0063] It must be noted that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a cell” includes a plurality of such cells and reference to “the peptide” includes reference to one or more peptides and equivalents thereof, e.g. polypeptides, known to those having skill in the art, and so forth.

[0064] In describing methods of the present invention, the terms “host”, “subject”, “individual” and “patient” are used interchangeably and refer to any mammal in need of such treatment according to the disclosed methods. Such mammals include, e.g., humans, ovines,

bovines, equines, porcines, canines, felines, non-human primate, mice, and rats. In certain aspects, the subject is a non-human mammal. In some aspects, the subject is a farm animal. In other aspects, the subject is a pet. In some aspects, the subject is mammalian. In certain instances, the subject is human. Other subjects can include domestic pets (e.g., dogs and cats), livestock (e.g., cows, pigs, goats, horses, and the like), rodents (e.g., mice, guinea pigs, and rats, e.g., as in animal models of disease), as well as non-human primates (e.g., chimpanzees, and monkeys). As such, subjects of the invention, include but are not limited to mammals, e.g., humans and other primates, such as chimpanzees and other apes and monkey species; and the like, where in certain aspects the subject are humans. The term subject is also meant to include a person or organism of any age, weight or other physical characteristic, where the subjects may be an adult, a child, an infant (including premature infants) or a newborn.

[0065] As used herein, “treatment” refers to any of (i) the prevention of the disease or disorder, or (ii) the reduction or elimination of symptoms of the disease or disorder. Treatment may be effected prophylactically (prior to the onset of disease) or therapeutically (following the onset of the disease). The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. Thus, the term “treatment” as used herein covers any treatment of an aging-related disease or disorder in a mammal, and includes: (a) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting the disease, i.e., arresting its development; or (c) relieving the disease, i.e., causing regression of the disease. Treatment may result in a variety of different physical manifestations, e.g., modulation in gene expression, rejuvenation of tissue or organs, etc. The therapeutic agent may be administered before, during or after the onset of disease. The treatment of ongoing disease, where the treatment stabilizes or reduces the undesirable clinical symptoms of the patient, is of particular interest. Such treatment may be performed prior to complete loss of function in the affected tissues. The subject therapy may be administered during the symptomatic stage of the disease, and in some cases after the symptomatic stage of the disease.

[0066] As used herein, the term “recommended frequency” refers to the rate of administration over time of a pharmaceutical agent approved by a regulatory agency which has been mandated or suggested whether by product label or other documentation. Said regulatory agency may be,

for example, the U.S. Food and Drug Administration, European Medicines Agency, Pharmaceuticals and Medical Devices Agency (Japan), China Food and Drug Administration or other comparable national or regional authority. For off-label or pre- or non-approved use, the “recommended frequency” refers to the rate of administration over time of a pharmaceutical agent as is generally recognized for an indication by those healthcare givers having ordinary skill in the art whether as recommended by experts in the field or used in general common practice. In both cases, the term also includes the frequency given in standard practice as understood by one having ordinary skill in the art.

[0067] As used herein, the term “loading dose” refers to the practice of administering monthly doses of an anti-VEGF agent to a subject for a period of time lasting no longer than six months, followed by a variable dosing regimen. Thus, the term “loading dose” encompasses (but is not limited to) administering six doses over the course of six months, five doses over the course of five months, four doses over the course of four months, three doses over the course of three months, or two doses over the course of two months. In an exemplary aspect of the disclosure, the method comprises administering an initial “loading dose” of anti-VEGF agent which comprises administering three monthly doses (i.e., one dose per month every three months) prior to administering subsequent additional doses of anti-VEGF agent at an altered frequency between doses (i.e., at an interval other than once a month, and/or which is less frequent than the recommended dosing of the anti-VEGF agent). The variable dosing regimen may be PRN (*pro re nata* or as needed) or follow a prescribed regimen such as bimonthly or trimonthly dosing. (Schmidt-Erfurth U *et al.*, Br J Ophthalmol., 98:1144-67 (2014) and Ba J *et al.*, Drug Design, Develop Therapy, 9:5397-405 (2015)). In various aspects, the variable dosing regimen does not comprise monthly administrations of the anti-VEGF agent.

Aflibercept

[0068] The recommended frequency derived from the FDA approved label (available at www.accessdata.fda.gov/drugsatfda_docs/label/2017/125387s054lbl.pdf under Reference ID 4102905 herein incorporated by reference) for aflibercept (Eylea®) for neovascular (wet) AMD is 2 mg (0.05 mL) administered by intravitreal injection every 4 weeks (monthly) for the first 3 months (a loading dose), followed by 2 mg (0.05 mL) via intravitreal injection every 8 weeks (2 months). Per the label, although it may be dosed as frequently as 2 mg every 4 weeks, additional efficacy has not been demonstrated in most patients when aflibercept is dosed every 4 weeks

compared to every 8 weeks. Some patients may require monthly dosing after the first 12 weeks (3 months).

[0069] For the most common uses of aflibercept, nAMD, the labeled dose would result in 7-8 doses per year (1 dose per month). As mentioned in the introduction, the labeled use of anti-VEGF agents does not always tie to the use of these agents in practice due to the burden of use. Two retrospective analyses assessed two large claims databases of treatment-naïve neovascular AMD patients treated with IVT anti-VEGF. The intensity of anti-VEGF therapy and its relationship with visual acuity change in nAMD patients was assessed. The mean number of injections per patient per year in the first year of treatment for aflibercept was 5.1 (.43 per month) to 7.3 (0.61 per month/ median being 7 (0.58 per month) reflecting the burden of treatment and the desire to extend treatment duration. In the second year, it was 4.8 injections (0.4 per month) yet vision still decreased. (MacCumber M, Yu, JS, Sagkriotis A, et al. Injection intervals in treatment-naïve neovascular AMD patients who received anti-VEGF agents: An analysis of the IRIS Registry. American Academy of Ophthalmology, San Francisco, 2019:PO471).

[0070] It was determined that patients who received fewer anti-VEGF injections experience worse visual outcomes compared with those receiving fixed, frequent anti-VEGF therapy. (Ciulla TA et al., Ophthalmology Retina, 4:19-30 (2020)). In addition, even in this suboptimal treatment paradigm, more than 33% of patients in the claims database required treatment frequency of less than 8 weeks with aflibercept in both the first and second year of treatment, showing the need for frequent follow-up and injections.

[0071] For macular edema following vein occlusion (RVO), the recommended dose for aflibercept is 2 mg (0.05 mL) administered by intravitreal injection every 4 weeks (monthly).

[0072] For diabetic macular edema (DME), the recommended dose for aflibercept is 2 mg (0.05 mL) administered by intravitreal injection every 4 weeks (monthly) for the first 5 injections, followed by 2 mg (0.05 mL) via intravitreal injection once every 8 weeks (2 months). Per the label, although aflibercept may be dosed as frequently as 2 mg every 4 weeks (monthly), additional efficacy has not not demonstrated in most patients when aflibercept was dosed every 4 weeks compared to every 8. Some patients need every 4-week (monthly) dosing after the first 20 weeks (5 months).

[0073] For diabetic retinopathy (DR) the recommended dose for aflibercept is 2 mg (0.05 mL or 50 microliters) administered by intravitreal injection every 4 weeks (monthly) for the first 5 injections, followed by 2 mg (0.05 mL) via intravitreal injection once every 8 weeks (2 months). Per the label, although aflibercept may be dosed as frequently as 2 mg every 4 weeks (monthly), additional efficacy has not been demonstrated in most patients when aflibercept was dosed every 4 weeks compared to every 8 weeks. Some patients may need every 4-week (monthly) dosing after the first 20 weeks (5 months).

Bevacizumab

[0074] In ophthalmology, oncology drug bevacizumab (Avastin®) is typically aliquoted by compounding pharmacies into microdoses and given by transconjunctival intravitreal injections into the posterior segment. Intravitreal injections for retinal pathologies are typically administered at 4-6-week intervals, although this varies widely based on disease and response. The typical dose is 1.25mg in 0.05ml in adults, and half that dose in premature infants. Importantly, bevacizumab has never been approved for ocular use in the US, for any indication. While other doses (2.5mg) have been evaluated in large trials, no significant benefit has been shown over the 1.25mg standard dose, although some advocate “super-doses” in certain situations. (*See Karth PA et al., Bevacizumab, EyeWiki, American Academy of Ophthalmology, (2020) (available at eyewiki.aao.org/Bevacizumab)*).

[0075] For the most common uses of bevacizumab, the labeled dose would result in 12 doses per year (1 dose per month). As mentioned in the introduction, the labeled use of anti-VEGF agents does not always tie to the use of these agents in practice due to the burden of use. A retrospective analysis assessed a large database of treatment-naïve neovascular AMD patients treated with IVT anti-VEGF. The intensity of anti-VEGF therapy and its relationship with visual acuity change in nAMD patients was assessed. The mean number of injections per patient per year for bevacizumab was 7.3 (0.61 per month), with the median being 8 (0.67 per month). It was determined that patients who received fewer anti-VEGF injections experience worse visual outcomes compared with those receiving fixed, frequent anti-VEGF therapy. (Ciulla TA et al., *Ophthalmology Retina*, 4:19-30 (2020)).

Ranibizumab

[0076] The recommended frequency derived from the FDA approved label (available at www.accessdata.fda.gov/drugsatfda_docs/label/2017/125156s111lbl.pdf under Reference ID 4037547 herein incorporated by reference) for ranibizumab (Lucentis®) for neovascular (wet) AMD is one intravitreal injection per month (approximately 28 days). Although not as effective, patients may be treated according to label with 3 monthly doses followed by less frequent dosing with regular assessment. In the nine months after 3 initial monthly doses, less frequent dosing with 4-5 doses on average is expected to maintain visual acuity while monthly dosing may be expected to result in an additional average 1-2 letter gain. Per the label, although not as effective, patients may also be treated with one dose every 3 months after 4 monthly doses. Compared with continued monthly dosing, dosing every 3 months over the next 9 months will lead to an approximate 5-letter (1-line) loss of visual acuity benefit, on average.

[0077] For macular edema following retinal vein occlusion (RVO), 0.5 mg (0.05 mL of Lucentis solution) is recommended IVT once per month (approximately 28 days). In Studies RVO-1 and RVO-2, patients received monthly injections of LUCENTIS for 6 months. In spite of being guided by optical coherence tomography and visual acuity re-treatment criteria, patients who were then not treated at Month 6 experienced on average, a loss of visual acuity at Month 7, whereas patients who were treated at Month 6 did not. Patients with RVO should be treated monthly.

[0078] For diabetic macular edema (DME), 0.3 mg of Lucentis is recommended to be administered by IVT once per month (approximately 28 days). For diabetic retinopathy 0.3 mg of Lucentis solution is recommended to be administered by IVT once per month (approximately 28 days). For myopic choroidal neovascularization (mCNV), 0.5 mg Lucentis is recommended to be administered by IVT once per month (approximately 28 days) for up to three months with patients being retreated if needed.

[0079] For the most common uses of ranibizumab, the labeled dose would result in 12 doses per year (1 dose per month). As mentioned in the introduction, the labeled use of anti-VEGF agents does not always tie to the use of these agents in practice due to the burden of use. Two retrospective analyses assessed two large databases of treatment-naïve neovascular AMD patients treated with IVT anti-VEGF. The intensity of anti-VEGF therapy and its relationship with visual acuity change in nAMD patients was assessed. The mean number of injections per

patient per year for the first year of treatment with ranibizumab was 4.9 (0.41) to 7.3 (0.61 per month, with the median being 7 (0.58 per month), reflecting the burden of treatment and the desire to extend treatment duration. In the second year, it was 4.5 injections (0.38 per month) yet vision still decreased. MacCumber M, Yu, JS, Sagkriotis A, et al. Injection intervals in treatment-naïve neovascular AMD patients who received anti-VEGF agents: An analysis of the IRIS Registry. American Academy of Ophthalmology, San Francisco, 2019:PO471. It was determined that patients who received fewer anti-VEGF injections experience worse visual outcomes compared with those receiving fixed, frequent anti-VEGF therapy. (Ciulla TA et al., Ophthalmology Retina, 4:19-30 (2020)).

Brolucizumab

[0080] The recommended frequency derived from the FDA approved label (available at www.accessdata.fda.gov/drugsatfda_docs/label/2019/761125s000lbl.pdf under BLA reference ID 761125 herein incorporated by reference) for brolucizumab (Beovu®) for neovascular (wet) age-related macular degeneration (wAMD) is 6 mg (0.05 mL of 120 mg/mL solution) monthly (approximately every 25-31 days) for the first three doses (loading dose), followed by one dose of 6 mg (0.05 mL) every 8-12 weeks (determined by the physician). Brolucizumab is a human vascular endothelial growth factor (VEGF) inhibitor administered by intravitreal injection in a single-dose vial. Brolucizumab binds to the three major isoforms of VEGF-A (e.g., VEGF110, VEGF121, and VEGF165) which prevents interaction with receptors VEGFR-1 and VEGFR-2. This inhibits endothelial cell proliferation, neovascularization, and vascular permeability. Brolucizumab is also being investigated for use as a treatment for diabetic macular edema (DME) and macular edema secondary to retinal vein occlusion. For DME, trials have used 6mg/0.05 mL every 4 weeks for 12 months (available at www.clinicaltrials.gov/ct2/show/NCT03917472 Accession No. NCT03917472 herein incorporated by reference). It is also being investigated for a treatment for diabetic retinopathy (DR) with a dose of 6 mg three times, 6 weeks apart (3 x q6w) and then once every 12 weeks for a total of 93 weeks (www.clinicaltrials.gov/ct2/show/NCT04278417 Accession No. NCT04278417 herein incorporated by reference). Brolucizumab has also been studied as a treatment for retinal vein occlusion, both branched (BRVO) and central (CRVO). For BRVO, treatment is 6 mg every 4 weeks for a total of 6 injections, follow by 48 weeks of individual flexible treatment (IFT) (www.clinicaltrials.gov/ct2/show/NCT03802630 Accession No.

NCT03802630 herein incorporated by reference). For CRVO, treatment is also 6 mg every 4 weeks for a total of 6 injections, followed by 48 weeks of individual flexible treatment (IFT) (www.clinicaltrials.gov/ct2/show/NCT03810313 Accession No. NCT03810313 herein incorporated by reference).

[0081] Thus, “at a frequency less than the recommended frequency” or “at a frequency less than the frequency given in standard practice” means, in various aspects of the disclosure, that the subsequent administrations of the anti-VEGF agent are administered at a timing interval that is longer than the time between doses employed by recommended frequency of doses or frequency of doses given in standard practice. In various aspects, the time between doses is at least 30 days longer than the time between doses in the recommended frequency of dosing of the anti-VEGF agent. For example, the time between doses is optionally at least (or greater than) four weeks, at least (or greater than) six weeks, at least (or greater than) eight weeks, at least (or greater than) 10 weeks, at least (or greater than) 12 weeks, at least (or greater than) 14 weeks, or at least (or greater than) 16 weeks. In various aspects, the time between doses is optionally at least four weeks longer than the recommended frequency of dosing, at least six weeks longer than the recommended frequency of dosing, at least eight weeks longer than the recommended frequency of dosing, at least 10 weeks longer than the recommended frequency of dosing, at least 12 weeks longer than the recommended frequency of dosing, at least 14 weeks longer than the recommended frequency of dosing, or at least 16 weeks longer than the recommended frequency of dosing. Thus, for illustration only, the FDA’s recommended dosing regimen for brolucizumab is one dose of 6 mg (0.05 mL) every 8-12 weeks, and the instant method comprises administering a CCR3 inhibitory agent, administering brolucizumab to the subject after the administering of the CCR3 inhibitory agent, and then administering additional subsequent doses of brolucizumab such that the time between doses is, e.g., more than 12 weeks (resulting in a frequency of dosing that is less than the recommended frequency).

[0082] As used herein, the term “refractory” in relation to anti-VEGF treatment refers to a subject who exhibits an incomplete, poor, or declining response to persistent IVT administration of anti-VEGF agents or who is resistant to treatment despite aggressive therapy over an extended period of time. The term also includes subjects classified as nonresponsive, unresponsive, recalcitrant, or resistant to anti-VEGF or who exhibit tolerance to anti-VEGF, exhibit tachyphylaxis to anti-VEGF treatment or are recalcitrant to anti-VEGF treatment. The term also

includes, for example, the eye(s) of subjects who are affected by nAMD show persistent fluid collection despite at least three to five monthly consecutive anti-VEGF injections, persistent exudation after at least 6-month regular anti-VEGF therapy, persistent fluid on spectral-domain OCT (SD-OCT) at greater than 30 days after the last of six IVT injections of an anti-VEGF agent at monthly intervals, and persistent exudation as evident on clinical examination and also imaging studies (leakage on fluorescein angiography, or fibrovascular pigment epithelial detachment (PED) with intraretinal fluid (IRF) or subretinal fluid (SRF) on SD-OCT) or increasing hemorrhage compared to baseline after six consecutive injections at monthly intervals. (See, e.g. Yang S *et al.*, Drug Design, Development and Therapy, 10:1857-67 (2016)).

d. Retina-Associated Disease Indications

i. Macular Degeneration

[0083] Macular degeneration is a clinical term that is used to describe a family of disease that are all characterized by a progressive loss of central vision associated with abnormalities of the choroid, Bruch's membrane, the retinal pigmental epithelium and/or the neural retinalium. These disorders include very common conditions that affect older subjects—such as age-related macular degeneration (AMD) as well as rarer, earlier-onset dystrophies that in some cases can be detected in the first decade of life. Other maculopathies include North Carolina macular dystrophy, Best disease, and Malattia leventinese.

[0084] AMD is the leading cause of permanent vision loss for individuals over the age of 65, currently affecting approximately 15 million Americans. AMD affects light-sensitive photoreceptor cells and pigmented epithelial cells in the macula, the center of the retina of the eye. While early cases may not cause total blindness, the disease destroys central vision, making reading, watching electronic monitor screens, and driving difficult to impossible. It has no documented cure, has never demonstrated spontaneous remission, and effective treatments are limited with substantial burden upon patient and caregiver as well as side effects, and must be continued for the duration of the life of the individual.

[0085] The retina is a complicated network of nerve cells that changes light into nerve impulses that travel to the brain where they are interpreted as visual images. The central part of the retina, called the macula, is responsible for vision that is needed for reading and other detailed work. Damage to the macula results in poor vision. The most common disease process

that affects the macula is AMD. In patients with AMD, retinal photoreceptor and pigment epithelial cells in the macula die over the course of several years. The cell death and gradual visual loss usually do not begin until age 60 or older, thus the name, age-related macular degeneration.

[0086] There are two types of AMD: dry macular degeneration and wet macular degeneration. Dry macular degeneration, although more common, typically results in a less severe, more gradual loss of vision. Patients who are affected by early or intermediate dry AMD have gradual loss of central vision due to the death of photoreceptor cells and their close associates, retinal pigmented epithelial (RPE) cells, with deposition of a complex waxy amyloid mixture, termed 'drusen'. Photoreceptors, the cells in the retina that actually 'see' light, are essential for vision. Macrophagic RPE cells are necessary for photoreceptor survival, function and renewal. The advanced form of dry AMD, called geographic atrophy, represents about 10-15% of AMD. Geographic Atrophy (GA) causes atrophy of photoreceptors, RPE and the underlying choriocapillaris. These atrophic scotoma typically start in the periphery, but over time form a ring around, and eventually can close around the fovea leading to complete blindness. Patients initially present with a blind spot in their peripheral vision which gradually grows, causing profound visual impairment. GA can lead to blindness without ever developing into neovascular or wet AMD. There is currently no treatment for Geographic Atrophy. Patients with wet macular degeneration develop new blood vessels under the retina. As the photoreceptor and RPE cells slowly degenerate, there is a tendency for blood vessels to grow from their normal location in the choroid into an abnormal location beneath the retina. This abnormal new blood vessel growth is called choroidal neovascularization (CNV). The abnormal blood vessels leak and bleed, causing hemorrhage, swelling, scar tissue, and severe loss of central vision. Only 10% of patients with AMD have the wet or exudative type, but it is responsible for 90% of all blindness resulting from AMD. One of the utilities of CCR3 inhibitory agents is the treatment of the inflammation and neovascularization (through a specific mechanism) resulting from disorders such as wAMD, dAMD, and Geographic Atrophy.

ii. Retinal Vein Occlusion (RVO)

[0087] Retinal Vein Occlusion, also known as RVO, occurs when venous occlusion prevents oxygen-depleted blood from flowing out of the eye's vasculature. As a result of reduced flow of oxygen-depleted blood in the eye, oxygen-rich blood is inhibited from reaching the retina's

surface layers, resulting in a hypoxic state. In response, the surface layers of the retina produce pro-angiogenic factors which contribute to the development of abnormal macular edema and neovascularization. RVO can be termed either CRVO (central) or BRVO (branch) for the location of the venous occlusion. One of the utilities of CCR3 inhibitory agents is the treatment of the macular edema and neovascularization resulting from RVO.

iii. Diabetic Retinopathy

[0088] Diabetic Retinopathy is a complication from diabetes that can cause blindness. Diabetic Retinopathy can develop in subjects with either Type 1 or Type 2 diabetes, which is associated with loss of control of blood sugar content. Long term lack of glycemic control causes damage to the blood vessels of the retina, eventually causing damage to the retinal tissue via the signaling of neovascularization, which can lead to inflammation, scarring, and edema. Improper regulation of the growth of these blood vessels ensues, resulting in vessels that leak readily. Symptoms, which may be subtle in early forms the disease, include spots or floaters in the subject's vision, blurred vision, fluctuating vision, impaired color vision, dark/empty areas of vision, and vision loss, usually affecting both eyes.

[0089] Two types of Diabetic Retinopathy exist: Early Diabetic Retinopathy, or Non-proliferative Diabetic Neuropathy (NPDR) and Advanced Diabetic Retinopathy, or Proliferative Diabetic Retinopathy. NPDR is marked by less pronounced neovascularization and vessel growth; however the walls of the retinal blood vessels weaken and the occurrence of microaneurysms can be present. These microaneurysms can protrude and leak fluid and blood into the retina. As more blood vessels are blocked, the NPDR gets more severe. Retinal nerve fibers as well as the macula (central part of the retina) can swell, a condition known as macular edema. In Advanced Diabetic Retinopathy (or Proliferative Diabetic Retinopathy), blood vessels that have been damaged close off, which causes new, abnormal blood vessel growth, and hemorrhage and leakage into the vitreous of the eye becomes more common. Scar tissue that results from the new blood vessel growth can cause retinal detachment as well as increased eye pressure—ultimately causing damage to the optic nerve and glaucoma. When there is leakage of fluid within the retina, this is called Diabetic Macular Edema (DME), a sequelae of PDR which accumulates fluid underneath or within the retina leading to a loss of vision.

[0090] DME although late in the DR cascade, can often be the first symptom noticed diabetic retinopathy. It is a frequent symptom that brings patients to the physician since it is an acute, common manifestation of diabetic retinopathy. Current Anti-VEGF medications have labeled indications for both DME and/or Diabetic Retinopathy. Although the two terms are sometimes used interchangeably in discussing the underlying disease of DR, DR encompasses a much wider range of symptomless to very severe and immediately vision threatening disease, similar to the term AMD. One of the utilities of CCR3 inhibitory agents is the treatment of the macular edema and neovascularization resulting from DR and DME.

iv. Myopic CNV

[0091] Pathological Myopia, or nearsightedness, can cause choroidal neovascularization due to a stretching of the layers of the retina, resulting in the creation of new blood vessels. The disease can happen in younger patients but is more common in elderly patients. Raecker ME *et al.*, *Diagnosis and Treatment of CNV in Myopic Macular Degeneration*, EyeNet Mag, (Apr 2015):35-37. One of the utilities of CCR3 inhibitory agents is the treatment of the neovascularization resulting from CNV.

v. Retinopathy of Prematurity

[0092] Affecting prematurely born babies, Retinopathy of Prematurity (ROP) is an eye disease associated with both oxygen toxicity and local hypoxia. These conditions are thought to contribute to the development of ROP. The underlying pathophysiology of the disease is that hypoxic conditions lead to stimulation of pro-angiogenic factors that cause disorganized growth of blood vessels with result in scarring and retinal detachment. Some patients with ROP can have it in a mild form and fully recover without therapeutic intervention, but in others it can lead to permanent blindness. The exact cause of the disease is unknown but leading hypotheses are that supplemental oxygen either causes local retinal hypoxia through vasoconstriction which triggers neovascularization, or that normal vascular processes are blunted by supplemental oxygen, but when it is suddenly removed results a rapid proliferation of vascular and fibrovascular disease. Surgery and therapeutic intervention are current therapies to treat the disease in its severe form. Surgical therapy can include sclera buckling and/or vitrectomy for retinal detachment, and some experiment with low dose anti-VEGF therapy, although the long-term impact of inhibiting VEGF in infants is unknown given its broad role in angiogenic growth throughout the quickly growing body of an infant. Laser induced photocoagulation is however

the mainstay of ROP treatment currently. The CCR3 inhibitory agents have utility in the prevention of neo-vascularization associated with ROP.

e. Methods of Diagnosing and Monitoring for Improvement of Retina-Associated Disease

i. Introduction

[0093] The disclosure further provides methods of diagnosing retina-associated disease. Such methods may include, by way of example, and not limitation, visual acuity (VA) tests including but limited to best corrected visual acuity (BCVA), macular degeneration or Amsler grids, retina examination with dilated pupils, fundus photography, fluorescein angiography, or optical coherence tomography (OCT) which can determine such endpoints as central retinal thickness (CRT). Any of the techniques described herein may be employed to examine the eye of a subject to determine whether treatment with an anti-VEGF agent is warranted.

ii. Visual Acuity (VA)

[0094] One method that can diagnose or determine disease progression/improvement is testing for visual acuity. Methods for testing for visual acuity are well-known to those having ordinary skill in the art. Visual acuity tests the sharpness of the subject's vision, often using an "eye chart" the most common of which is the Snellen eye chart. Other methods of testing for visual acuity include use of the Early Treatment Diabetic Retinopathy Study (ETDRS) chart, which, as with other VA tests, can be used to diagnose and measure progression/improvement in visual acuity of subjects with retina-associated diseases such as, by way of example and not limitation, macular degeneration, central retinal vein occlusion, retinopathy of prematurity, and diabetic retinopathy. (See Bokinni, Y, *et al.*, Eye 29:1085-91 (2015)).

[0095] A preferred method of determining improvement in visual acuity in a patient is determining whether the subject, after treatment, is able to identify more letters on the Snellen, ETDRS, or other similar charts than they were able to before treatment. Since such visual acuity tests require communication between the subject and the medical professional (e.g., reading letters out loud), it is difficult to get analogous readouts in visual acuity when testing on animal models in pre-clinical studies. Visual acuity tests used in clinical retinal practice typically test for best corrected visual acuity (BCVA) or the visual acuity obtained with correction (which can be glasses, contact lenses, etc.) as the testing is not concerned with underlying refractive correction, but the underlying health of the retina.

[0096] Visual acuity has the advantage of being a clinical endpoint that can be independent of other visual tests that depend upon observation of retinal vascularization or neovascularization such as fundus photography/observation, fluorescein angiography, or even optical coherence tomography. That is, if an improved effect on visual acuity is due not to a mechanism affecting the retinal vasculature, then this test can still reveal the efficacy of a treatment and is an important and real-world measurement of patient function.

iii. Macular Degeneration/Amsler Grids

[0097] One method that is commonly used to diagnose macular degeneration and to determine disease progression is the use Amsler (macular degeneration) grids, the methods of which are well known to those having ordinary skill in the art. The grid comprises a square similar in appearance to graph paper, with dark lines forming a square grid and a dark dot in the middle of the square. Covering each eye in succession, the subject focuses each individual eye on the dark dot and takes note if any of the lines of the grid are broken, distorted, wavy, or blurry.

iv. Comprehensive Retinal Examination with Pupil Dilation

[0098] Comprehensive retinal examination with pupil dilation is a method through which the retina may be observed directly by a practitioner such as an optometrist or ophthalmologist and is well-known to those having ordinary skill in the art. The practitioner administers dilating eye drops to the subject. The drops can be of two types of mydriatic medications, either administered together or separately. One stimulates contraction of the muscles that dilate the pupil (e.g., phenylephrine), and the other type relaxes the muscles that make the pupil constrict (e.g., cyclopentolate). Pupil dilation allows the practitioner to better observe a larger field of the retina during eye examination.

[0099] Comprehensive retinal examination with pupil dilation allows ophthalmologists to diagnose and determine disease progression of various eye and retina-associated diseases such as, by way of example and not limitation, glaucoma, diabetic retinopathy, retinopathy of prematurity, retinal vein occlusion, and age-related macular degeneration. Telltale signs of these diseases that can be determined by fundus photography include swell or leaking of blood vessels in the retina, abnormal growth of blood vessels in or beneath the retina, and deterioration of the macula of the retina.

v. Fundus Photography

[0100] Similar to retinal examination with pupil dilation, fundus photography is a method through which the retina may be photographed directly and is well-known to those having ordinary skill in the art. (Saine, PJ, *et al.*, *Fundus Photography Overview*, OPTHALMIC PHOTOGRAPHY: RETINAL PHOTOGRAPHY, ANGIOGRAPHY, AND ELECTRONIC IMAGING, Butterworth-Heinemann Medical (2nd ed.)). The procedure includes pupil dilation, with the patient sitting before the fundus camera. A flash sends light into the patient's eye, creating a fundus photograph or image of the retina. The photography can be performed with various colored filters, or the patient can be administered dyes such as fluorescein to aid in imaging.

[0101] A fundus camera is a specialized, low power microscope attached to a camera. The angle of acceptance of the lens can create different outputs. A 30-degree angle is considered by those having ordinary skill in the art to be the normal view of the retina. Wide angle fundus cameras are capable of capturing images between 45 and 140 degrees, and narrow angle fundus cameras have angle views of 20 degrees or less.

[0102] As with comprehensive retinal examination with pupil dilation, fundus photography allows ophthalmologists to diagnose and determine disease progression of various eye and retina-associated diseases such as, by way of example and not limitation, glaucoma, retinopathy of prematurity, diabetic retinopathy, retinal vein occlusion and age-related macular degeneration. Telltale signs of these diseases that can be determined by fundus photography include swell or leaking of blood vessels in the retina, abnormal growth of blood vessels in or beneath the retina, scotomas or fibrotic scarring and deterioration of the macula of the retina.

vi. Fluorescein Angiography

[0103] Fluorescein angiography is a method through which the blood vessels of the retina can be evaluated and is well-known to those having ordinary skill in the art. It is used most commonly for diagnosing or measuring progression of choroidal neovascularization in any of the retinal diseases marked by new vessel growth, including but not limited to wAMD, DME, RVO, and ROP.

[0104] Fluorescein dye is injected into the vein of a subject (whose eyes have been dilated prior) in order for the dye to travel to the eye and the vasculature of the retina. Before the dye is injected, baseline photos of the retina are taken. When it is determined that the dye has entered retinal vasculature, additional photos are taken of the retina over the span of one to several

minutes. Viewing the photographs, the ophthalmologist can determine if any vessel growth has occurred and whether any of the dye leaked from the vessels, which helps them understand where new and fragile blood vessels have developed, and if leakage into the retina is occurring.

vii. Optical Coherence Tomography (OCT)

[0105] OCT is a non-invasive test that provides high-resolution cross-sectional images of a retina and employs light waves to produce the images. (Fujimoto, JG, *et al.*, *Neoplasia*, 2(1-2):9-25 (Jan. 2000)). OCT allows for each of the distinctive layers of the retina to be imaged. Accordingly, an ophthalmologist is given the means through which they can map the retina and determine its thickness. Central thickness of the retina, if increased, can represent fluid accumulation under or within the central retina, 'retinal swelling,' and can be measured by OCT. The value obtained is often the CSF thickness, which refers to the retinal thickness in the central 1 mm subfield of the retina. By way of example, and not limitation, OCT allows for: the central retinal thickness (CRT) of the subject's retina to be precisely measured anywhere in the retina; the central macular thickness (CMT) of the subject's retina to be precisely measured in the central part of the retina (macula); and the central subfield thickness (CSF or CSFT or CST) of the subject's retina to be precisely measured in the central 1 mm subfield of the retina. The methods of performing an OCT test as well as determining CRT, CMT, and CST are well-known to those of skill in the art.

[0106] OCT can be performed using eye drops which dilate the pupils and allow better examination of the subject's retinas. Once the pupils are fully dilated, the subject the OCT scanner may scan the subject's eyes in a non-invasive fashion. OCT can help to diagnose many retina-associated conditions/diseases including macular edema, age-related macular degeneration, glaucoma, diabetic retinopathy, and retinopathy of prematurity.

viii. Angiography OCT (angio-OCT/aOCT/OCT-A)

[0107] Angiography OCT is a non-invasive technique that images retinal and choroidal microvasculature. (Fingler J *et al.*, *Investig Ophthalmol Vis Sci*, 49(11):5055-59 (2008)). Laser light reflectance is used on the surface of the moving red blood cells which results in a depiction of microvessels. Thus, injectable dyes are unnecessary in order to obtain a scan of the subject's retina. Multiple scans are taken over time which allows detection of areas with high, medium, and low to no blood flow rates.

[0108] Angio-OCT has been useful in diagnosing and researching several retinal conditions. (See Sousa DC *et al.*, *Optical Coherence Tomography Angiography*, EyeWiki, American Academy of Ophthalmology, (2019) (available at eyewiki.aao.org/Optical_Coherence_Tomography_Angiography). These include both dry and wet age-related macular degeneration (Waheed NK *et al.*, *Developments in Ophthalmology* 56:91-100 (2016) and de Carlo TE *et al.* *Int J Rein Vitro*. 1(1):5 (2015)), diabetic retinopathy (Samara WA *et al.*, *Ophthalmology*, 124(2):235-44 (2017)), central serous chorioretinopathy (de Carlo TE *et al.*, *Ophthalmic Surgery, Lasers Imaging Rein.* 47(2):128-33 (2016)), central retinal vein occlusion (Wakabayashi T *et al.*, *Investig Ophthalmology Vis Sci*, 58(4):2087 (2017)), macular telangiectasia (Zhang Q *et al.*, *Retina*, 35(11):2285-99 (2015)), choroidal neovascular membranes (Querques L *et al.*, *Br J Ophthalmol*, 309162 (2016)), glaucoma (de Carlo TE *et al.* *Int J Rein Vitro*. 1(1):5 (2015)), and uveitis (Kim AY *et al.*, *Am J Ophthalmol*, 171:101-12 (2016)). Both Angio-OCT and OCT can also be utilized to detect subretinal fluid (SRF), intra-retinal fluid (IRF), choroidal neovascularization (CNV), and retinal pigmental epithelium (RPE) thickness and detachment (Faridi A *et al.*, *Ophthalmol Retina*, 1(4):294-303 (2017)) which may be used to monitor morphological characteristics of a subject's retina. Angio-OCT is rapidly developing, and wide-field aOCT can provide an even larger cross section of the retina, and various types of OCT and aOCT can provide higher levels of detail for better resolution of images.

f. Methods of Determining Whether Treatment or Retreatment with Anti-VEGF Therapy is Warranted

[0109] The methods of the disclosure optionally further comprise examining the eye of the subject to determine whether a subject who has been treated with a CCR3 inhibitory agent should be treated with an anti-VEGF therapy. A determination step may be included in the method regardless of whether the subject is naïve (i.e., not previously treated with an anti-VEGF agent), has been treated previously and responded to anti-VEGF therapy, or is refractory to anti-VEGF therapy. In various aspects, the method further comprises administering anti-VEGF therapy to the subject based on the determination of whether the subject should be treated. Methods of determining whether a subject should be treated with an anti-VEGF agent involve examination of the eye of the subject using any one or more suitable techniques, such as the techniques described herein.

[0110] For example, the method may comprise determining whether the central retinal thickness (CRT) of the eye with the retinal disorder/condition has increased using, e.g., OCT. In an aspect of the disclosure, an additional dose of anti-VEGF agent is provided in response to increased CRT of 50 μm , 100 μm , 150 μm , 200 μm or more. (*See e.g.*, Patel AV et al., Int Ophthalmol Clin, 55(4):103-12 (2015) which is incorporated herein by reference in its entirety). Alternatively or in addition, the method may comprise determining whether an anti-VEGF therapy should be administered based on a decrease in visual acuity or best corrected visual acuity by 1 letter, 2 letters, 3 letters, 4 letter, 5 letters, or more. (*See e.g.*, *Id.* and Martin DF et al., Ophthalmology, 119(7):1388-98 (2012) which are incorporated herein by reference in their entirety). Alternatively or in addition, the method may comprise determining whether an anti-VEGF therapy should be administered based on new persistent hemorrhage, increased fluid on OCT, dye leakage on leakage on fluorescein angiography, reports of metamorphopsia (distorted images) or scotoma (blind spots) or other changes in vision reported by the subject. Alternatively or in addition, the method may comprise determining whether there are signs of active choroidal neovascularization, the presence of subretinal, intraretinal, or sub-RPE fluid on OCT, the presence of subretinal or intraretinal hemorrhage, the presence of persistent subretinal or intraretinal hemorrhage, decreased visual acuity relative to the last visit without another explanation, increased lesion size on fluorescein angiography relative to the last angiogram, or leakage on fluorescein angiogram. (See Martin supra and the CATT Manual of Procedures, 2011 available at www.med.upenn.edu/cpob/assets/user-content/documents

/CATTManualofProceduresJan2011.pdf which is herein incorporated by reference in its entirety.). The method may then comprise administering one or more subsequent doses of anti-VEGF agent.

[0111] Determining whether the subject should be treated with the anti-VEGF agent can, for example, be performed at follow-up visits to a physician, ophthalmologist, or other qualified healthcare professional. Such visits may be regular (e.g., monthly), as requested by the patient, or otherwise as needed.

g. Reagents, Devices, and Kits

[0112] Also provided are reagents, devices, and kits thereof for practicing one or more of the above-described methods. The subject reagents, devices, and kits thereof may vary greatly. Reagents and devices of interest include those mentioned above with respect to the methods of administering the CCR3 inhibitory agents in the subject.

[0113] In addition to the above components, the subject kits will further include instructions for practicing the subject methods. These instructions may be present in the subject kits in a variety of forms, one or more of which may be present in the kit. One form in which these instructions may be present is as printed information on a suitable medium or substrate, e.g., a piece or pieces of paper on which the information is printed, in the packaging of the kit, in a package insert, etc. Yet another means would be a computer readable medium, e.g. diskette, CD, portable flash drive, etc., on which the information has been recorded. Yet another means that may be present is a website address which may be used via the internet to access the information at a remote site. Any convenient means may be present in the kits.

[0114] The following examples are provided by way of illustration and not by way of limitation.

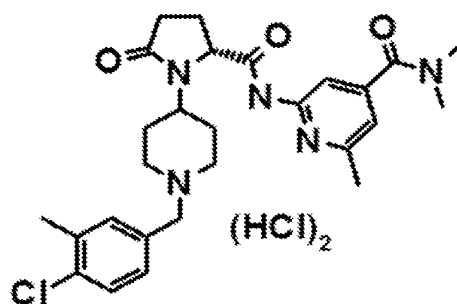
h. EXAMPLES

i. Pharmaceutical Preparation

[0115] The pharmaceutical compositions that are administered to subjects with retina-associated disease that are comprised of the compounds, co-crystals, and salts described above can be synthesized, made, and formulated using the examples disclosed in U.S. Patent Application Publication Nos. 2013/0266646, 2016/0081998, U.S. Patent Nos. 8,278,302, 8,653,075, RE 45323, 8,742,115, 9,233,950, and 8,680,280, which are herein incorporated by reference in their entirety. Additional CCR3 inhibitory agents are also described in detail in Section 0 above.

ii. The Investigational Product

[0116] The investigational product of the invention ("Compound 1") conforms to the following chemical structure:



[0117] Those having ordinary skill in the relevant art would recognize that the compounds, co-crystals, salts, and formulations described previously in US patent application nos. 12/727,318, 13/596,225, 14/175,082, 13/267,417, 14/160,646, 14/572,889, 13/851,547, 13/851,564, 13/851,538 and PCT patent application number WO2018/187473 can also be used in these examples. Those having ordinary skill in the relevant art would also recognize that any CCR3 antagonists described previously above can also be used in these examples.

[0118] The investigational product of the invention was made available as 100 mg, 200 mg, and 400 mg film-coated tablets with a biconvex, round or oval shape and a dull red color. The tablets were produced by a dry granulation process and contained microcrystalline cellulose, hydrogen phosphate, croscarmellose sodium, magnesium stearate, polyvinyl alcohol, titanium dioxide, polyethylene glycol, talc, iron oxide red and iron oxide yellow as inactive ingredients. Placebo tablets matching the investigational product were produced by a direct compression process and contained the same inactive ingredients.

i. Clinical Examples

i. Refractory Subject Eyes

[0119] Subjects with refractory wet Age-Related Macular Degeneration (wAMD) were administered 400 mg of Compound 1 CCR antagonist orally twice per day (BID) for a total dose of 800 mg per day. The treatment regimen of Compound 1 lasted 6-weeks. Refractory subjects for this 6-week regimen were described as having persistent subretinal fluid, intraretinal fluid, and absence of improvement in visual acuity after at least 3 consecutive (approximately 4-6 weeks apart) IVT anti-VEGF injections.

[0120] After the end of a clinical trial consisting of 6-weeks of Compound 1 CCR antagonist treatment, and four weeks of clinical trial follow up with no additional treatment, a subject with heavily pre-treated bilateral disease entered back into normal treatment with their physician and was treated or re-treated with α -VEGF agents per physician judgment guided by standard criteria such as new CNV activity on OCT / angiography OCT (angio-OCT or aOCT) or foveal subretinal fluid (SRF) / intra-retinal fluid (IRF) or PED. Different α -VEGF agents (described in **Figures 4-5, 9-10, 14-15, 19-21** and **Tables D and H**) were administered by intra-vitreous injection. For aflibercept, 2 mg in 0.05 mL was given per single intravitreal injection. For bevacizumab, 1.25 mg in 0.05 mL was given per single intravitreal injection. For ranibizumab, 0.5 mg in 0.05 mL was given per single intravitreal injection. Brolucizumab was not approved at the time and was not used in these clinical examples.

[0121] All OCT tests were performed using REVO NX (Optopol Technology, Poland). A 3-dimensional (3D) scanning protocol covering a 7x7 mm area was used (1024 A-scans x 64 tomograms). The central retinal thickness (CRT) was measured automatically (software version 9.6). The detection of the fovea and the accuracy of layers segmentation was checked by the operator. If necessary, the fovea location and the outer retinal boundaries were corrected manually by moving the pointer to the desired position. In such cases, the central macular thickness value was recalculated. Subretinal and intraretinal fluid presence was assessed manually. SRF was defined as a hyporeflective space between retinal pigment epithelium and the sensory retina, whereas IRF as hyporeflective ovoid or round spaces within the retina. SRF and IRF was distinguished from the outer retinal tubulations (ORTs) and degenerative IRF. The latter were not recognized as a CNV activity sign. CNV activity signs such as neovascular vasculature seen on OCT angiography were monitored. Additionally, lipid exudates and enlargement of RPE elevation were noted.

[0122] Different methods are currently being explored to overcome refractory nAMD. These include switching anti-VEGF agents resulting in very limited success in resensitization, increasing anti-VEGF dose resulting in only modest improvement in resensitization, and *increasing* anti-VEGF dosing frequency resulting in improved resolution of SRF but limited functional improvement. (Fowler SC and Schneider EW, *More frequent dosing for refractory nAMD?*, Retina Specialist, May 13, 2020).

[0123] Despite clear data highlighting the value of standardized dosing or frequency of dosing, the results described in **Figures 4-5, 9-10, 14-15, 19-21** and **Tables D and H** were completely unexpected and surprising. The dose amounts for anti-VEGF agents used in these examples were unchanged, but resolution of SRF, IRF, and CNV was more pronounced. More surprisingly refractory eyes required a significantly *decreased* frequency of IVT anti-VEGF administrations (e.g., 0.69 to 1.37 doses required per year compared to a standard medium of 7.3 across all eyes, and a likely higher frequency of dosing per year in practice of these non-responsive patients. (See **Figures 21** and **22** and the retrospective analysis of Jang L *et al.*, Graefes Arch Clin Exp Ophthalmol, 253(8):1211-16 (2015), *see also* Fowler SC and Schneider EW, More frequent dosing for refractory nAMD?, Retina Specialist, May 13, 2020). Further, resensitization of refractory eyes was achieved to an unexpected degree. For example, the numbers of ETDRS letters read improved and stabilized (**Figures 9-10** and **Table D**). This was unexpected as well as refractory patients tend not to show improvement with the standard of care, rather they typically require monthly (or more frequent) injection merely to maintain their visual or anatomic status, plus these particular subjects were already severely impaired.

[0124] The CRT stabilized and become responsive once again to anti-VEGF IVT injections (**Figures 14-15** and **Table D**). Central retinal pigmental epithelium detachment height was also reduced and could be maintained with infrequent anti-VEGF IVT injections (**Figures 19-21** and **Table D**). Additionally, fluid exudation as determined by the presence of IRF and SRF as well as presence of CNV were once again responsive and successfully managed by anti-VEGF IVT administration even with a significantly *reduced* frequency of dosing (**Table H**). The combination of a regimen of Compound 1 CCR3/Eotaxin-1 pathway antagonist followed by IVT anti-VEGF appeared to be complimentary and function in a synergistic manner. It is unusually surprising that a selective anti-inflammatory agent administered prior to anti-VEGF injection produced a significantly more pronounced and durable effect.

ii. Naïve Subject Eyes

[0125] Newly diagnosed subjects with choroidal neovascularization (CNV) secondary to wAMD were administered 400 mg of Compound 1 CCR antagonist orally twice per day (BID) for a total dose of 800 mg per day. The clinical trial treatment regimen included 6 weeks of Compound 1 followed by 4 weeks of safety follow up (weeks 7 and 8 as in office visits and follow up by phone at week 10) with no additional Compound 1 treatment. Subject treatment

visits took place once per week where mean changes in BCVA as measured by ETDRS were noted, slit lamp and SD-OCT performed, and intraocular pressure taken. Subjects then returned to routine treatment with their physician post clinical trial and were treated or re-treated with α -VEGF agents per physician judgment guided by standard criteria such as new CNV activity on OCT / angio-OCT (aOCT) or increased foveal subretinal fluid (SRF) / intra-retinal fluid (IRF). Different α -VEGF agents (described in **Figures 1-3, 6-8, 11-13, and 16-18, 21** and **Tables A-C and E-G**) were administered by intra-vitreous injection. For aflibercept, 2 mg in 0.05 mL was given per single intravitreal injection. For bevacizumab, 1.25 mg in 0.05 mL was given per single intravitreal injection. For ranibizumab, 0.5 mg in 0.05 mL was given per single intravitreal injection.

[0126] All OCT tests were performed using REVO NX (Optopol Technology, Poland). A 3-dimensional (3D) scanning protocol covering a 7x7 mm area was used (1024 A-scans x 64 tomograms). The central retinal thickness (CRT) was measured automatically (software version 9.6). The detection of the fovea and the accuracy of layers segmentation was checked by the operator. If necessary, the fovea location and the outer retinal boundaries were corrected manually by moving the pointer to the desired position. In such cases, the central macular thickness value was recalculated. Subretinal and intraretinal fluid presence was assessed manually. SRF was defined as a hyporeflective space between retinal pigment epithelium and the sensory retina, whereas IRF as ovoid or round spaces within the retina, with a low reflectivity content. SRF and IRF was distinguished from the outer retinal tubulations (ORTs) and degenerative IRF. The latter were not recognized as a CNV activity sign. CNV activity signs such as neovascular vasculature seen on OCT angiography were monitored. Additionally, lipid exudates and enlargement of RPE elevation were noted.

[0127] In real-world studies, the average number of injections per patient per year in initial anti-VEGF IVT therapy is 7.3 for bevacizumab, aflibercept, and ranibizumab, or per-label would be 12 injections for bevacizumab or ranibizumab. (Ciulla TA et al., *Ophthalmology Retina*, 4:19-30 (2020)). In comparison, the combination of Compound 1 CCR3/Eotaxin-1 pathway antagonist and subsequent anti-VEGF IVT treatment produced a markedly lower average number of injections, independent of the identity of the agent (i.e., aflibercept, bevacizumab, or ranibizumab). Patient Case 1, treated with bevacizumab required an average of less than one dose per year (0.667) (*See Figures 1, 6, 11, 16, and 22 plus Tables A and E*). Patient Case 2,

treated with aflibercept required an average of at most, less than 3 doses per year (2.36). (See **Figures 2, 7, 12, 17, and 22** plus **Tables B and F**). Patient Case 3 treated once with ranibizumab and subsequently with bevacizumab required an average of approximately 1 dose per year (1.23). (See **Figures 3, 8, 13, 18, and 22** plus **Tables C and G**).

[0128] The required frequency of IVT anti-VEGF injections is dramatically reduced in these naïve patients, compared to retrospective analysis. (Ciulla, *supra*). In another retrospective study, even when the timing of IVT anti-VEGF injection was to a PRN dosing regimen (*pro re nata*, PRN, or “as needed” dosing), dosing averages ranged from 4.9 to 8 doses per year (Wykof CC *et al.*, JMCP, 24(2-a):S3-S15 (2018)). Thus, the effect of this combination of the CCR3/Eotaxin-1 pathway antagonist (Compound 1) and various anti-VEGF agents was to unexpectedly reduce the recommended frequency of anti-VEGF IVT injections to a dramatic degree. Such a drop in the frequency of IVT injections will result in significantly less cost, physician visits, and unpleasant and invasive procedures for patients.

[0129] In retrospective analyses where anti-VEGF agents are administered less frequently, CRT, intraretinal and subretinal fluid occurrence as well as pigment epithelial detachment tend to produce a sawtooth curve over time. That is, a semi-regular pattern of peaks and troughs as opposed to a smoother curve produced in populations receiving more frequent dosing. (See, Waldstein SM *et al.*, Ophthalmology 123:1521-29 (2016) and JaffeGJ, *et al.*, Ophthalmology, 123:1856-64 (2016)). Unexpectedly, the naïve patients from Patient Cases 1 through 3 all experienced a relatively flattened visual and anatomic pattern in comparison to the commonly observed semi-regular sawtooth patterns of standard of care anti-VEGF treatment in these measures despite a significantly greater reduction in frequency of anti-VEGF IVT treatment.

iii. Tables

Table A, Patient Case 1
Naïve Unilateral Disease

Days After Last Dose of Compound 1	Drug Treatment	Snellen Ratio (Right Eye)	ETDRS Letters Read (Right Eye)	Central Retinal Thickness (CRT; µm) (Right Eye)	Central Retinal Pigmentary Epithelium (RPE; µm) (Right Eye)
-44	Compound 1	0.125	40	374	148

-41	Compound 1	0.125	40	349	150
-35	Compound 1	0.125	40	329	143
-28	Compound 1	0.2	50	323	140
-21	Compound 1	0.125	40	360	153
-14	Compound 1	0.1	35	375	174
-8	Compound 1	0.1	35	401	189
0	Compound 1	0.08	30	382	198
7		0.0625	25	434	231
15		0.08	30	453	264
47	bevacizumab (right eye)				
68		0.2	50	332	139
124		0.16	45	328	115
194		0.16	45	285	103
271		0.16	45	322	93
318		0.16	45	313	91
348		0.2	50	296	93
383		0.2	50	289	97
426		0.25	55	285	100
491		0.2	50	290	104
540		0.2	50	286	102

Table B, Patient Case 2
Naïve Unilateral Disease

Days After Last Dose of Compound 1	Drug Treatment	Snellen Ratio (Right Eye)	ETDRS Letters Read (Right Eye)	Central Retinal Thickness (CRT; μm) (Right Eye)	Central Retinal Pigmental Epithelium Detachment Height (RPE; μm) (Right Eye)
-42	Compound 1	0.200	50	517	413
-35	Compound 1	0.160	45	489	400
-28	Compound 1	0.160	45	509	395
-21	Compound 1	0.200	50	501	397
-14	Compound 1	0.200	50	520	409
-7	Compound 1	0.200	50	530	425
0	Compound 1	0.160	45	521	403
7		0.160	45	531	427
19	aflibercept (right eye)				
40		0.320	50	339	280
72		0.250	55	247	157
130		0.317	60	256	147
184		0.317	60	229	139
194	aflibercept (right eye)				
236		0.200	50	291	190
253		0.200	50	315	209
329		0.160	45	320	214
348	aflibercept (right eye)				
376		0.200	50	343	160
407		0.200	50	221	151
456		0.250	55	210	139

Table C, Patient Case 3
Naive Unilateral Disease

Days After Last Dose of Compound 1	Drug Treatment	Snellen Ratio (Left Eye)	ETDRS Letters Read (Left Eye)	Central Retinal Thickness (CRT; μm) (Left Eye)	Central Retinal Pigmental Epithelium Detachment Height (RPE; μm) (Left Eye)
-41	Compound 1	0.080	30	398	210
-35	Compound 1	0.080	30	392	208
-28	Compound 1	0.050	20	357	194
-20	Compound 1	0.063	25	450	267
-13	Compound 1	0.087	25	353	235
-6	Compound 1	0.080	30	315	192
0	Compound 1	0.063	25	309	193
7		0.100	35	323	179
48		0.100	35	319	175
50	ranibizumab (left eye)				
79		0.125	40	216	115
103		0.160	45	206	99
148		0.160	45	204	100
168		0.160	45	207	101
220		0.125	40	224	110
293		0.125	40	214	120
320		0.125	40	212	121
365		0.125	40	211	126
398	bevacizumab (left eye)				
418		0.125	40	165	88
448		0.125	40	159	86
467		0.125	40	157	85
526		0.125	40	166	88
585		0.160	45	167	91

Table D, Patient Case 4
Refractory Bilateral Disease

Days After Last Dose of Compound 1	Drug Treatment*	Snellen Ratio (Right Eye)	Snellen Ratio (Left Eye)	ETD RS Letters Read (Right Eye)	ETD RS Letters Read (Left Eye)	Central Retinal Thickness (CRT; μm) (Right Eye)	Central Retinal Thickness (CRT; μm) (Left Eye)	Central Retinal Pigmental Epithelium (RPE; μm) (Right Eye)	Central Retinal Pigmental Epithelium Detachment Height (RPE; μm) (Left Eye)
-49		0.160	0.200	45	50	471	655	253	291
-42	Compd 1	0.125	0.250	40	55	598	715	355	347
-34	Compd 1	0.160	0.200	45	50	445	694	231	277
-28	Compd 1	0.160	0.250	45	55	446	677	226	291
-22	Compd 1	0.125	0.250	40	55	407	672	203	299
-14	Compd 1	0.125	0.250	40	55	426	665	205	275
-7	Compd 1	0.160	0.200	45	50	395	663	207	253
0	Compd 1	0.160	0.200	45	50	440	670	227	243
7		0.200	0.160	50	45	558	713	354	323
14		0.125	0.160	40	45	576	766	386	365
34	ranibizumab (right eye)								
36	ranibizumab (left eye)								
48		0.250	0.400	55	65	313	238	216	50
90		0.250	0.400	55	65	299	208	199	65
125		0.200	0.317	50	60	303	217	203	63
196		0.250	0.317	55	60	307	247	225	72
229		0.250	0.317	55	60	318	225	227	69
299		0.125	0.400	40	65	503	210	339	74
303	ranibizumab (right eye)								
334		0.250	0.317	55	60	342	218	230	69
348		0.250	0.317	55	60	325	223	221	70
377		0.250	0.400	50	65	358	215	229	66
524		0.200	0.317	50	60	439	206	181	64

*Prior to treatment with Compound 1 (Compd 1), the subject's right eye had received 11 injections of ranibizumab, and the left eye received 18 injections of ranibizumab.

Table E, Patient Case 1

Naïve Unilateral Disease

Presence of Sub-retinal fluid (**SRF**), Intraretinal Fluid (**IRF**), or Choroidal Neovascularization (**CNV**)

*Angio-OCT performed these visits only, remainder ETDRS, OCT, Clinical Exam only

Days After Last Dose of Compound 1	Drug Treatment*	Presence of SRF or IRF (Right Eye)	Presence of Other CNV Activity (Right Eye)	Atrophy/Fibrosis Noted (Right Eye)
-44	Compound 1	SRF		Some atrophy and fibrosis
-41	Compound 1			
-35	Compound 1			
-28	Compound 1	slightly less SRF		
-21	Compound 1			
-14	Compound 1			
-8	Compound 1			
0	Compound 1	less SRF		
7		no SRF		
15		Extra-foveal SRF only, IRF		
47	bevacizumab (right eye)			
68				
124				
194				
271				
318				
348				
383				
426				
491				
540				

Table F, Patient Case 2

Naïve Unilateral Disease

Presence of Sub-retinal fluid (SRF), Intraretinal Fluid (IRF), or Choroidal Neovascularization (CNV)

*Angio-OCT performed these visits only, remainder ETDRS, OCT, Clinical Exam only

Days After Last Dose of Compound 1	Drug Treatment	Presence of SRF or IRF (Right Eye)	Presence of Other CNV Activity (Right Eye)	Atrophy/Fibrosis Noted (Right Eye)
-42	Compound 1	SRF, extensive IRF		
-35	Compound 1			
-28	Compound 1			
-21	Compound 1			
-14	Compound 1			
-7	Compound 1			
0	Compound 1			
7		SRF, IRF		
19	aflibercept (right eye)			
40		no SRF, few small IRF, scarring in the fovea		Scarring in the fovea
72		no SRF, no IRF, smaller scarring in the fovea		Smaller scarring in the fovea
130		no SRF, no IRF		
184*		no SRF, no IRF	CNV vessels visible in angio-OCT, no activity in OCT	
194	aflibercept (right eye)			
236*		few cysts of IRF, shallow extra-foveal SRF	small CNV vessels visible in angio-OCT - no injection (patient did not want to have injection)	
253*		more cysts of IRF, SRF	small CNV vessels visible in angio-OCT - no injection	

Days After Last Dose of Compound 1	Drug Treatment	Presence of SRF or IRF (Right Eye)	Presence of Other CNV Activity (Right Eye)	Atrophy/Fibrosis Noted (Right Eye)
329*		IRF, SRF	small CNV vessels visible in angio-OCT - no injection	
348	aflibercept (right eye)			
376		no SRF, no IRF		
407		no SRF, few extra-foveal IRF cysts		
456		no SRF, few cysts of IRF outside the fovea		

Table G, Patient Case 3

Naïve Unilateral Disease

Presence of Sub-retinal fluid (SRF), Intraretinal Fluid (IRF), or Choroidal Neovascularization (CNV)

*Angio-OCT performed these visits only, remainder ETDRS, OCT, Clinical Exam only

Days After Last Dose of Compound 1	Drug Treatment	Presence of SRF or IRF (Left Eye)	Presence of Other CNV Activity (Left Eye)	Atrophy/Fibrosis Noted (Left Eye)
-41	Compound 1	SRF, IRF		
-35	Compound 1	SRF, IRF		
-28	Compound 1	SRF, IRF		
-20	Compound 1	SRF, IRF		
-13	Compound 1	no IRF, less SRF		
-6	Compound 1	no IRF, less SRF		
0	Compound 1	no SRF, two small cysts of IRF		
7		no SRF, less IRF		
48*		no SRF, less IRF	CNV vessels visible in angio-	

Days After Last Dose of Compound 1	Drug Treatment	Presence of SRF or IRF (Left Eye)	Presence of Other CNV Activity (Left Eye)	Atrophy/Fibrosis Noted (Left Eye)
			OCT, no new activity in OCT	
50	ranibizumab (left eye)			
79		no SRF, no IRF		
103		no SRF, no IRF		
148		no SRF, no IRF		
168*		no SRF, no IRF	only few larger CNV vessels (with fibrosis)	only few larger CNV vessels (with fibrosis)
220		no SRF, no IRF		
293		no SRF, no IRF		
320		no SRF, no IRF		
365		very small amount of SRF outside the fovea, no IRF		
398	bevacizumab (left eye)			
418		no SRF, no IRF		
448		no SRF, no IRF		
467		no SRF, no IRF		
526		no SRF, no IRF		
585		no SRF, no IRF		

Table H, Patient Case 4

Refractory Bilateral Disease

Presence of Sub-retinal fluid (SRF), Intraretinal Fluid (IRF), or Choroidal Neovascularization (CNV)

*Angio-OCT performed these visits only, remainder ETDRS, OCT, Clinical Exam only

Days After Last Dose of Compound 1	Drug Treatment *	Presence of SRF or IRF (Right Eye)	Presence of SRF or IRF (Left Eye)	Presence of Other CNV Activity (Right Eye)	Presence of Other CNV Activity (Left Eye)	Atrophy / Fibrosis (Right Eye)	Atrophy / Fibrosis (Left Eye)
-49		large amount of SRF, IRF	SRF, Large foveal IRF space				
-42	Compound 1	large amount of SRF, IRF	SRF, Large foveal IRF space				
-34	Compound 1	SRF, IRF	SRF, Large foveal IRF space				
-28	Compound 1	SRF, IRF					
-22	Compound 1	SRF, IRF					
-14	Compound 1	SRF, IRF	Decrease d Foveal IRF				
-7*	Compound 1	SRF, IRF		CNV vessels in angio-OCT	CNV vessels in angio-OCT		
0	Compound 1	SRF, IRF	Decrease d Foveal IRF				
7		SRF, IRF	SRF, IRF				Large Atrophic IRF

Days After Last Dose of Compound 1	Drug Treatment *	Presence of SRF or IRF (Right Eye)	Presence of SRF or IRF (Left Eye)	Presence of Other CNV Activity (Right Eye)	Presence of Other CNV Activity (Left Eye)	Atrophy / Fibrosis (Right Eye)	Atrophy / Fibrosis (Left Eye)
14		SRF, IRF	SRF, IRF				Large Atrophic IRF
34	ranibizumab (right eye)						
36	ranibizumab (left eye)						
48		no SRF, no IRF	no SRF, no IRF	no activity in OCT	no activity in OCT		
90*		no SRF, no IRF	no SRF, no IRF, single central atrophic space	small CNV vessels in angio-OCT, no activity in OCT	no activity in angio-OCT		Single central atrophic space
125		no SRF, no IRF	no SRF, no IRF, single central atrophic space				Single central atrophic space
196		no SRF, no IRF	no SRF, no IRF, single central atrophic space				Single central atrophic space
229		no SRF, no IRF	no SRF, no IRF, single central atrophic space				Single central atrophic space

Days After Last Dose of Compound 1	Drug Treatment *	Presence of SRF or IRF (Right Eye)	Presence of SRF or IRF (Left Eye)	Presence of Other CNV Activity (Right Eye)	Presence of Other CNV Activity (Left Eye)	Atrophy / Fibrosis (Right Eye)	Atrophy / Fibrosis (Left Eye)
299		SRF, IRF	no SRF, no IRF, single central atrophic space				Single central atrophic space
303	ranibizumab (right eye)						
334		no SRF, no IRF	no SRF, no IRF, single central atrophic space				Single central atrophic space
348			no SRF, no IRF, single central atrophic space				Single central atrophic space
377			no SRF, no IRF, single central atrophic space				Single central atrophic space
524		no SRF, IRF	no SRF, small IRF, single central atrophic space		minimal activity of CNV		

WHAT IS CLAIMED:

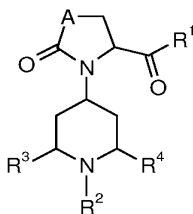
1. A method of reducing the frequency of administration of an anti-VEGF agent to a subject diagnosed with wet age-related macular degeneration, the method comprising: administering a CCR3 inhibitory agent to the subject; administering the anti-VEGF agent to the subject after the administering of the CCR3 inhibitory agent; and administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency.
2. The method of **Claim 1** wherein the CCR3 inhibitory agent is a CCR3 antagonist.
3. The method of **Claim 1** wherein the CCR3 inhibitory agent is an Eotaxin-1 antagonist.
4. The method of **Claim 2** wherein the CCR3 antagonist is a small molecule.
5. The method of **Claim 4** wherein the small molecule is Compound 1.
6. The method of **Claim 1** wherein the anti-VEGF agent is an antibody or antibody fragment that selectively binds to a vascular endothelial growth factor (VEGF).
7. The method of **Claim 6** wherein the antibody or antibody fragment is selected from bevacizumab and ranibizumab.
8. The method of **Claim 1** wherein the anti-VEGF agent is a recombinant fusion protein.
9. The method of **Claim 8** wherein the recombinant fusion protein is aflibercept.
10. The method of **Claim 1** wherein the frequency less than the recommended frequency is less than seven doses every twelve months.
11. The method of **Claim 1** wherein the frequency less than the recommended frequency is less than one dose every three months.
12. The method of **Claim 1** wherein the frequency less than the recommended frequency is less than one dose every two months.
13. The method of **Claim 1** wherein the administering of an anti-VEGF agent to a subject is performed intravitreally.
14. A method of reducing the frequency of administration of an anti-VEGF agent to a subject diagnosed with wet age-related macular degeneration, the method comprising: administering a CCR3 inhibitory agent to the subject;

administering the anti-VEGF agent to the subject after the administering of the CCR3 inhibitory agent;

administering additional subsequent doses of the anti-VEGF agent to the subject at a frequency less than the recommended frequency; and

determining whether to administer the additional subsequent doses of the anti-VEGF agent by diagnosing a morphological change in an eye of the subject diagnosed with wet age-related macular degeneration.

15. The method of **Claim 14** wherein the morphological change is selected from clinically relevant increased intraretinal fluid, clinical-relevant increased intraretinal fluid, clinically relevant increased choroidal neovascularization, and clinically relevant increased central retinal pigmental epithelium detachment height.
16. The method of **Claim 1** wherein the subject is refractory to the anti-VEGF agent.
17. The method of **Claim 1** wherein the subject has not yet been treated with the anti-VEGF agent.
18. The method of **Claim 1** wherein the administering the anti-VEGF agent to the subject after the administering of the CCR3 inhibitory agent commences with administration of a loading dose of the anti-VEGF agent.
19. The method of **Claim 4** wherein the CCR3 agonist is a compound of Formula 1:



Formula 1

wherein

A is CH₂, O or N-C₁₋₆-alkyl;

R¹ is selected from

- NHR^{1.1}, NMeR^{1.1};
- NHR^{1.2}, NMeR^{1.2};
- NHCH₂-R^{1.3};

- NH-C₃₋₆-cycloalkyl, whereas optionally one carbon atom is replaced by a nitrogen atom, whereas the ring is optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, O-C₁₋₆-alkyl, NHSO₂-phenyl, NHCONH-phenyl, halogen, CN, SO₂-C₁₋₆-alkyl, COO-C₁₋₆-alkyl;
- a C_{9 or 10}-bicyclic-ring, whereas one or two carbon atoms are replaced by nitrogen atoms and the ring system is bound via a nitrogen atom to the basic structure of formula **1** and whereas the ring system is optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, COO-C₁₋₆-alkyl, C₁₋₆-haloalkyl, O-C₁₋₆-alkyl, NO₂, halogen, CN, NHSO₂-C₁₋₆-alkyl, methoxy-phenyl;
- a group selected from NHCH(pyridinyl)CH₂COO-C₁₋₆-alkyl, NHCH(CH₂O-C₁₋₆-alkyl)-benzimidazolyl, optionally substituted with halogen or CN;
- or 1-aminocyclopentyl, optionally substituted with methyl-oxadiazole;

R^{1.1} is phenyl, optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, C₂₋₆-alkenyl, C₂₋₆-alkynyl, C₁₋₆-haloalkyl, C₁₋₆-alkylene-OH, C₂₋₆-alkenylene-OH, C₂₋₆-alkynylene-OH, CH₂CON(C₁₋₆-alkyl)₂, CH₂NHCONH-C₃₋₆-cycloalkyl, CN, CO-pyridinyl, CONR^{1.1.1}R^{1.1.2}, COO-C₁₋₆-alkyl, N(SO₂-C₁₋₆-alkyl)(CH₂CON(C₁₋₄-alkyl)₂) O-C₁₋₆-alkyl, O-pyridinyl, SO₂-C₁₋₆-alkyl, SO₂-C₁₋₆-alkylen-OH, SO₂-C₃₋₆-cycloalkyl, SO₂-piperidinyl, SO₂NH-C₁₋₆-alkyl, SO₂N(C₁₋₆-alkyl)₂, halogen, CN, CO-morpholinyl, CH₂-pyridinyl or a heterocyclic ring optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, NHC₁₋₆-alkyl and =O;

R^{1.1.1} is H, C₁₋₆-alkyl, C₃₋₆-cycloalkyl, C₁₋₆-haloalkyl, CH₂CON(C₁₋₆-alkyl)₂, CH₂CO-azetindinyl, C₁₋₆-alkylen-C₃₋₆-cycloalkyl, CH₂-pyranyl, CH₂-tetrahydrofuranyl, CH₂-furanyl, C₁₋₆-alkylen-OH or thiadiazolyl, optionally substituted with C₁₋₆-alkyl;

R^{1.1.2} is H, C₁₋₆-alkyl, SO₂C₁₋₆-alkyl;

or R^{1.1.1} and R^{1.1.2} together are forming a four-, five- or six-membered carbocyclic ring, optionally containing one N or O, replacing a carbon atom of the ring, optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, C₁₋₄-alkylene-OH, OH, =O;

or

R^{1.1} is phenyl, wherein two adjacent residues are together forming a five- or six-membered carbocyclic aromatic or non-aromatic ring, optionally containing independently from each other one or two N, S, or SO₂, replacing a carbon atom of the ring, wherein the ring is optionally substituted with C₁₋₄-alkyl or =O;

R^{1.2} is selected from

- heteroaryl, optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, C₂₋₆-alkenyl, C₂₋₆-alkynyl, C₃₋₆-cycloalkyl, CH₂COO-C₁₋₆-alkyl, CONR^{1.2.1}R^{1.2.2}, COR^{1.2.3}, COO-C₁₋₆-alkyl, CONH₂, O-C₁₋₆-alkyl, halogen, CN, SO₂N(C₁₋₆-alkyl)₂ or heteroaryl optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl;
- heteroaryl, optionally substituted with a five- or six-membered carbocyclic non-aromatic ring containing independently from each other two N, O, S, or SO₂, replacing a carbon atom of the ring;
- a aromatic or non-aromatic C₉ or ₁₀-bicyclic-ring, whereas one or two carbon atoms are replaced by N, O or S each optionally substituted with one or two residues selected from the group consisting of N(C₁₋₆-alkyl)₂, CONH-C₁₋₆-alkyl, =O;
- a heterocyclic non-aromatic ring, optionally substituted with pyridinyl;
- 4,5-dihydro-naphtho[2,1-d]thiazole, optionally substituted with NHCO-C₁₋₆-alkyl,

R^{1.2.1} is H, C₁₋₆-alkyl, C₁₋₆-alkylene-C₃₋₆-cycloalkyl, C₁₋₄-alkylene-phenyl, C₁₋₄-alkylene-furanyl, C₃₋₆-cycloalkyl, C₁₋₄-alkylene-O-C₁₋₄-alkyl, C₁₋₆-haloalkyl or a five- or six-membered carbocyclic non-aromatic ring, optionally containing independently from each other one or two N, O, S,

or SO₂, replacing a carbon atom of the ring, optionally substituted with 4-cyclopropylmethyl-piperazinyl

R^{1.2.2} is H, C₁₋₆-alkyl;

R^{1.2.3} is a five- or six-membered carbocyclic non-aromatic ring, optionally containing independently from each other one or two N, O, S, or SO₂, replacing a carbon atom of the ring;

R^{1.3} is selected from phenyl, heteroaryl or indolyl, each optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, C₃₋₆-cycloalkyl, O-C₁₋₆-alkyl, O-C₁₋₆-haloalkyl, phenyl, heteroaryl;

R² is selected from the group consisting of C₁₋₆-alkylene-phenyl, C₁₋₆-alkylene-naphthyl, and C₁₋₆-alkylene-heteroaryl; each optionally substituted with one, two or three residues selected from the group consisting of C₁₋₆-alkyl, C₁₋₆-haloalkyl, O-C₁₋₆-alkyl, O-C₁₋₆-haloalkyl, halogen;

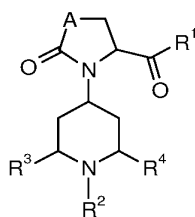
R³ is H, C₁₋₆-alkyl;

R⁴ is H, C₁₋₆-alkyl;

or **R**³ and **R**⁴ together are forming a CH₂-CH₂ group.

20. A method of treating a subject suffering from a retina-associated disease, the method comprising administering an anti-VEGF agent to a subject suffering from a retina-associated disease and previously treated with a CCR3 inhibitory agent, wherein the anti-VEGF agent is administered at a frequency less than the recommended frequency for the anti-VEGF agent.
21. The method of **Claim 20** wherein the subject was treated with the CCR3 inhibitory agent within six months of administration of the anti-VEGF agent.
22. The method of **Claim 20** wherein the CCR3 inhibitory agent is a CCR3 antagonist.
23. The method of **Claim 20** wherein the CCR3 inhibitory agent is an Eotaxin-1 antagonist.
24. The method of **Claim 22** wherein the CCR3 antagonist is a small molecule.
25. The method of **Claim 24** wherein the small molecule is Compound 1.
26. The method of **Claim 20** wherein the anti-VEGF agent is an antibody or antibody fragment that selectively binds to a vascular endothelial growth factor (VEGF).

27. The method of **Claim 27** wherein the antibody or antibody fragment is selected from bevacizumab and ranibizumab.
28. The method of **Claim 20** wherein the anti-VEGF agent is a recombinant fusion protein.
29. The method of **Claim 28** wherein the recombinant fusion protein is aflibercept.
30. The method of **Claim 20** wherein the frequency less than the recommended frequency is less than seven doses every twelve months.
31. The method of **Claim 20** wherein the frequency less than the recommended frequency is less than one dose every three months.
32. The method of **Claim 20** wherein the frequency less than the recommended frequency is less than one dose every two months.
33. The method of **Claim 20** wherein the administering of an anti-VEGF agent to a subject is performed intravitreally.
34. The method of **Claim 24** wherein the CCR3 antagonist is a compound of Formula 1:

**Formula 1**

wherein

A is CH₂, O or N-C₁₋₆-alkyl;

R¹ is selected from

- NHR^{1.1}, NMeR^{1.1};
- NHR^{1.2}, NMeR^{1.2};
- NHCH₂-R^{1.3};
- NH-C₃₋₆-cycloalkyl, whereas optionally one carbon atom is replaced by a nitrogen atom, whereas the ring is optionally substituted with one or two residues selected

from the group consisting of C₁₋₆-alkyl, O-C₁₋₆-alkyl, NHSO₂-phenyl, NHCONH-phenyl, halogen, CN, SO₂-C₁₋₆-alkyl, COO-C₁₋₆-alkyl;

- a C₉ or 10-bicyclic-ring, whereas one or two carbon atoms are replaced by nitrogen atoms and the ring system is bound via a nitrogen atom to the basic structure of formula **1** and whereas the ring system is optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, COO-C₁₋₆-alkyl, C₁₋₆-haloalkyl, O-C₁₋₆-alkyl, NO₂, halogen, CN, NHSO₂-C₁₋₆-alkyl, methoxy-phenyl;
- a group selected from NHCH(pyridinyl)CH₂COO-C₁₋₆-alkyl, NHCH(CH₂O-C₁₋₆-alkyl)-benzimidazolyl, optionally substituted with halogen or CN;
- or 1-aminocyclopentyl, optionally substituted with methyl-oxadiazole;

R^{1.1} is phenyl, optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, C₂₋₆-alkenyl, C₂₋₆-alkynyl, C₁₋₆-haloalkyl, C₁₋₆-alkylene-OH, C₂₋₆-alkenylene-OH, C₂₋₆-alkynylene-OH, CH₂CON(C₁₋₆-alkyl)₂, CH₂NHCONH-C₃₋₆-cycloalkyl, CN, CO-pyridinyl, CONR^{1.1.1}R^{1.1.2}, COO-C₁₋₆-alkyl, N(SO₂-C₁₋₆-alkyl)(CH₂CON(C₁₋₄-alkyl)₂) O-C₁₋₆-alkyl, O-pyridinyl, SO₂-C₁₋₆-alkyl, SO₂-C₁₋₆-alkylen-OH, SO₂-C₃₋₆-cycloalkyl, SO₂-piperidinyl, SO₂NH-C₁₋₆-alkyl, SO₂N(C₁₋₆-alkyl)₂, halogen, CN, CO-morpholinyl, CH₂-pyridinyl or a heterocyclic ring optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, NHC₁₋₆-alkyl and =O;

R^{1.1.1} H, C₁₋₆-alkyl, C₃₋₆-cycloalkyl, C₁₋₆-haloalkyl, CH₂CON(C₁₋₆-alkyl)₂, CH₂CO-azetindinyl, C₁₋₆-alkylen-C₃₋₆-cycloalkyl, CH₂-pyranyl, CH₂-tetrahydrofuranyl, CH₂-furanyl, C₁₋₆-alkylen-OH or thiadiazolyl, optionally substituted with C₁₋₆-alkyl;

R^{1.1.2} H, C₁₋₆-alkyl, SO₂C₁₋₆-alkyl;

or R^{1.1.1} and R^{1.1.2} together are forming a four-, five- or six-membered carbocyclic ring, optionally containing one N or O, replacing a carbon atom of the ring,

optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, C₁₋₄-alkylene-OH, OH, =O;

or

R^{1.1} is phenyl, wherein two adjacent residues are together forming a five- or six-membered carbocyclic aromatic or non-aromatic ring, optionally containing independently from each other one or two N, S, or SO₂, replacing a carbon atom of the ring, wherein the ring is optionally substituted with C₁₋₄-alkyl or =O;

R^{1.2} is selected from

- heteroaryl, optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, C₂₋₆-alkenyl, C₂₋₆-alkynyl, C₃₋₆-cycloalkyl, CH₂COO-C₁₋₆-alkyl, CONR^{1.2.1}R^{1.2.2}, COR^{1.2.3}, COO-C₁₋₆-alkyl, CONH₂, O-C₁₋₆-alkyl, halogen, CN, SO₂N(C₁₋₆-alkyl)₂ or heteroaryl optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl;
- heteroaryl, optionally substituted with a five- or six-membered carbocyclic non-aromatic ring containing independently from each other two N, O, S, or SO₂, replacing a carbon atom of the ring;
- a aromatic or non-aromatic C₉ or C₁₀-bicyclic-ring, whereas one or two carbon atoms are replaced by N, O or S each optionally substituted with one or two residues selected from the group consisting of N(C₁₋₆-alkyl)₂, CONH-C₁₋₆-alkyl, =O;
- a heterocyclic non-aromatic ring, optionally substituted with pyridinyl;
- 4,5-dihydro-naphtho[2,1-d]thiazole, optionally substituted with NHCO-C₁₋₆-alkyl,

R^{1.2.1} H, C₁₋₆-alkyl, C₁₋₆-alkylene-C₃₋₆-cycloalkyl, C₁₋₄-alkylene-phenyl, C₁₋₄-alkylene-furanyl, C₃₋₆-cycloalkyl, C₁₋₄-alkylene-O-C₁₋₄-alkyl, C₁₋₆-haloalkyl or a five- or six-membered carbocyclic non-aromatic ring, optionally containing independently from each other one or two N, O, S, or SO₂, replacing a carbon atom of the ring, optionally substituted with 4-cyclopropylmethyl-piperazinyl

- $R^{1.2.2}$ H, C₁₋₆-alkyl;
- $R^{1.2.3}$ a five- or six-membered carbocyclic non-aromatic ring, optionally containing independently from each other one or two N, O, S, or SO₂, replacing a carbon atom of the ring;
- $R^{1.3}$ is selected from phenyl, heteroaryl or indolyl, each optionally substituted with one or two residues selected from the group consisting of C₁₋₆-alkyl, C₃₋₆-cycloalkyl, O-C₁₋₆-alkyl, O-C₁₋₆-haloalkyl, phenyl, heteroaryl;
- R^2 is selected from the group consisting of C₁₋₆-alkylene-phenyl, C₁₋₆-alkylene-naphthyl, and C₁₋₆-alkylene-heteroaryl; each optionally substituted with one, two or three residues selected from the group consisting of C₁₋₆-alkyl, C₁₋₆-haloalkyl, O-C₁₋₆-alkyl, O-C₁₋₆-haloalkyl, halogen;
- R^3 is H, C₁₋₆-alkyl;
- R^4 is H, C₁₋₆-alkyl;
- or R^3 and R^4 together are forming a CH₂-CH₂ group.

Figure 1

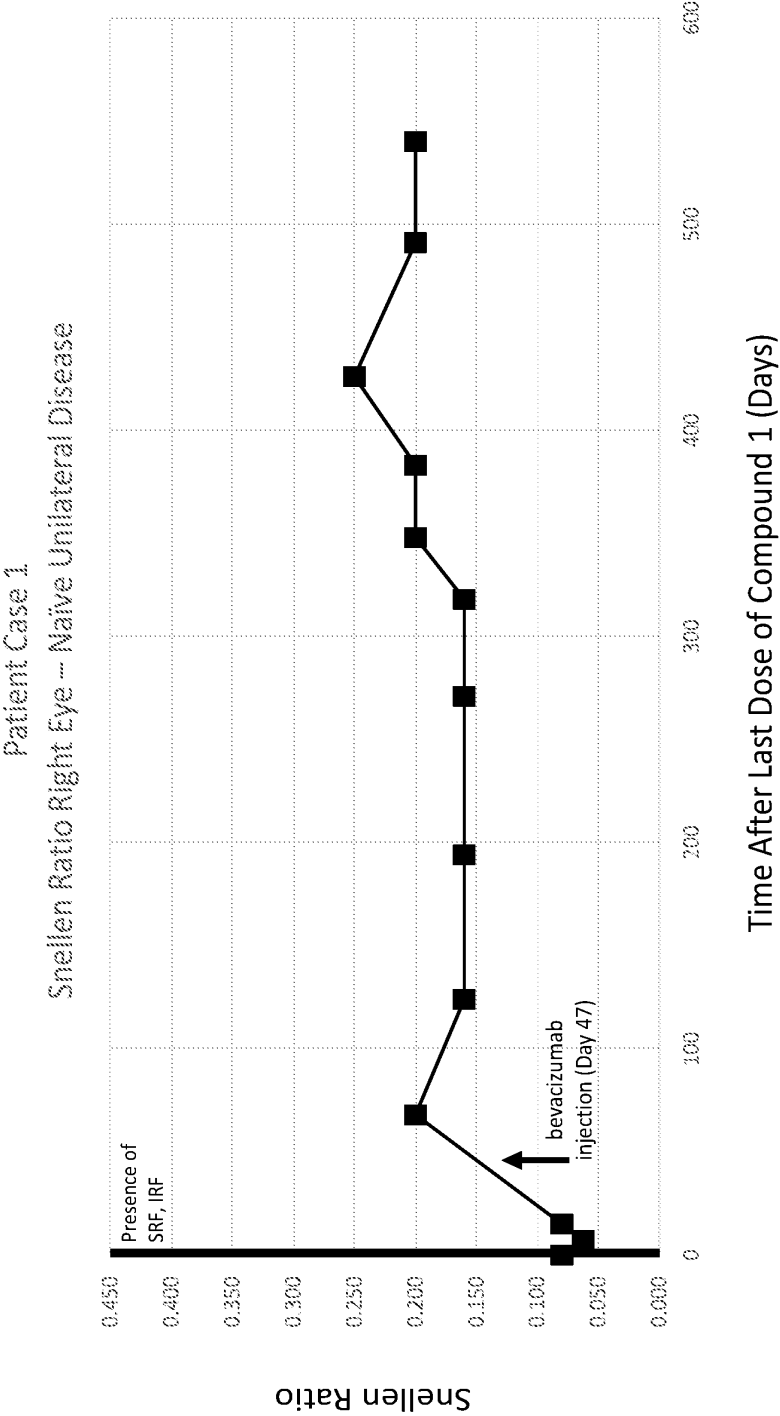


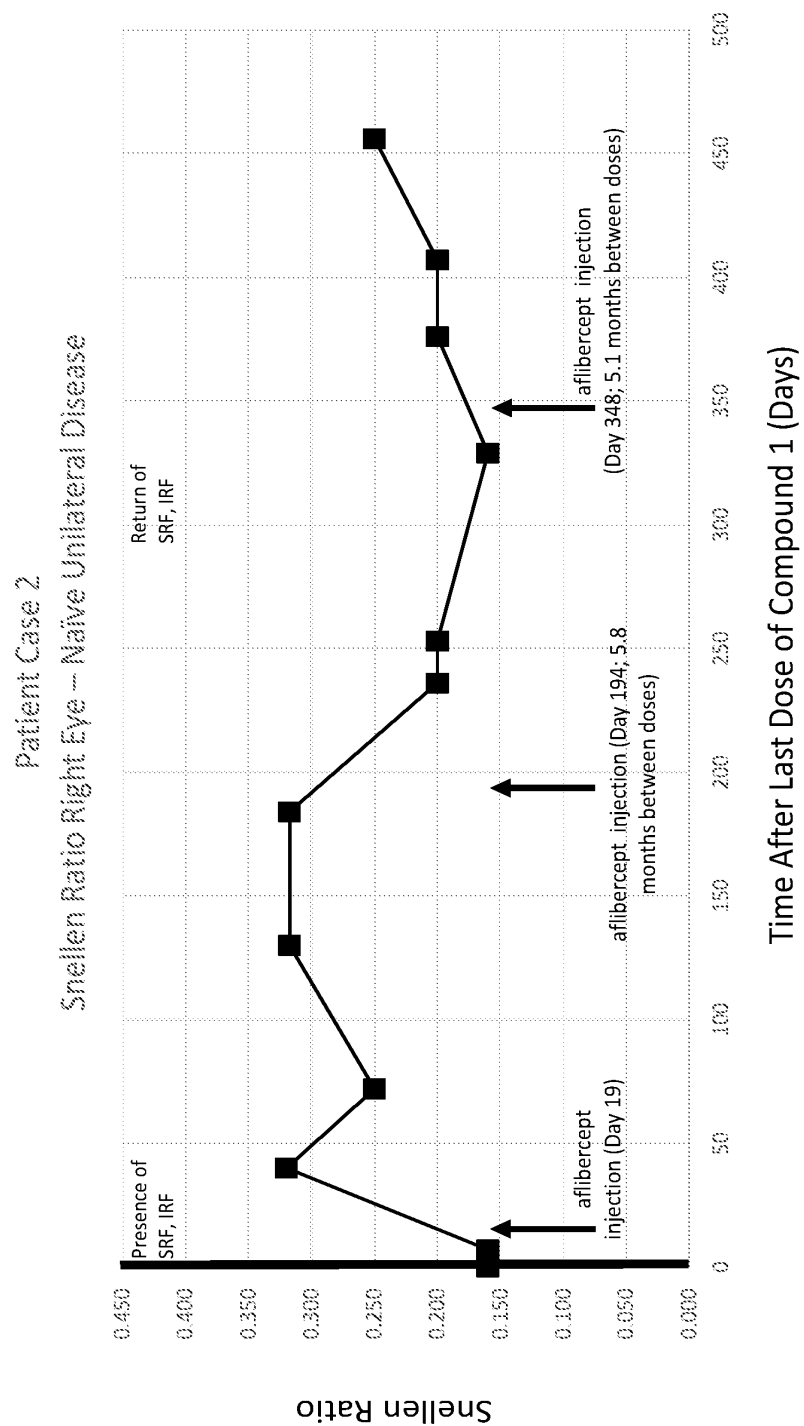
Figure 2

Figure 3

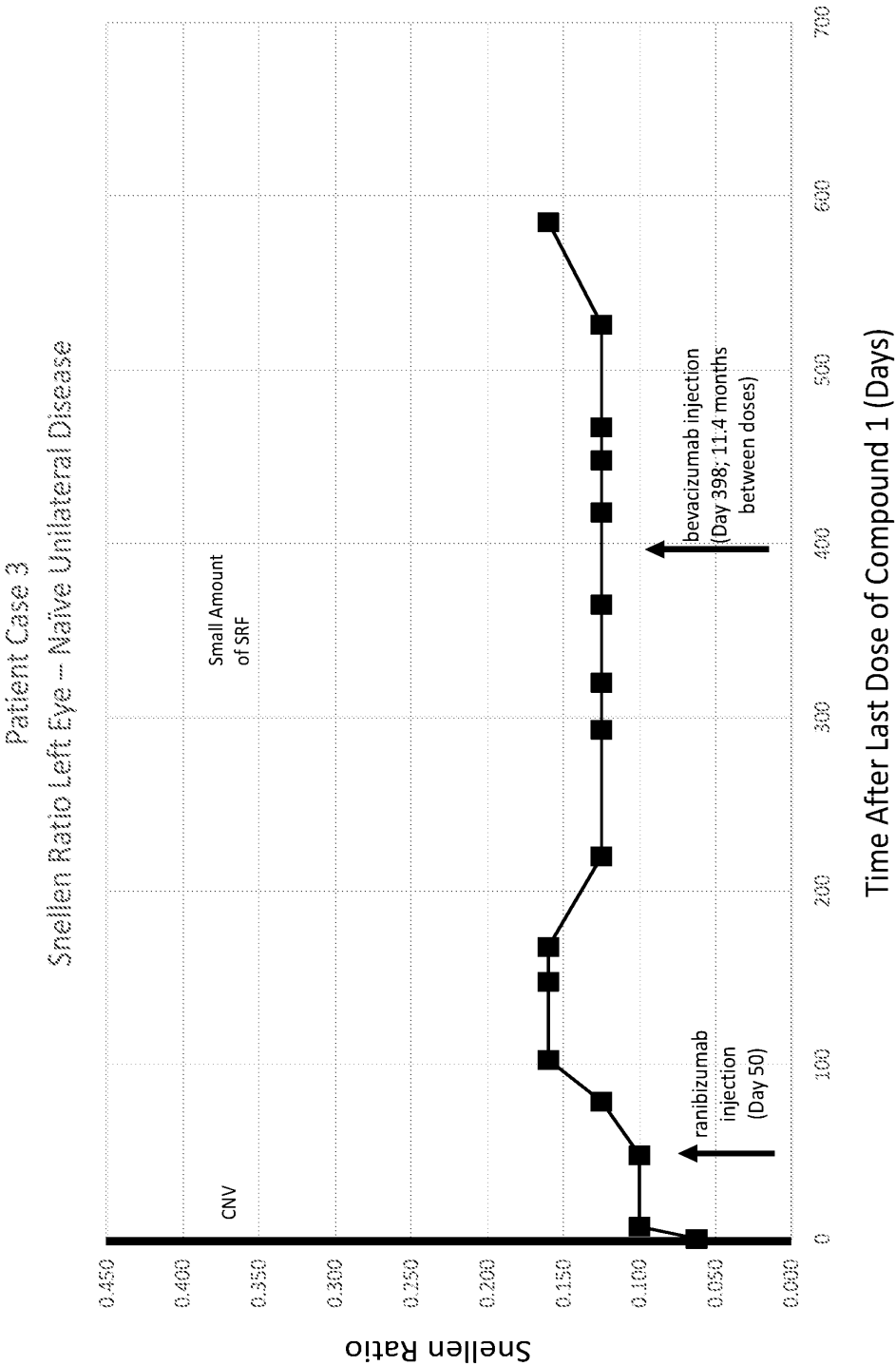


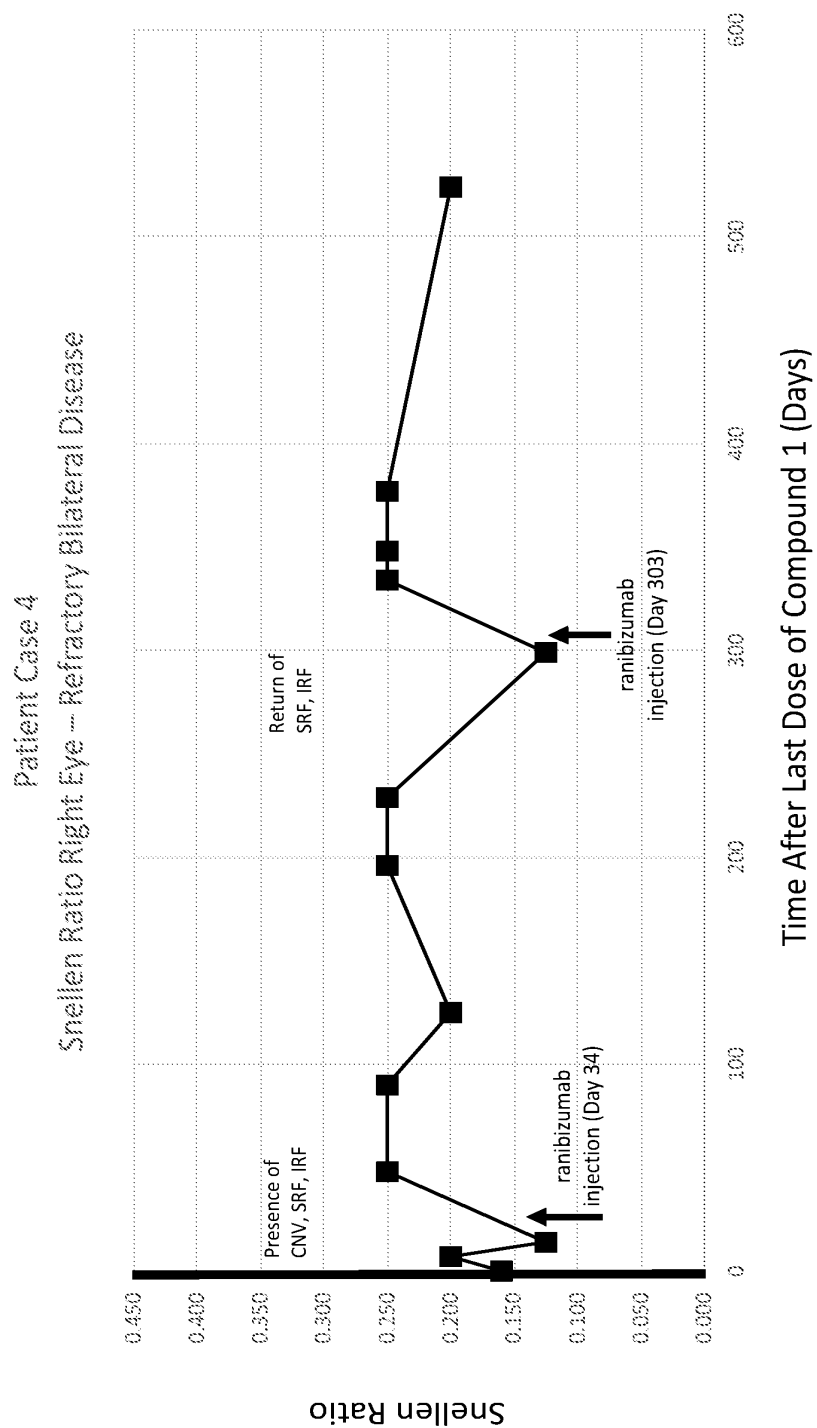
Figure 4

Figure 5

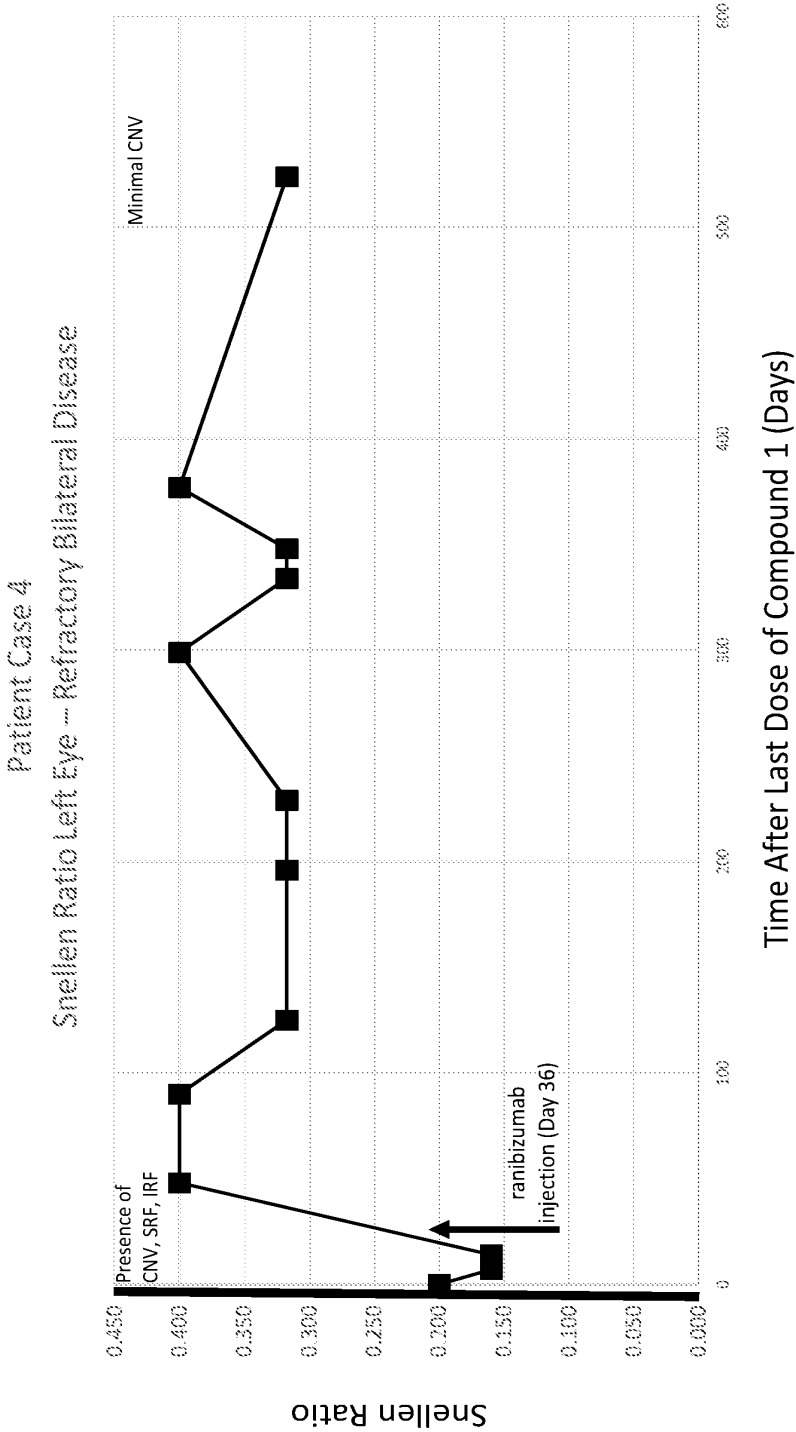


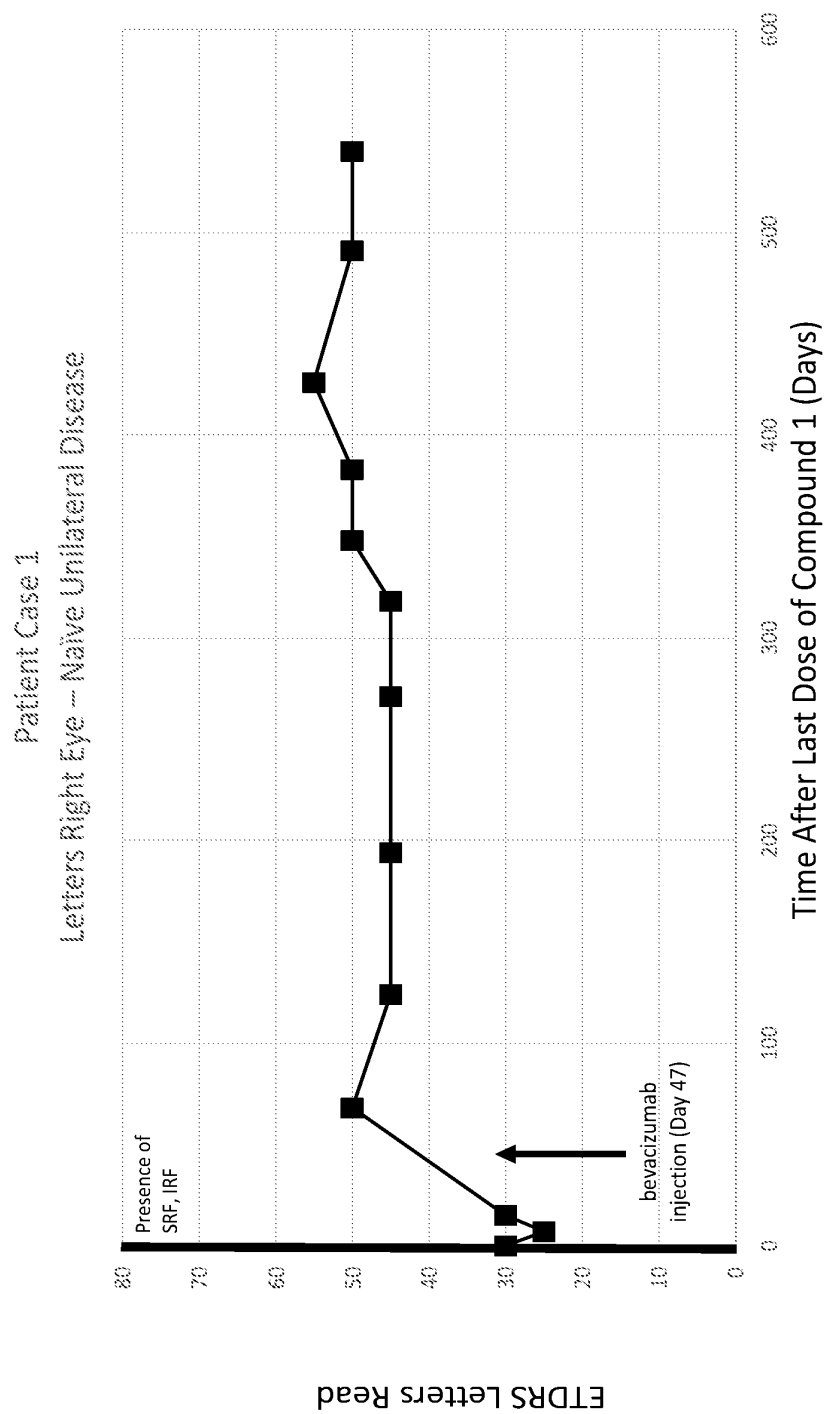
Figure 6

Figure 7

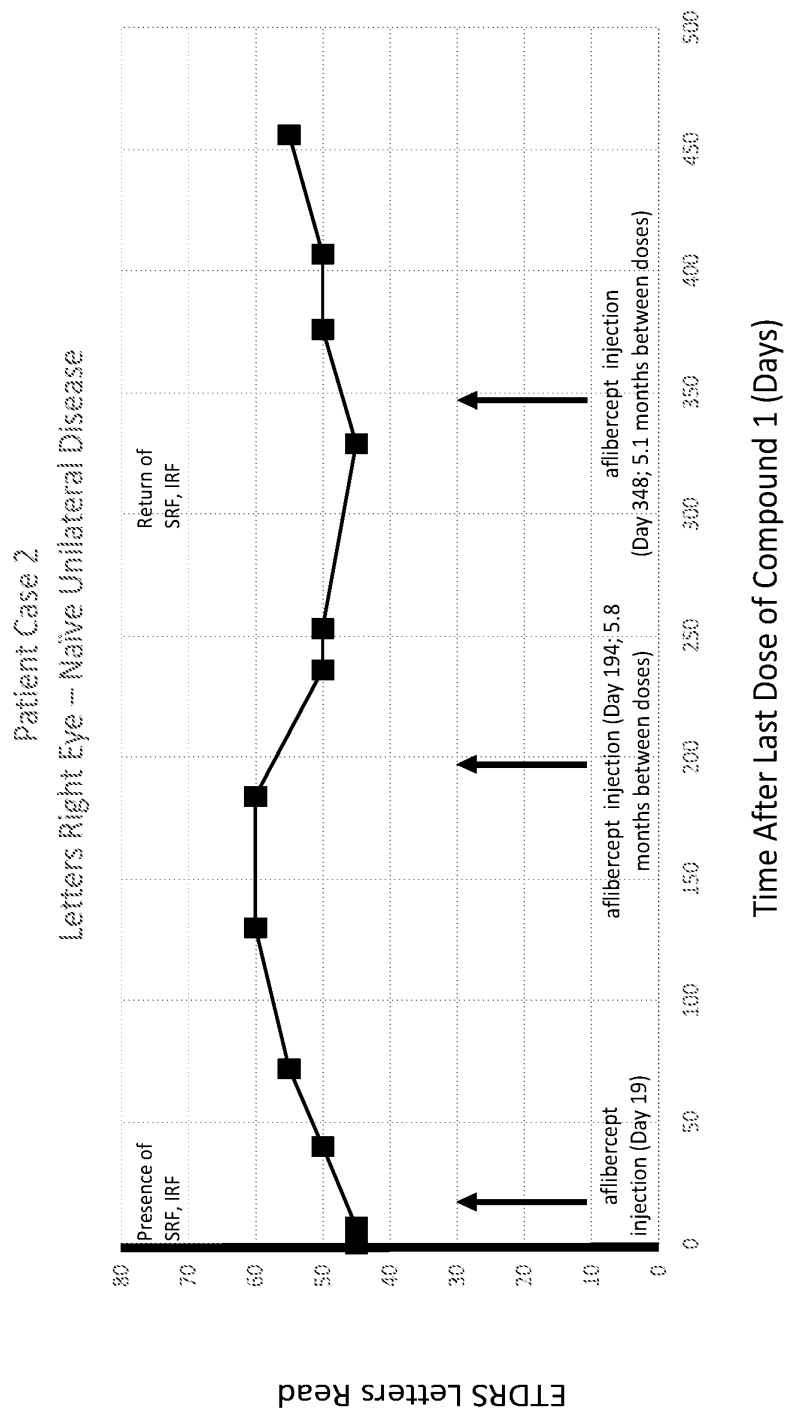


Figure 8

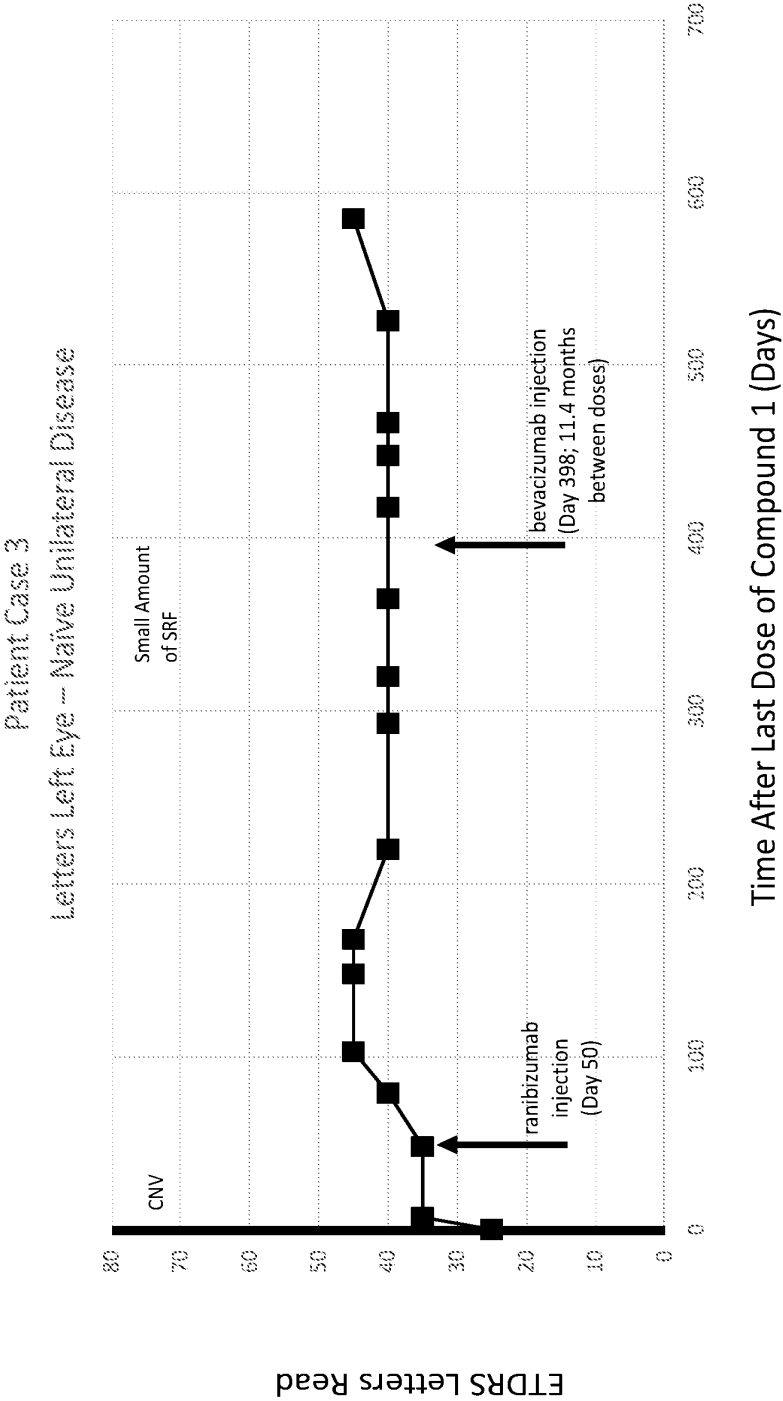


Figure 9

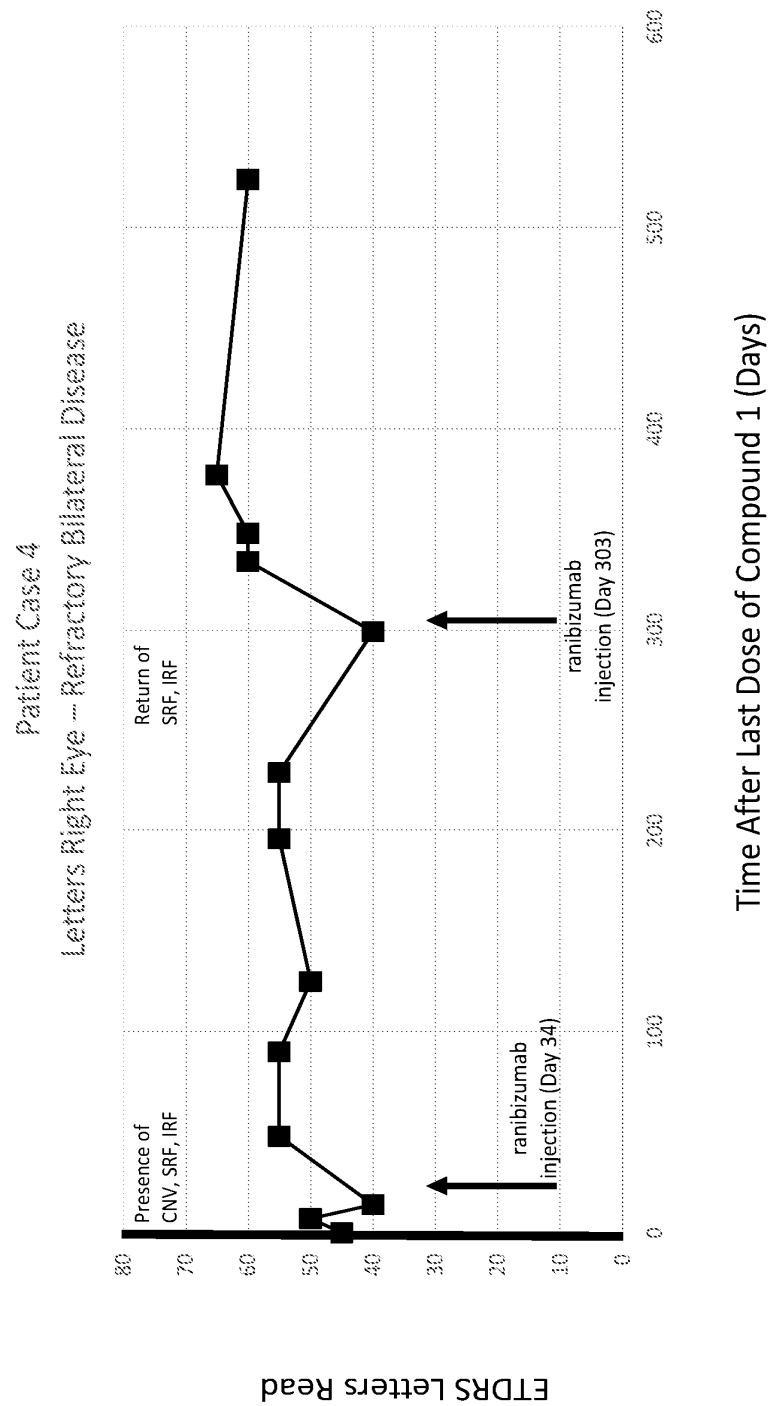


Figure 10

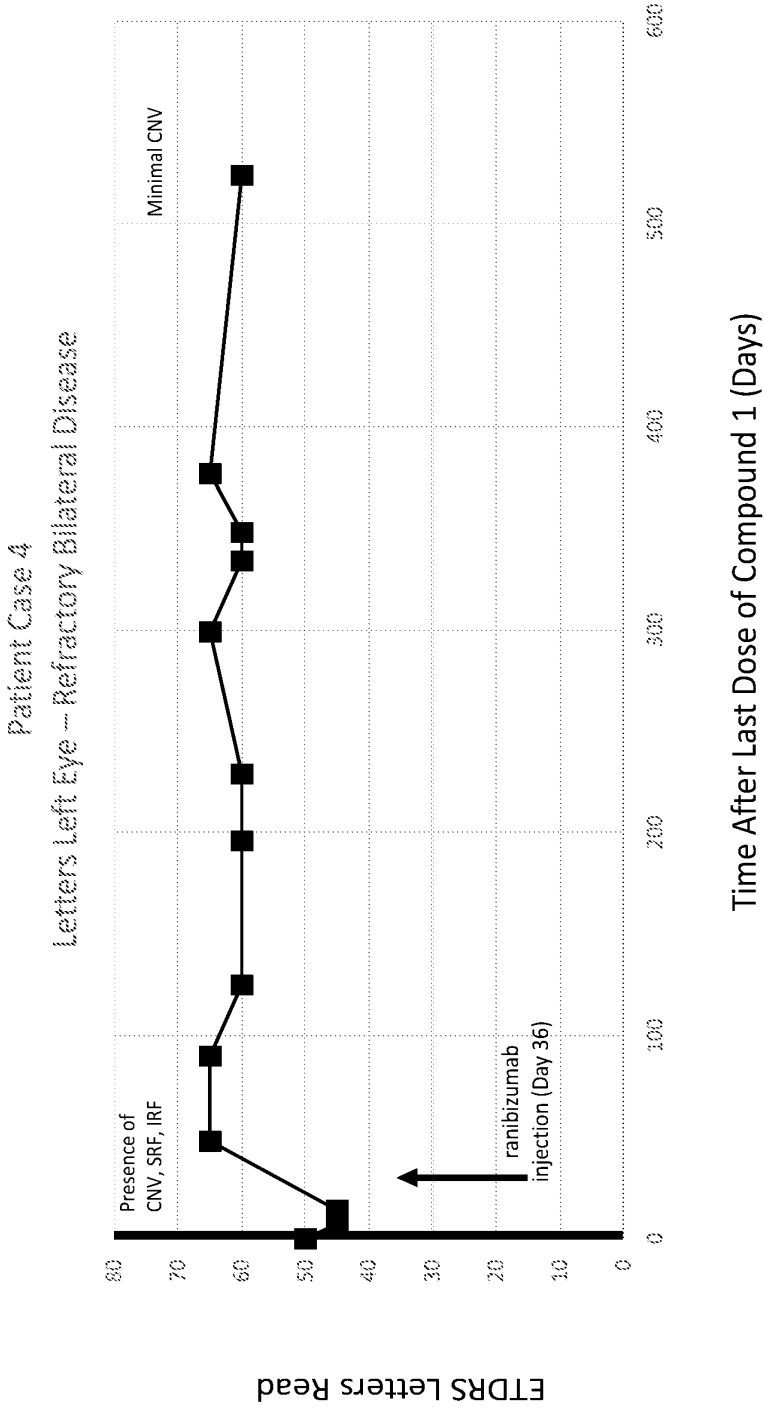


Figure 11

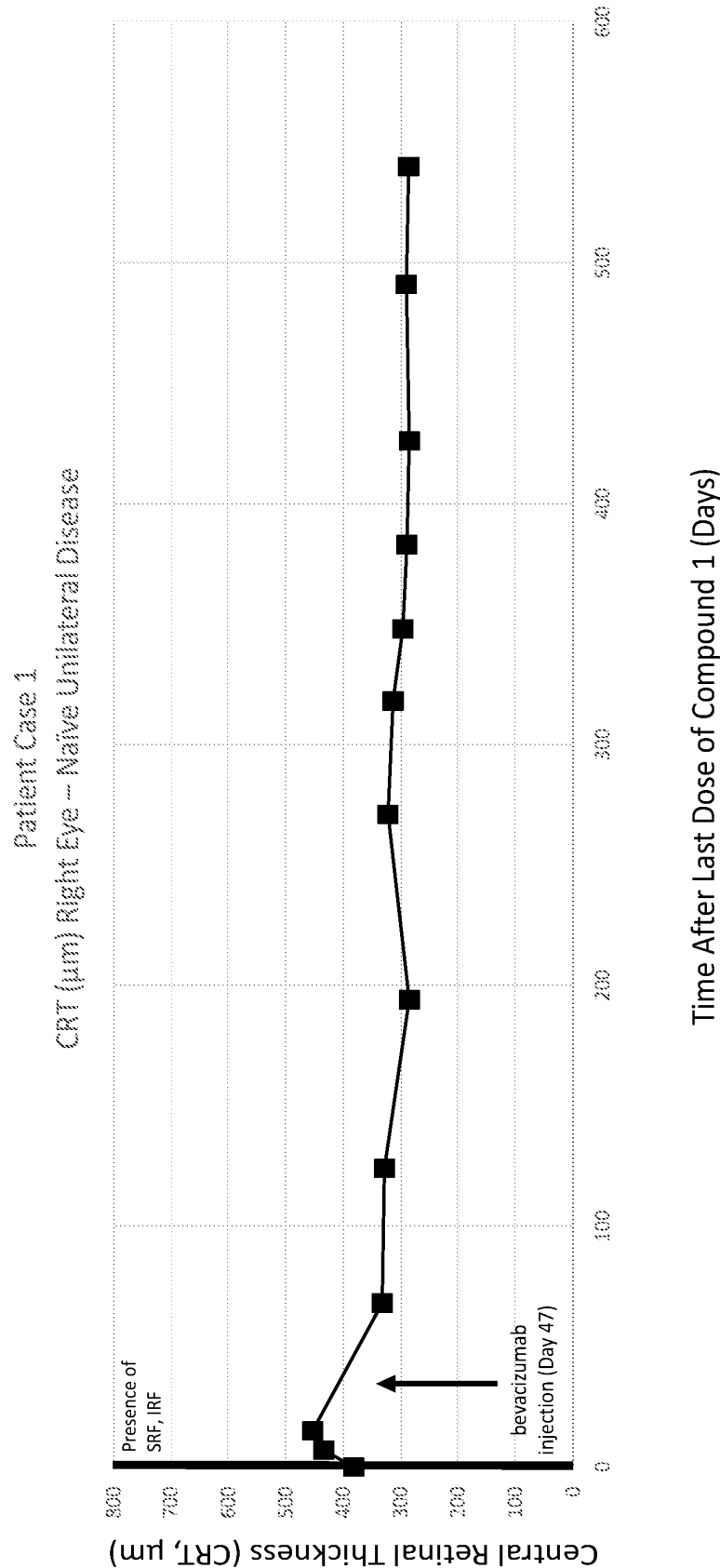


Figure 12

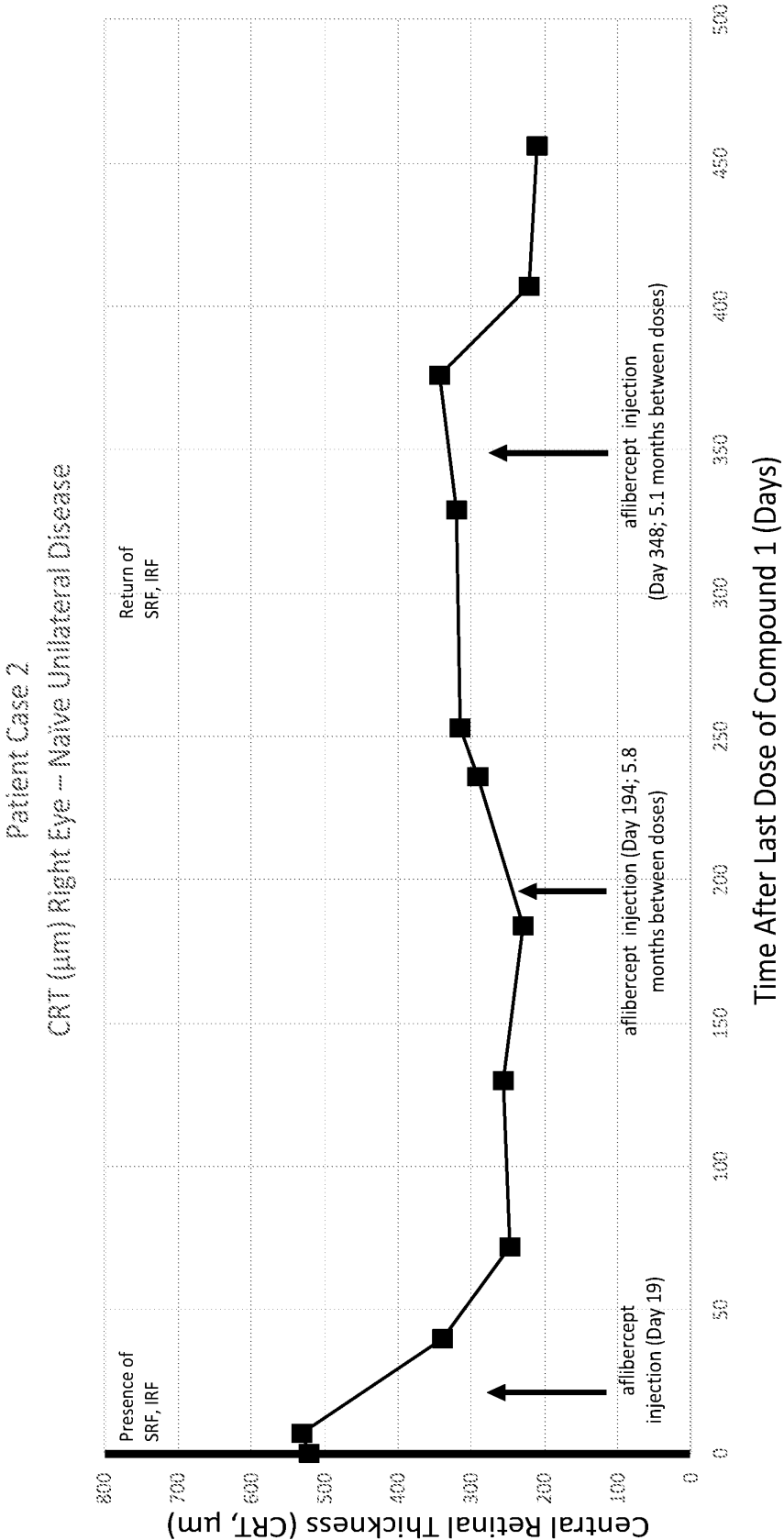


Figure 13

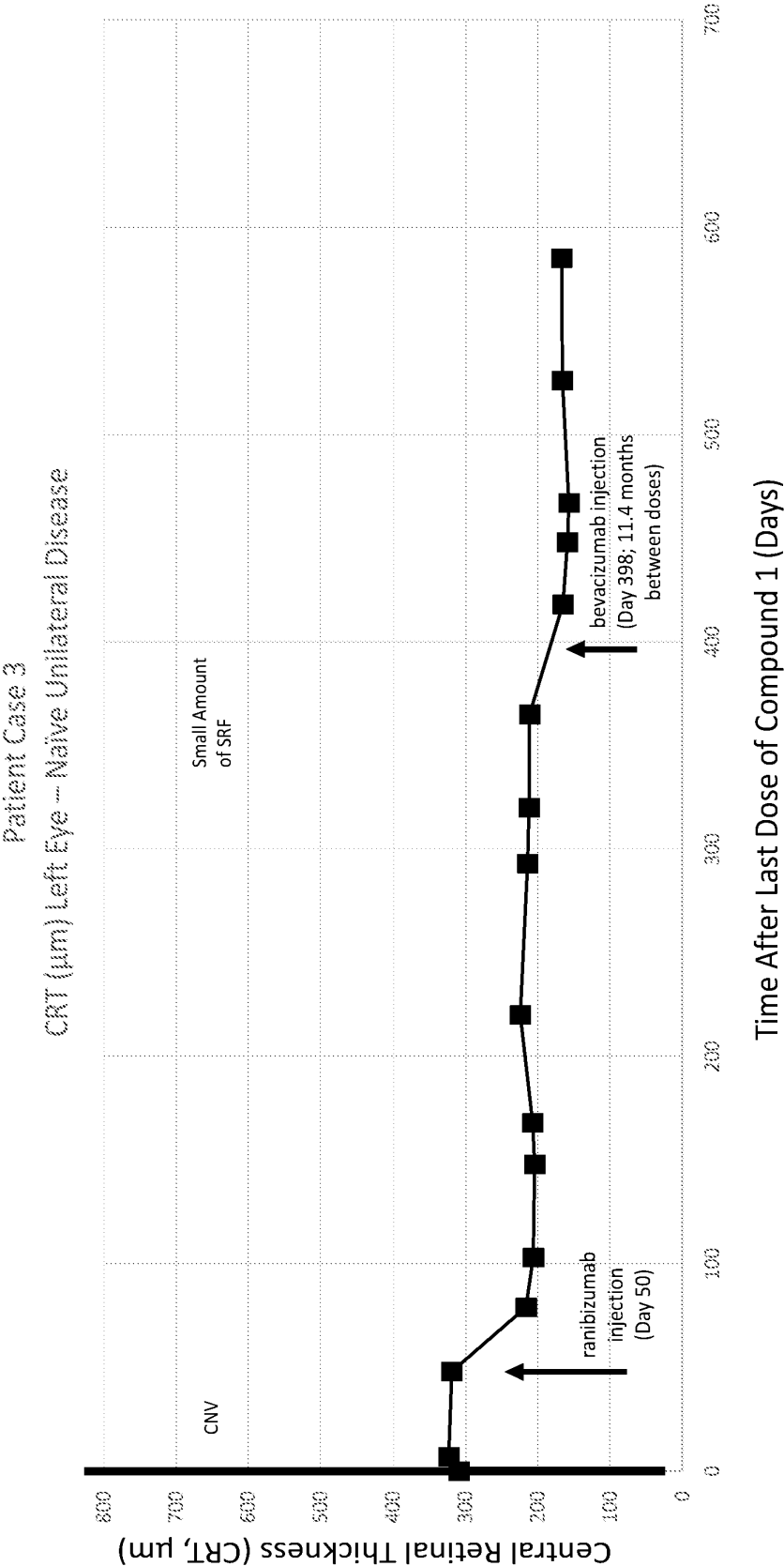


Figure 14

Patient Case 4
CRT (μm) Right Eye – Refractory Bilateral Disease

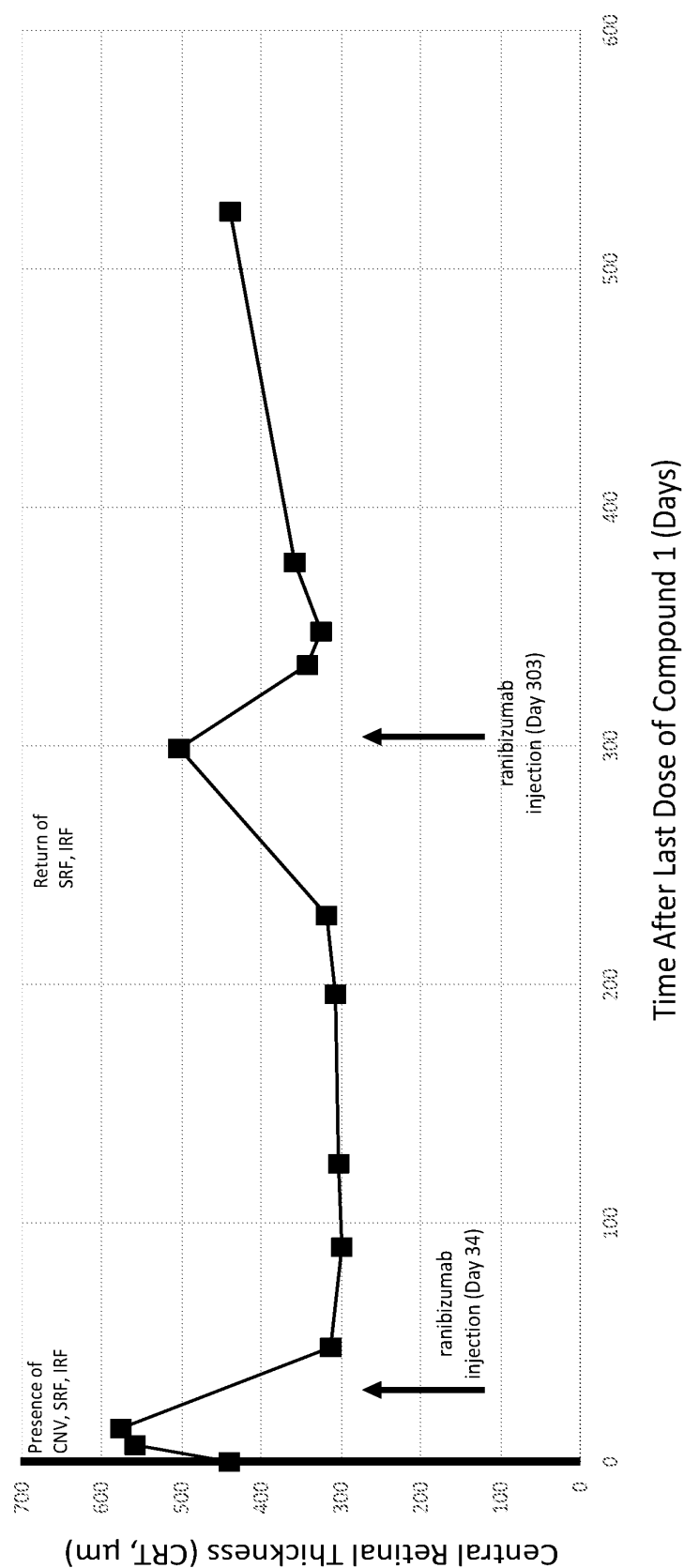


Figure 15

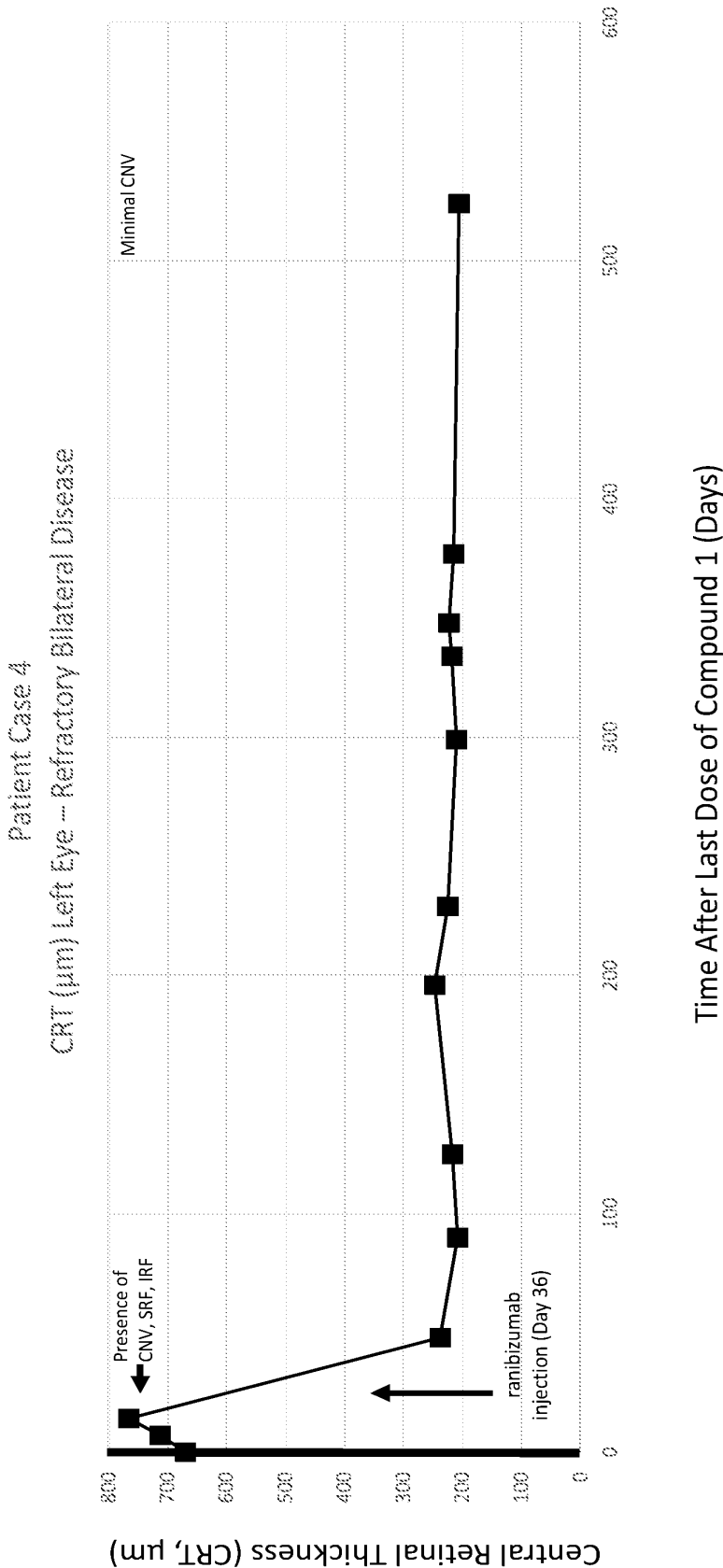


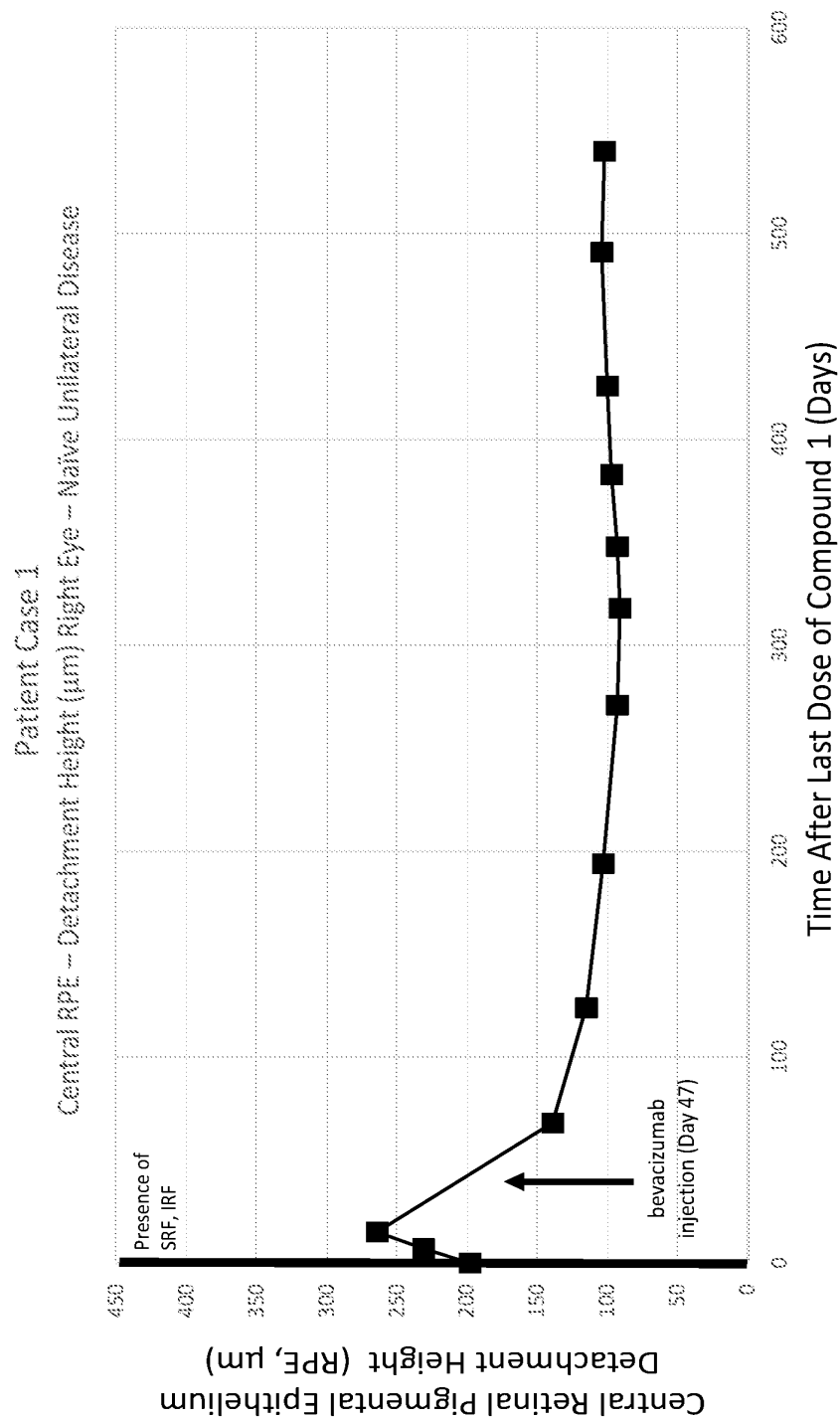
Figure 16

Figure 17

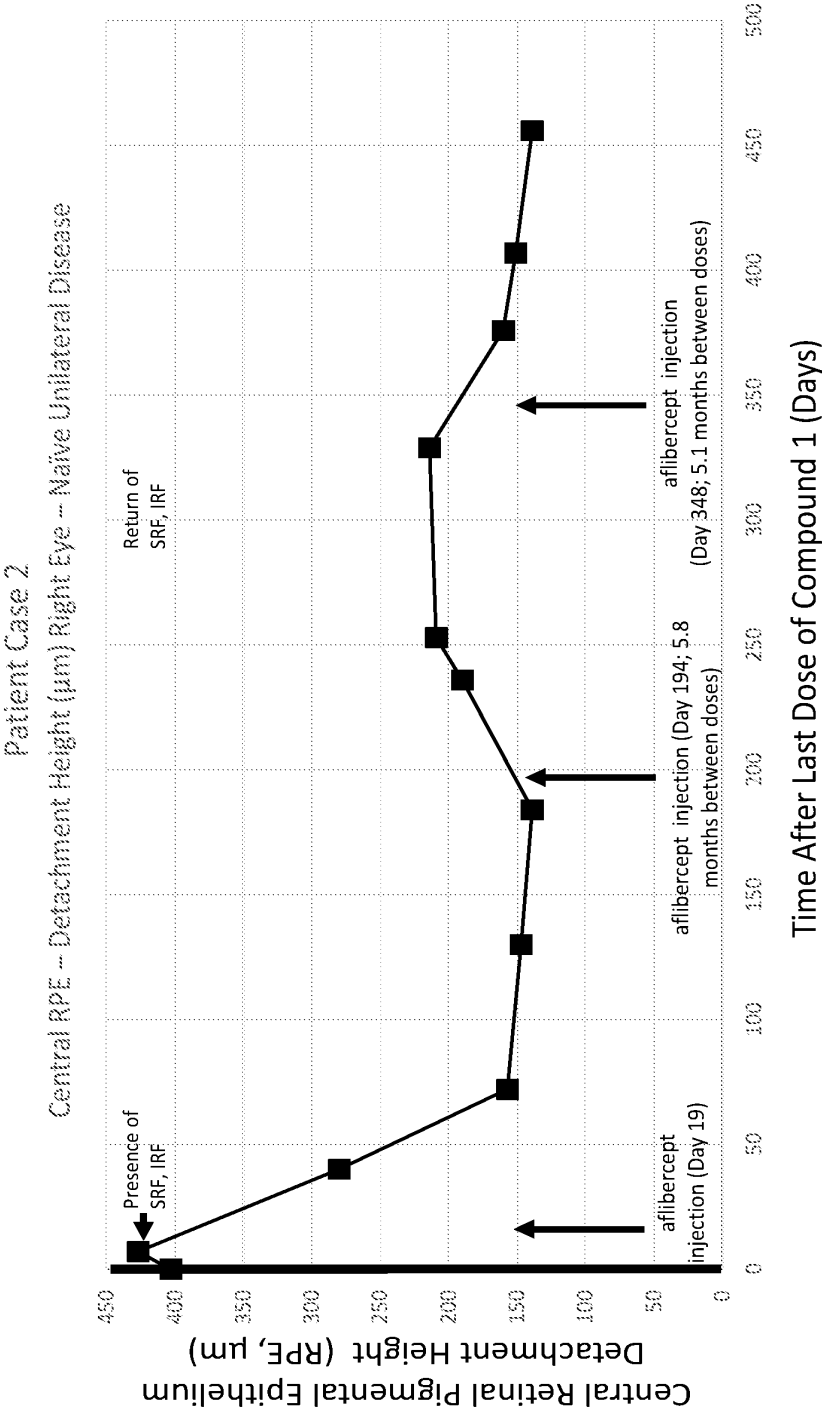


Figure 18

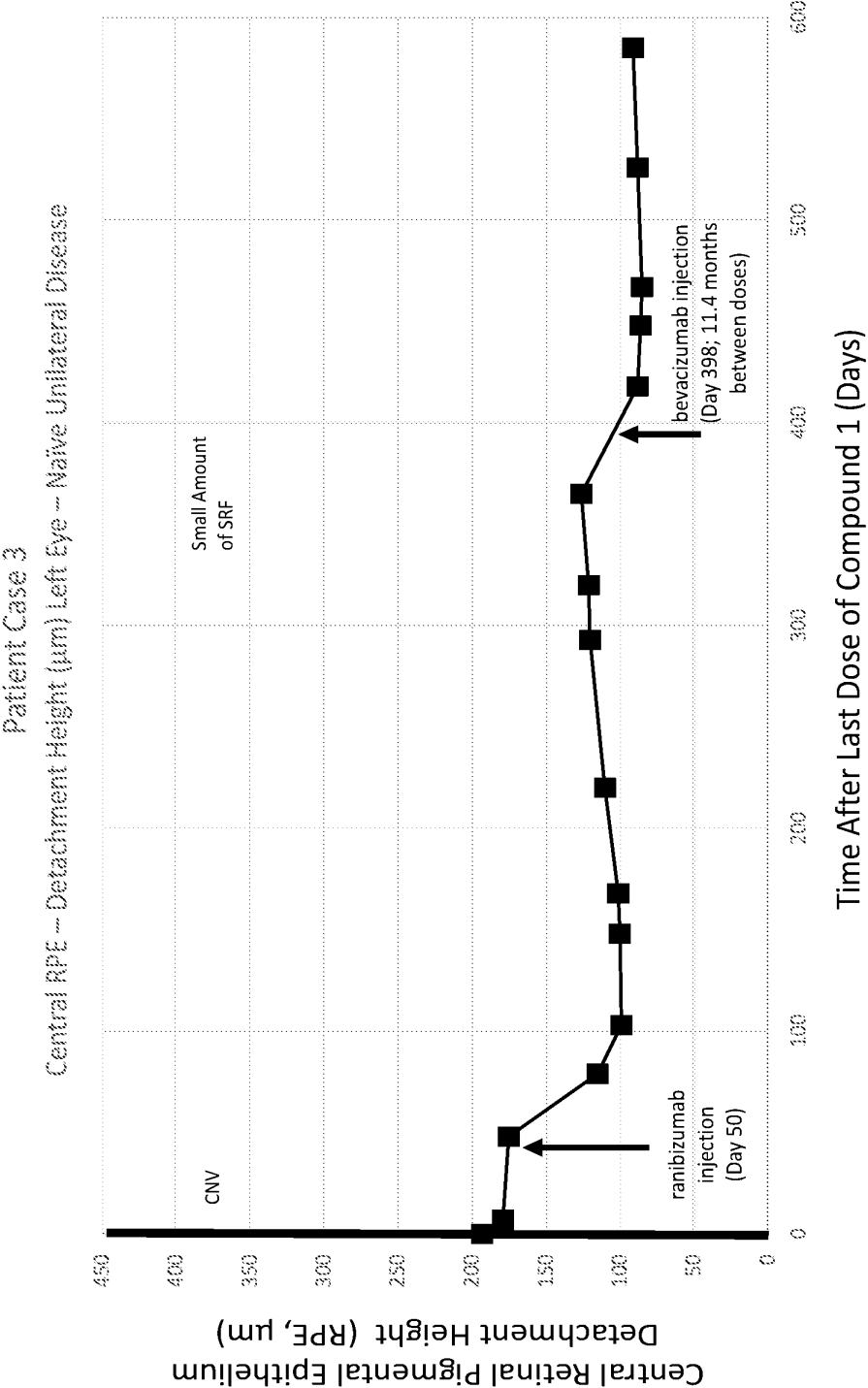


Figure 19

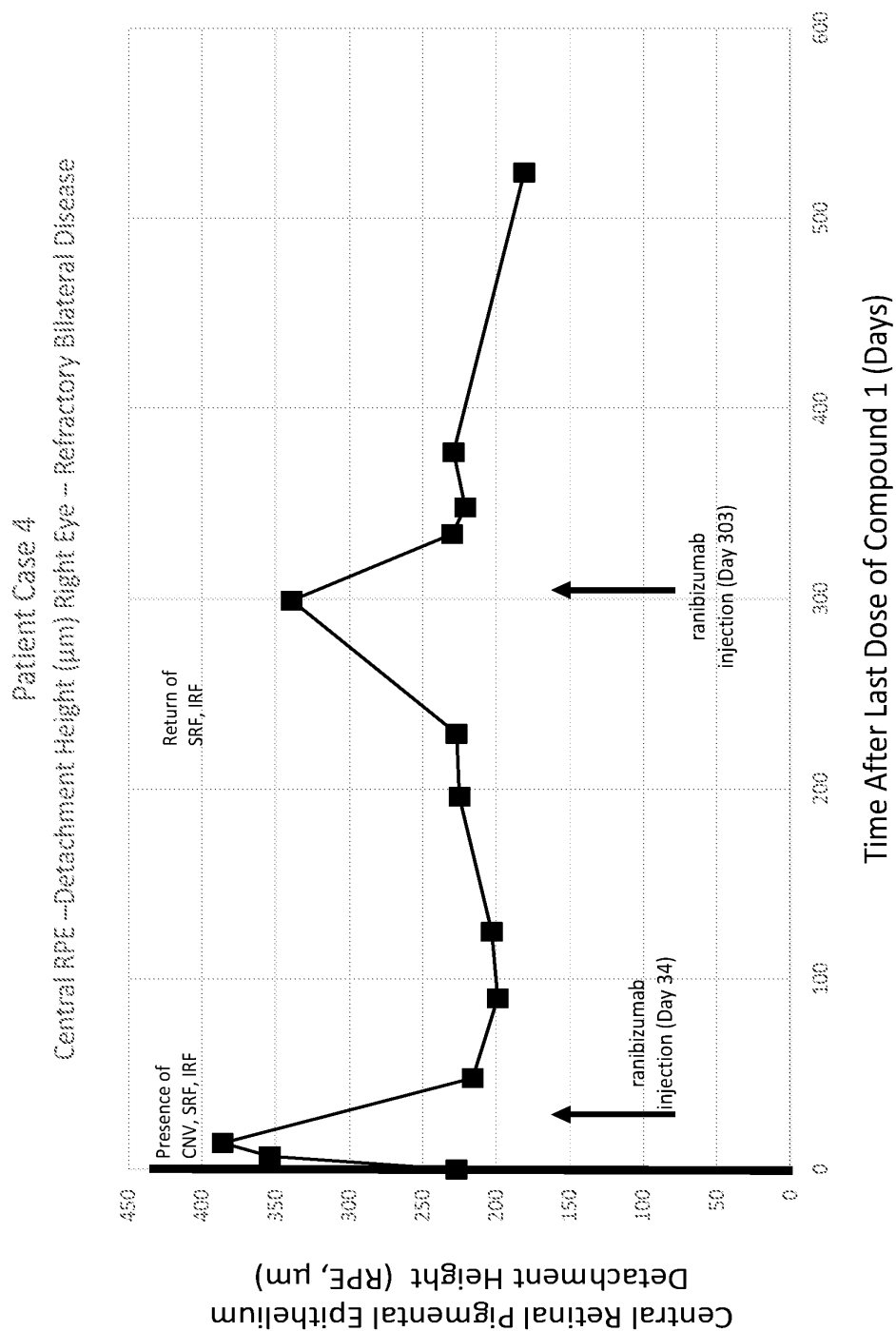


Figure 20

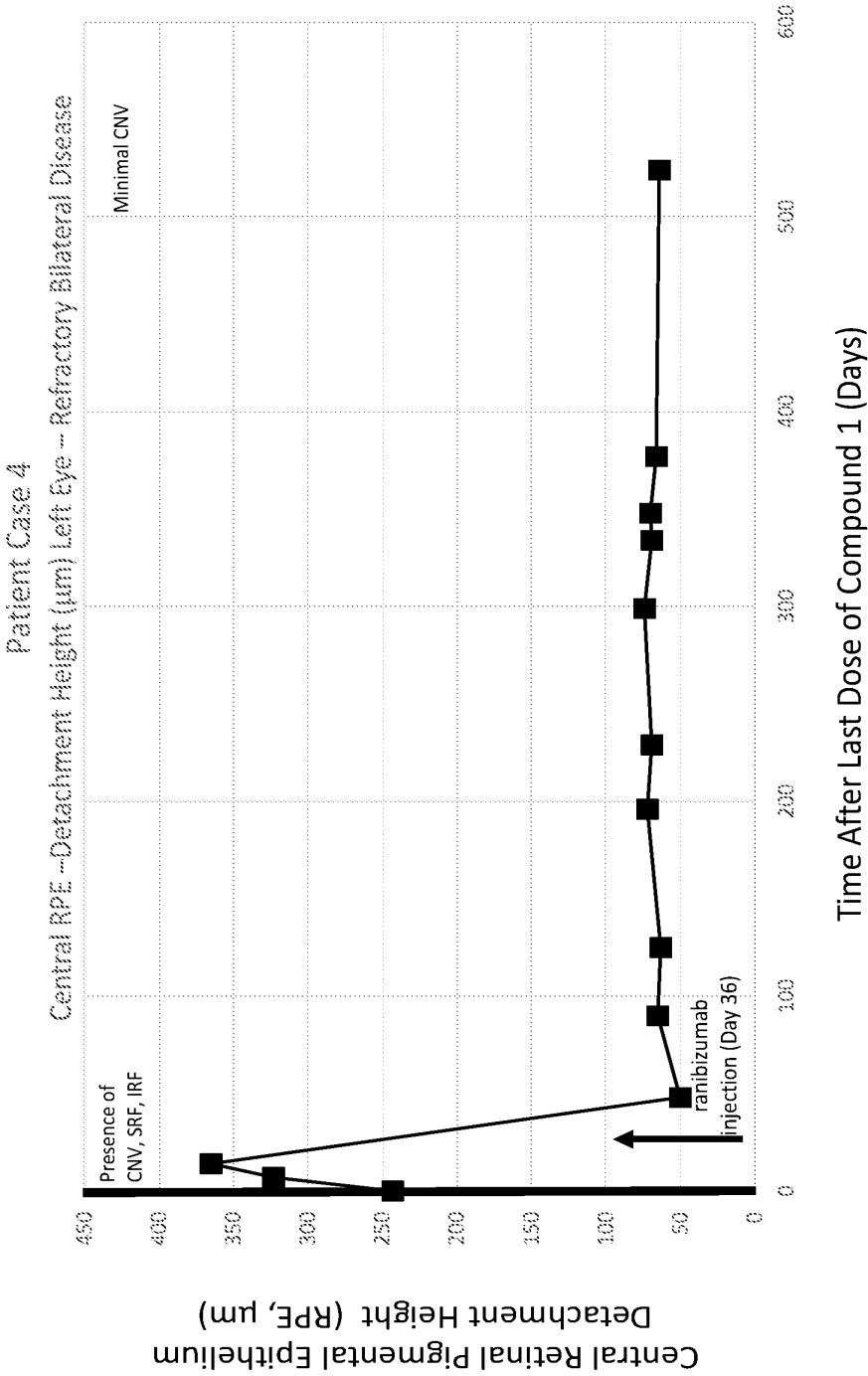
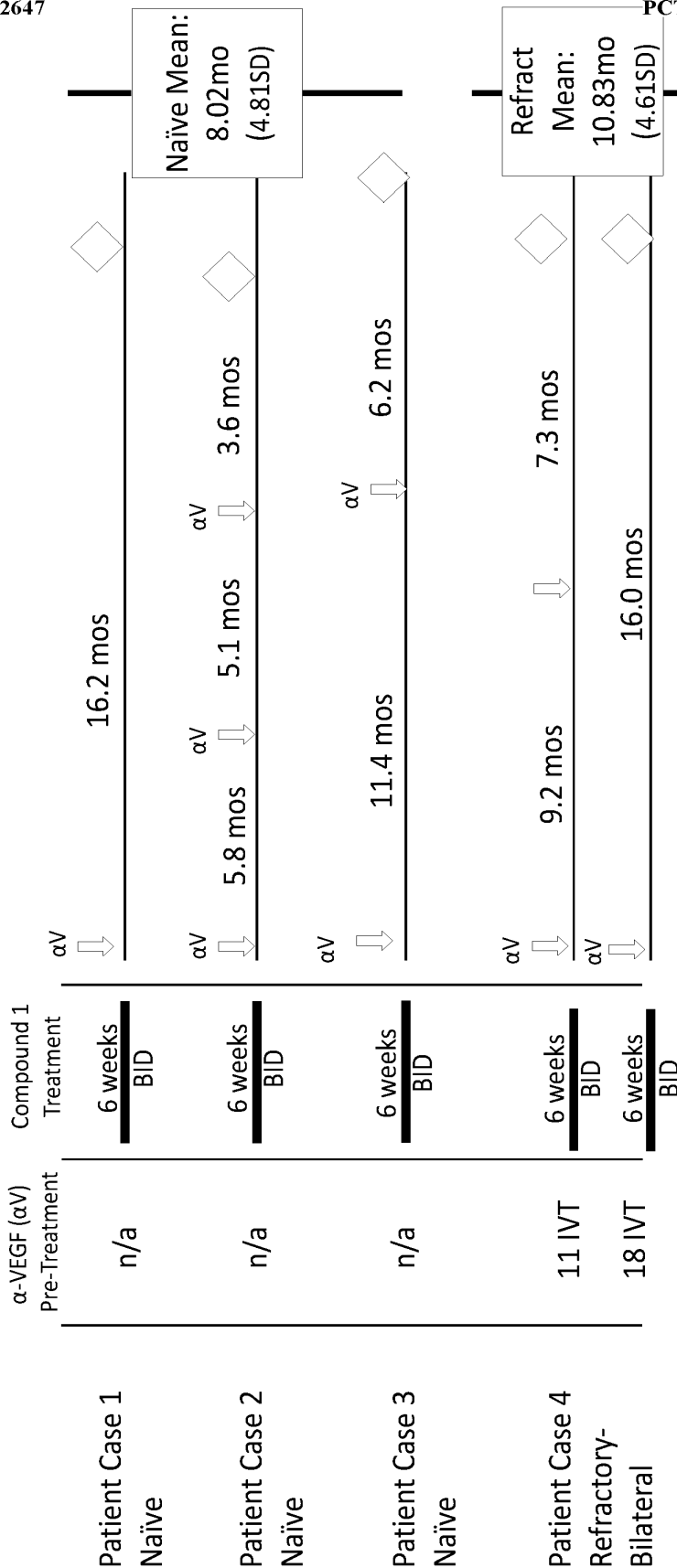


Figure 21

Treatment Sequencing



Patients Re-treated per Physician Judgment guided by Standard Criteria: New CNV Activity on OCT/angio-OCT, or foveal SRF/IRF

◇ End of Follow Up

Figure 22

Patient Case Number	Eye	Days Between Doses	Months Between Doses or Since Last Dose	Notes	Mean Months Without anti-VEGF Injection	Std Dev	Range	Doses Per Month	Doses Per Year
Refractory									
Patient Case 4	Right Eye	279	9.17						
Patient Case 4	Right Eye	221	7.27	No 3rd dose				0.115	1.374
Patient Case 4	Left Eye	488	16.04	No 2nd dose	10.83	4.61	7.27 - 16.04	0.057	0.687
Naïve									
Patient Case 1	Right Eye	493	16.2	No 2nd dose				0.056	0.667
Patient Case 2	Right Eye	175	5.75						
Patient Case 2	Right Eye	154	5.06						
Patient Case 2	Right Eye	108	3.55	No 4th dose				0.197	2.368
Patient Case 3	Left Eye	348	11.41						
Patient Case 3	Left Eye	187	6.15	No 3rd dose	8.02	4.81	3.55 - 16.2	0.103	1.231
			*Bold-Italics indicate time since last dose where a next follow-up dose had not yet been administered at the time this data was compiled.						

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International application No.

PCT/US 21/36641

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 31/4523; A61K 31/454; A61K 31/4545 (2021.01)

CPC - A61K 31/4545; A61K 31/5377; A61K 45/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2018/187473 A1 (ALKAHEST INC.) 11 October 2018 (11.10.2018); para [0008], [0010], [0017], [0021], [0162], [0310], [0312]-[0314]	1-34
A	Nagai et al. "Novel CCR3 Antagonists Are Effective Mono- and Combination Inhibitors of Choroidal Neovascular Growth and Vascular Permeability" American Journal of Pathology. July 2015, vol 185. pg. 2534-2549; entire document	1-34
A	US 2020/0054745 A1 (Lashkari) 20 February 2020 (20.02.2020); entire document	1-34
A	US 2016/0193217 A1 (MITSUBISHI TANABE PHARMA CORPORATION) 07 July 2016 (07.07.2016); entire document	1-34

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

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"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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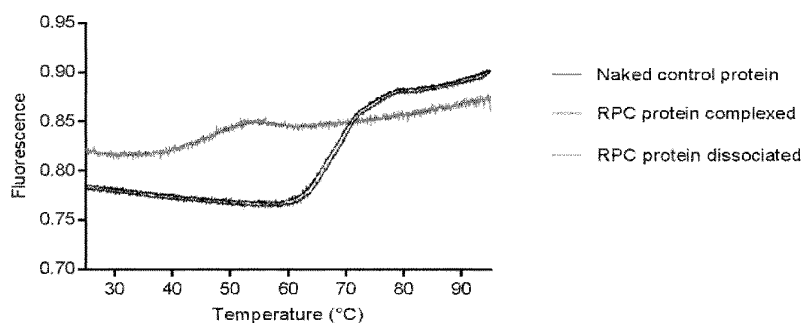
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(54) Title: CONCENTRATED COMPOSITIONS OF PROTEINS, THEIR PREPARATION AND USE THEREOF

FIG.2



(57) Abstract: The invention relates to a method for producing a composition comprising reversible protein complexes (RPCs), the method comprising the steps of contacting a protein and a complexing agent in a buffer solution, wherein the complexing agent is dextran sulphate or chondroitin sulphate, and wherein the protein and the complexing agent have opposite net charges when comprised in the buffer solution; formation of RPCs between the protein and the complexing agent in the buffer solution; and obtaining a suspension comprising the RPCs. Provided herein are also compositions, including pharmaceutical compositions/ formulations comprising the reversible protein complexes (RPCs) of the invention, in particular as obtained by the method provided herein.

[Continued on next page]

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CONCENTRATED COMPOSITIONS OF PROTEINS, THEIR PREPARATION AND USE THEREOF

BACKGROUND OF THE INVENTION

In the last decades, biologics have been increasingly taking over small molecules in terms of FDA drug approvals. Indeed, while new biological entities (NBE) represented 10% of the total approved new molecular entities (NME) from 1993 to 1999, they raised up to 17% from 2000 to 2009 and to 24% from 2010 to 2019 (Mullard, Nature Reviews Drug Discovery, 2020, ISSN 1474-1784). This trend is attributed to the higher tolerability of biologics owing to their biological origin over the chemically synthesized small molecules but also for their higher selectivity, particularly the last antibody generations, procuring them a very specific targeting of the disease area, hence increasing safety and efficacy (Buvailo, <https://www.biopharmatrend.com/post/67-will-small-molecules-sustain-pharmaceutical-race-with-biologics/>; July 11, 2018).

Biologics are predominantly commercialized as solutions for parenteral administration. They are formulated in an aqueous medium containing certain excipients necessary to ensure protein stability, prevent oxidation and ensure isotonicity. Biologics drug products are most frequently stored at 5 ± 3 °C that results in a complicated and costly supply chain to maintain the cold chain, decreasing the drug accessibility; especially in the developing countries. In certain cases, protein solutions can be lyophilized in order to increase the stability and shelf-life of the drug product and enables easier transportation and storage compared to a cold chain. In this case, additional excipients such as cryoprotectants are used to protect the protein during the freeze-drying cycle and others to facilitate the reconstitution of the lyophilized formulation prior administration to the patient.

These standard dosage forms of biologics have been widely studied during these last decades and the scientific community witnessed the emergence of many limitations related to the current existing formulations. The first limitation is the particle formation in the biologic solutions, which is a major problem that the pharmaceutical industry is facing nowadays since these particles are considered immunogenic by the health authorities and hence may lead to product withdrawal which represent a considerable cost for the pharmaceutical industry. Recently,

many efforts have been made to understand the mechanism underlying particle formation and results showed a surfactant-related origin; mainly polysorbates degradation and poloxamers interaction with silicon oils present in the primary packaging. Surfactants are among the excipients widely used in biologic formulations and are essential to prevent protein aggregation due to stresses experienced while processing, transporting and storage of the drug product (Gervasi et al., *Eur J Pharm Biopharm*, 2018, 131, 8-24). Hence, the pharmaceutical industry is aiming to find alternative excipients or ultimately, develop novel surfactant-free formulations of biologics.

Another limitation encountered by the current biologics formulations is the limitation in terms of concentration due to the high viscosity associated with the highly concentrated protein formulations. In parallel, lately, there is a clear trend in biologics formulation development when it comes to the route of administration by moving from intravenous infusion to subcutaneous administration to improve patient compliance as they can auto-inject the drug. To support this, formulations need to be highly concentrated (> 100 mg/mL) in order to allow the delivery of the therapeutic dose via a single injection. However, such highly concentrated formulations (>100 mg/mL) are associated with excessive viscosity complicating their administration via injection. Development of high concentration formulations of biologics may thus be very beneficial to improve the patient compliance.

In the past, reversible protein-polyion complexation concepts have been used to develop highly concentrated formulation of biologics and to tackle the aforementioned formulation challenges. Reversible protein complexes (RPCs), also referred to as hydrophobic ion pairing (HIP), protein-polyelectrolyte complexes (PPCs) or polyion complexes (PICs), have already been reported widely in the literature and was first introduced by Morawetz and Hughes in 1952 as a purification method, its use has since then evolved to other applications, including drug delivery systems for biologics (Mimura et al., *J Chem Phys*, 2019, 150(6), 064903; Morawetz and Hughes, *The Journal of Physical Chemistry*, 1952, 56(1), 64-49).

This concept relies on mixing oppositely charged molecules under specific physico-chemical conditions (e.g. pH, ionic strength, mole-charge ratio) leading to the formation of a complex via electrostatic interactions. Since the charges are neutralized and masked, the complex is not soluble anymore and precipitates as whitish particles. Importantly, this complexation is reversible by decreasing the electrostatic interactions, which are sensitive to the surrounding pH and ionic strength. Indeed, increasing the ionic strength causes an electrostatic shielding (or screening) between the oppositely charged molecules whereas changing the pH results in reducing the charge distribution on the molecules leading to decrease in the electrostatic

interactions and consequent dissociation of the two molecules (Chamieh et al., In J Pharm, 2019, 559, 228-234; Matsuda et al., J Pharm Sci, 2018, 107(10), 2713-2719).

This concept has already been assessed with biological molecules; including enzymes, hormones, peptides and proteins, and an oppositely charged polymer for protein purification, dissolving enzymes in organic solvents without losing activity and also as a drug delivery strategy to enhance bioavailability Mimura et al., J Chem Phys, 2019, 150(6), 064903; Chamieh et al., In J Pharm, 2019, 559, 228-234; Matsuda et al., J Pharm Sci, 2018, 107(10), 2713-2719; Ristroph and Prud'homme, Nanoscale Advances, 2019, 1(11), 4207-4237). Indeed, most of the biological molecules are hydrophilic. The RPC concept was used to render them hydrophobic and increase their incorporation efficiency in already established drug delivery systems including micelles, liposomes and Self-emulsifying drug delivery systems (SEDDS) (Chamieh et al., In J Pharm, 2019, 559, 228-234). RPC has been shown to improve protein stability towards physico-chemical stress including heat, agitation and oxidation (Matsuda et al., J Pharm Sci, 2018, 107(10), 2713-2719).

It has been reported that to maximize the complexation efficiency, pH of the buffer should be adjusted to two pH units below or above the isoelectric point (pI) of the protein to maximize the charge distribution on the protein. Though, proteins are generally unstable at basic pH, thus mild acidic pH favoring positively charged protein is more suitable, and the oppositely charged polymer should be negatively charged to form a complex (Mimura et al., J Chem Phys, 2019, 150(6), 064903; Matsuda et al., J Pharm Sci, 2018, 107(10), 2713-2719).

It has also been mentioned that in addition to the electrostatic interactions between both polyions, hydrophobic interactions are involved in stabilizing the complex. Hence, another important parameter to take into consideration when selecting the complexing agent (CA) is the hydrophobicity. Indeed, the hydrophobic domains of the counterion such as an alkyl tail or aromatic group would coat the proteins' surface area with hydrophobic domains that exclude water (Mimura et al., J Chem Phys, 2019, 150(6), 064903; Ristroph and Prud'homme, Nanoscale Advances, 2019, 1(11), 4207-4237).

While complexation of proteins can be efficiently achieved with a variety of complexing agents and under various conditions, subsequent dissociation of protein complexes still remains challenging. In particular, (partial) degradation of the protein or the formation of irreversible complexes negatively affects the dissociation efficiency. Especially when administered subcutaneously to a patient, compositions comprising protein complexes that do not efficiently dissociate at physiological conditions may pose a serious threat in terms of tolerability and risks of immunogenicity.

Accordingly, there is a need in the art for highly concentrated compositions comprising protein complexes that efficiently dissociate under physiological conditions into native, functional therapeutic proteins.

Thus, one objective of the present invention is to provide methods for producing compositions comprising reversible protein complexes that efficiently dissociate under physiological conditions.

A further objective of the invention is to provide compositions comprising reversible protein complexes that efficiently dissociate under physiological conditions.

A further objective of the invention is to provide pharmaceutical formulations comprising reversible protein complex for use as a medicament, in particular wherein the reversible protein complexes comprise a therapeutic protein and/or wherein the pharmaceutical formulation is administered subcutaneously.

SUMMARY OF THE INVENTION

The technical problem is solved by the embodiments provided herein and as characterized in the claims. That is, the present invention relates, inter alia, to the following items:

1. A method for producing a composition comprising reversible protein complexes (RPCs), the method comprising the steps of:
 - a) contacting a protein and a complexing agent in a buffer solution, wherein the complexing agent is dextran sulphate or chondroitin sulphate, and wherein the protein and the complexing agent have opposite net charges when comprised in the buffer solution;
 - b) formation of RPCs between the protein and the complexing agent in the buffer solution; and
 - c) obtaining a suspension comprising the RPCs formed in step (b).
2. The method according to item 1, wherein the complexing agent is dextran sulphate, in particular dextran sulphate with 40 kDa molecular weight.
3. The method according to item 1 or 2, wherein the pH of the buffer solution is adjusted to be lower than the isoelectric point of the protein.

4. The method according to any one of items 1 to 3, wherein the pH of the buffer solution is adjusted to 2 to 5 pH units below the isoelectric point of the protein, in particular 3 pH units below the isoelectric point of the protein.
5. The method according to item 1 or 2, wherein the buffer solution has a pH ranging from 1 to 6, in particular wherein the buffer solution has a pH ranging from 3 to 6, in particular wherein the buffer solution has a pH ranging from 4.5 to 5.5.
6. The method according to any one of items 1 to 5, wherein the buffer solution has an ionic strength ranging from 20 to 50 mM, in particular wherein the buffer solution has an ionic strength ranging from 20 to 30 mM.
7. The method according to any one of items 1 to 6, wherein the buffer solution comprises histidine or citrate as buffering agent.
8. The method according to any one of items 1 to 7, wherein the buffer solution comprising the protein and the complexing agent is obtained by mixing a first solution comprising the protein and a second solution comprising the complexing agent.
9. The method according to item 8, wherein the first solution comprising the protein and/or the second solution comprising the complexing agent comprises a buffering agent.
10. The method according to any one of items 1 to 9, wherein the protein and the complexing agent are contacted at a mole-charge ratio ranging from 1:0.2 to 1:2, in particular wherein the protein and the complexing agent are contacted at a mole-charge ratio ranging from 1:0.2 to 1:1.
11. The method according to any one of items 1 to 10, wherein the protein is contacted with the complexing agent in the buffer solution at a protein concentration ranging from 1 - 40 mg/mL, in particular from 1 - 5 mg/mL.
12. The method according to any one of items 1 to 11, wherein the protein is an antibody, a growth factor, a hormone, a cytokine, an enzyme, or a fragment and/or fusion protein of any of the foregoing.
13. The method according to item 12, wherein the antibody is an antibody, in particular wherein the antibody is a monoclonal antibody, a polyclonal antibody, a chimeric antibody, a multispecific antibody, an antibody fusion protein, an antibody-drug-

conjugate or an antibody fragment.

14. The method according to any one of items 1 to 13, wherein the complexing agent has a negative net charge when comprised in the buffer solution.
15. The method according to any one of items 1 to 14, wherein the complexing agent comprises a hydrophobic moiety.
16. The method according to any one of items 1 to 15, wherein the composition comprising the RPCs comprises at least one excipient.
17. The method according to item 16, wherein the at least one excipient is added to the composition before and/or after the formation of the RPCs.
18. The method according to item 17 or 18, wherein the at least one excipient is a stabilizer and/or a surfactant.
19. The method according to any one of items 1 to 18, wherein the method comprises a further step of exchanging the liquid fraction of the suspension comprising the RPCs.
20. The method according to item 19, wherein the liquid fraction of the suspension comprising the RPCs is exchanged by centrifugation of the suspension comprising the RPCs and resuspension of the sedimented RPCs in a buffer solution or water.
21. The method according to item 19, wherein the liquid fraction of the suspension comprising the RPCs is exchanged by dialysis of the suspension comprising the RPCs against a buffer solution or water.
22. The method according to any one of items 1 to 21, wherein the method comprises a further step of enriching the RPCs in the suspension to obtain an enriched RPC suspension.
23. The method according to item 22, wherein enriching the RPCs in the suspension comprises the steps of:
 - a) centrifuging the suspension comprising the RPCs to obtain a supernatant and a precipitate comprising an enriched RPC suspension; and
 - b) removing the supernatant from the precipitate to obtain an enriched RPC suspension.
24. The method according to item 22 or 23, wherein the liquid fraction of the enriched RPC

suspension is at least in part replaced with a non-aqueous solvent during the enrichment step.

25. The method according to item 24, wherein the non-aqueous solvent is triacetin, diethylene glycol monoethyl ether or ethyl oleate.
26. The method according to any one of items 1 to 25, wherein the method comprises a further step of lyophilizing the suspension comprising the RPCs or the enriched RPC suspension to obtain a lyophilisate.
27. The method according to item 26, wherein at least one cryoprotectant is added to the suspension comprising the RPCs or the enriched RPC suspension before the lyophilisation step.
28. The method according to item 27, wherein the at least one cryoprotectant is selected from a group consisting of: sugars, amino acids, methylamines, lyotropic salts, polyols, propylene glycol, polyethylene glycol and pluronics.
29. The method according to any one of items 26 to 28, wherein the protein concentration of the suspension comprising the RPCs or the enriched RPC suspension is adjusted to 10 to 100 mg/mL, in particular to 40 to 80 mg/mL, prior to the lyophilisation step.
30. The method according to any one of items 1 to 25, wherein the method comprises a further step of spray drying the suspension comprising the RPCs or the enriched RPC suspension to obtain a spray dried powder.
31. The method according to item 30, wherein the protein concentration of the suspension comprising the RPCs or the enriched RPC suspension is adjusted to 1 to 10 mg/mL, in particular to 1 to 5 mg/mL, prior to the spray drying step.
32. The method according to item 30 or 31, wherein the liquid fraction of the suspension comprising the RPCs or the enriched RPC suspension is exchanged prior to the spray drying step.
33. The method according to item 32, wherein exchanging the liquid fraction of the suspension comprising the RPCs or the enriched RPC suspension reduces the concentration of at least one buffering agent, complexing agent and/or excipient in the suspension.

34. The method according to item 33, wherein the suspension comprising the RPCs or the enriched RPC suspension is substantially free of buffering agent after exchanging the liquid fraction of the suspension.
35. The method according to item 33 or 34, wherein the liquid fraction of the suspension is exchanged before the spray-drying step to obtain a mole-charge ratio between the protein and the complexing agent between 1:0.2 to 1:1, in particular between 1:0.4 to 1:0.8.
36. The method according to any one of items 30 to 35, wherein spray drying is performed at an inlet temperature 115°C and/or an outlet temperature of 48°C.
37. The method according to any one of items 30 to 36, wherein spray drying is performed at a feed rate of 17 mL/min.
38. The method according to any one of items 30 to 37, wherein the method comprises a further step of resuspending the spray dried powder in a non-aqueous solvent (NAS) to obtain an RPC-NAS suspension.
39. The method according to item 38, wherein the non-aqueous solvent is at least one selected from a group consisting of: diethylene glycol monoethyl ether, ethyl oleate, triacetin, isosorbide dimethyl ether and glycofurol.
40. The method according to item 39, wherein the spray dried powder is resuspended to obtain a RPC-NAS suspension with a protein concentration ranging from 50 to 300 mg/mL, in particular ranging from 100 – 250 mg/mL.
41. A composition comprising reversible protein complexes (RPCs), wherein the composition is obtained by the method according to any one of items 1 to 40.
42. A composition comprising reversible protein complexes (RPCs), wherein the RPCs comprise a protein and a complexing agent, and wherein the complexing agent is dextran sulphate or chondroitin sulphate.
43. The composition according to item 42, wherein the complexing agent is dextran sulphate, in particular dextran sulphate with 40 kDa molecular weight.
44. The composition according to item 42 or 43, wherein the protein has a positive net charge when comprised in the RPCs.

45. The composition according to item 44, wherein the protein is an antibody, a growth factor, a hormone, a cytokine, an enzyme, or a fragment and/or fusion protein of any of the foregoing.
46. The composition according to item 45, wherein the antibody is a monoclonal antibody, a polyclonal antibody, a chimeric antibody, a multispecific antibody, an antibody fusion protein, an antibody-drug-conjugate or an antibody fragment.
47. The composition according to any one of items 42 to 46, wherein the complexing agent has a negative charge when comprised in the RPCs.
48. The composition according to any one of items 42 to 47, wherein the complexing agent comprises a hydrophobic moiety.
49. The composition according to any one of items 42 to 48, wherein the composition comprises at least one excipient.
50. The composition according to item 49, wherein the at least one excipient is a stabilizer and/or a surfactant.
51. The composition according to any one of items 42 to 50, wherein the protein has a higher melting temperature when comprised in the RPC compared to the uncomplexed protein.
52. The composition according to any one of items 42 to 51, wherein the RPCs comprising the protein and the complexing agent dissociate at physiological pH and ionic strength.
53. The composition according to any one of items 42 to 51, wherein the RPCs comprising the protein and the complexing agent dissociate in 10 mM to 100 mM PBS (pH 7.4, 137 mM NaCl) when diluted to a protein concentration of 0.1 to 10 mg/mL.
54. The composition according to any one of items 42 to 53, wherein the composition is a suspension.
55. The composition according to item 54, wherein the suspension is obtained with the method according to any one of items 1 to 25.
56. The composition according to items 54 or 55, wherein the protein concentration in the suspension ranges from 50 to 250 mg/mL, in particular wherein the protein concentration

in the suspension ranges from 100 to 200 mg/mL.

57. The composition according to any one of items 54 to 56, wherein the suspension comprises uncomplexed complexing agent.
58. The composition according to any one of items 54 to 57, wherein the RPCs comprised in the suspension have a mean particle size ranging from 5 to 20 μm , in particular wherein the RPCs comprised in the suspension have a mean particle size ranging from 6 to 12 μm .
59. The composition according to any one of items 54 to 57, wherein the RPCs comprised in the suspension have a mean particle size ranging from 100 to 4000 nm, in particular wherein the RPCs comprised in the suspension have a mean particle size ranging from 150 to 2000 nm.
60. The composition according to any one of items 54 to 57, wherein the RPCs comprised in the suspension have a mean particle size ranging from 0.1 to 20 μm , in particular wherein the RPCs comprised in the suspension have a mean particle size ranging from 0.1 to 12 μm .
61. The composition according to any one of items 54 to 60, wherein the suspension is injectable through a 26G needle.
62. The composition according to any one of items 54 to 61, wherein the suspension is stable for at least 4 weeks at 4°C and/or 25°C
63. The composition according to any one of items 54 to 62, wherein the suspension has a viscosity ranging from 2 to 20 cP, in particular ranging from 3 to 15 cP, when measured at 20°C.
64. The composition according to any one of items 54 to 63, wherein the pH of the suspension is lower than the isoelectric point of the protein.
65. The composition according to any one of items 54 to 64, wherein the pH of the suspension is 1 to 3 pH units lower than the isoelectric point of the protein, in particular wherein the pH of the suspension is 2 pH units lower than the isoelectric point of the protein.
66. The composition according to any one of items 54 to 64, wherein the pH of the suspension ranges from 1 to 6, in particular wherein the pH of the suspension ranges from 4.5 to 5.5.

67. The composition according to any one of items 54 to 66, wherein the suspension comprises a buffering agent.
68. The composition according to item 67, wherein the buffering agent is histidine or citrate.
69. The composition according to any one of items 54 to 68, wherein the suspension has an ionic strength ranging from 20 to 50 mM, in particular wherein the suspension has an ionic strength ranging from 20 to 30 mM.
70. The composition according to any one of items 54 to 66, wherein the suspension is substantially free of buffering agents.
71. The composition according to any one of items 54 to 70, wherein the suspension further comprises a non-aqueous solvent.
72. The composition according to item 71, wherein the non-aqueous solvent is diethylene glycol monoethyl ether, triacetin or ethyl oleate.
73. The composition according to any one of items 42 to 53, wherein the composition is a lyophilisate.
74. The composition according to item 73, wherein the lyophilisate is obtained with the method according to any one of items 26 to 29.
75. The composition according to item 73 or 74, wherein the lyophilisate comprises a buffering agent.
76. The composition according to item 75, wherein the buffering agent is histidine or citrate.
77. The composition according to any one of items 73 to 76 wherein the lyophilisate comprises at least one cryoprotectant.
78. The composition according to item 77, wherein the at least one cryoprotectant is selected from a group consisting of: sugars, amino acids, methylamines, lyotropic salts, polyols, propylene glycol, polyethylene glycol and pluronics.
79. The composition according to any one of items 73 to 78, wherein the lyophilisate is stable for at least 4 weeks at 40°C.

80. The composition according to any one of items 73 to 79, wherein the lyophilisate is reconstituted in a liquid to a protein concentration ranging from 50 to 250 mg/mL, in particular wherein the lyophilisate is reconstituted in a liquid to a protein concentration ranging from 100 to 200 mg/mL.
81. The composition according to item 80, wherein the liquid is PBS.
82. The composition according to item 80 or 81, wherein the resuspended lyophilisate has a viscosity ranging from 2 to 20 cP, in particular ranging from 10 to 20 cP.
83. The composition according to any one of items 42 to 53, wherein the composition is a spray dried powder.
84. The composition according to item 83, wherein the protein content of the spray dried powder is at least 40% by weight (w/w), at least 50% by weight (w/w), at least 60% by weight (w/w)
85. The composition according to item 83 or 84, wherein the spray dried powder is obtained with the method according to any one of items 30 to 37.
86. The composition according to any one of items 83 to 85, wherein the spray dried powder comprises a buffering agent.
87. The composition according to item 86, wherein the buffering agent is histidine or citrate.
88. The composition according to any one of items 83 to 85, wherein the spray dried powder is substantially free of buffering agents.
89. The composition according to any one of items 83 to 88, wherein the RPCs comprised in the spray dried powder have a mean particle size ranging from 5 to 50 μm , in particular ranging from 10 to 40 μm , in particular ranging from 20 to 35 μm .
90. The composition according to any one of items 83 to 89, wherein the spray dried powder is re-suspended in a liquid to a protein concentration in the suspension ranging from 50 to 300 mg/mL, in particular wherein the spray dried powder is re-suspended in a liquid to a protein concentration in the suspension ranging from 100 to 250 mg/mL.
91. The composition according to item 90, wherein the liquid is a non-aqueous solvent.

92. The composition according to item 91, wherein the non-aqueous solvent is at least one selected from a group consisting of: diethylene glycol monoethyl ether, ethyl oleate, triacetin, isosorbide dimethyl ester and glycofurol, preferably diethylene glycol monoethyl ether, ethyl oleate or triacetin.
93. The composition according to any one of items 90 to 92, wherein the reconstituted spray dried powder has a viscosity ranging from 10 to 100 cP, in particular ranging from 20 to 80 cP.
94. A pharmaceutical formulation comprising the composition according to any one of items 41 to 93.
95. The pharmaceutical formulation according to item 94, wherein the pharmaceutical formulation comprises the suspension according to any one of items 54 to 72, the reconstituted lyophilisate according to any one of items 80 to 82, or the re-suspended spray dried powder according to any one of items 90 to 93.
96. The pharmaceutical formulation according to items 94 or 95 for use as a medicament.
97. The pharmaceutical formulation according to any one of items 94 to 96 for use in the treatment of an autoimmune disease, an immune dysregulation disease, carcinoma, sarcoma, glioma, melanoma, lymphoma, leukemia, chronic lymphocytic leukemia, follicular lymphoma, diffuse large B cell lymphoma, multiple myeloma, non-Hodgkin's lymphoma, Alzheimer's disease, type 1 or type 2 diabetes, amyloidosis, or atherosclerosis.
98. The pharmaceutical formulation for use according to item 97, wherein the pharmaceutical formulation is administered subcutaneously, intramuscularly, transdermally, ocularly, such as subconjunctivally, intracamerally, intravitreally, subretinally, or suprachoroidally, to the brain, such as intralumbally, intrathecally, or intraventricularly, intra-articularly, or by inhalation.
99. Use of the pharmaceutical formulation according to items 94 or 95 for the treatment of a disease selected from the group consisting of autoimmune disease, immune dysregulation disease, carcinoma, sarcoma, glioma, melanoma, lymphoma, leukemia, chronic lymphocytic leukemia, follicular lymphoma, diffuse large B cell lymphoma, multiple myeloma, non-Hodgkin's lymphoma, Alzheimer's disease, type 1 or type 2 diabetes, amyloidosis, and atherosclerosis.

100. Use of the pharmaceutical formulation according to items 94 or 95 in the preparation of a medicament for the treatment of a disease selected from the group consisting of autoimmune disease, immune dysregulation disease, carcinoma, sarcoma, glioma, melanoma, lymphoma, leukemia, chronic lymphocytic leukemia, follicular lymphoma, diffuse large B cell lymphoma, multiple myeloma, non-Hodgkin's lymphoma, Alzheimer's disease, type 1 or type 2 diabetes, amyloidosis, and atherosclerosis.
101. A method of treating a subject suffering from a disease selected from the group consisting of: an autoimmune disease, an immune dysregulation disease, carcinoma, sarcoma, glioma, melanoma, lymphoma, leukemia, chronic lymphocytic leukemia, follicular lymphoma, diffuse large B cell lymphoma, multiple myeloma, non-Hodgkin's lymphoma, Alzheimer's disease, type 1 or type 2 diabetes, amyloidosis, and atherosclerosis, the method comprising the steps of (a) producing the pharmaceutical formulation according to items 94 or 95; and (b) administering the pharmaceutical formulation to a subject in need thereof.
102. The method according to item 101, wherein the pharmaceutical composition is administered subcutaneously, intramuscularly or transdermally, in particular wherein the pharmaceutical composition is administered subcutaneously.
103. A method of subcutaneous, intramuscular or transdermal administration of a pharmaceutical formulation, the method comprising the steps of (a) producing the pharmaceutical formulation according to items 94 or 95; and (b) administering the pharmaceutical formulation to a subject by subcutaneous, intramuscular or transdermal delivery.

Accordingly, in one aspect, the invention relates to a method for producing a composition comprising reversible protein complexes (RPCs), the method comprising the steps of: (a) contacting a protein and a complexing agent in a buffer solution, wherein the complexing agent is dextran sulfate and/or chondroitin sulfate, and wherein the protein and the complexing agent have opposite charges when comprised in the buffer solution, preferably resulting in a mole-charge ratio of protein to complexing agent of about 1:1 or higher than 1:1; (b) formation of RPCs between the protein and the complexing agent in the buffer solution; and (c) obtaining a suspension comprising the RPCs formed in step (b).

That is, the invention is based at least in part on the surprising finding that the complexing agents dextran sulfate and chondroitin sulfate can be used to produce high concentration protein compositions comprising reversible protein complexes (RPCs) that efficiently dissolve under

physiological conditions. Further, it was unexpectedly found that formulations comprising the RPCs of the invention have a particularly low viscosity even at high protein concentrations and are thus suitable for subcutaneous administration.

It has been demonstrated by the inventors that both dextran sulfate and chondroitin sulfate can be used to complex proteins with nearly 100% efficiency (Fig.9). Surprisingly, RPCs comprising these complexing agents dissolve with nearly 100% efficiency in PBS (pH 7.4, 137 mM NaCl), which simulates physiological conditions in humans. Thus, formulations comprising the RPCs of the invention can be administered to a patient, preferably subcutaneously, where they dissolve *in situ* such that the protein becomes available to the patient.

Producing the reversible protein complexes of the invention requires a first step of contacting the protein and the complexing agent in a buffer solution. That is, the protein and the complexing agent may be mixed in a buffer solution such that a reversible protein complex forms between the protein and the complexing agent. Without being bound to theory, formation of the reversible protein complex is at least partially driven by electrostatic interactions between the oppositely charged proteins and complexing agents in the buffer solution. It is to be understood that a single protein molecule usually undergoes complex formation with multiple molecules of the complexing agent.

That is, within the present invention, the complexing agent and the protein have opposite charges when comprised in the buffer solution. Thus, in certain embodiments, the complexing agent may be positively charged, and the protein may be negatively charged when comprised in the buffer solution. However, it is preferred that the protein is positively charged when comprised in the buffer solution and that the complexing agent is negatively charged when comprised in the buffer solution.

It is to be understood that both the protein and the complexing agent may be dissolved in the buffer solution before the contacting step. Contacting of the oppositely charged proteins and complexing agents in the buffer solution results in precipitation of reversible protein complexes, thereby turning the buffered solution into a suspension comprising the reversible protein complexes.

A “composition”, as used herein, refers to a mixture comprising the reversible protein complexes of the invention and at least one further compound. That is, the composition of the invention preferably comprises reversible protein complexes comprising the complexing agents dextran sulfate and/or chondroitin sulfate. The composition may have any form. However, in

certain embodiments, the composition is a suspension. In other embodiments, the composition is a powder, in particular a lyophilized powder or a spray dried powder, or a reconstituted or re-suspended powder.

In certain embodiments, the composition of the invention is a suspension. The term “suspension” as used herein refers to a dispersion with continuous liquid phase and a discontinuous solid phase in form of particles, such as RPCs, that have a number average diameter of 5 to 300 μm . However, it is important to understand that the term “suspension” may also encompass dispersions comprising smaller particles, such as RPCs having a number average diameter in the nanometer range.

The term “complex” as used herein refers to the association of two or more molecules, usually by non-covalent bonding, e.g., the association between a positively charged group of a first molecule and a negatively charged group of a second molecule. Further, complex formation may be facilitated by hydrophobic interactions between the first molecule and the second molecule. Within the present invention, the first molecule is preferably a protein and the second molecule is preferably a complexing agent.

A complex is said to be reversible, if the association between the two molecules, i.e., the protein and the complexing agent, can be reversed without significantly modifying the protein. That is, a protein complex is said to be reversible, if the protein complex can dissociate and the protein retains its original size, structure and/or function after dissociation of the complex.

Preferably, a protein complex is determined to be a reversible protein complex, if both the formation of the complex and the dissociation of the complex can be achieved with high efficiency.

The term “complexing efficiency”, as used herein, refers to the efficiency with which a complex involving a protein and a complexing agent is formed under a specific condition, i.e., a specific pH and/or ionic strength. Complexing efficiency is defined as the percentage of proteins in a sample that have undergone complex formation after contacting with a complexing agent. In particular, a complex is said to form with high complexing efficiency, if at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% of the proteins in a sample have undergone complex formation following a contacting step with a complexing agent.

The term “dissociation efficiency”, as used herein, refers to the efficiency with which a complex involving a protein and a complexing agent is dissociated under a specific condition, i.e., a

specific pH and/or ionic strength. Dissociation efficiency is defined as the percentage of protein complexes in a sample that dissolved under the specific conditions such that the protein is released from the complex and goes back in solution. In particular, a complex is said to dissociate with a high dissociation efficiency, if at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% of the complexes in a sample dissociate into soluble proteins under the specific conditions in the sample.

Thus, a protein complex is defined to be a “reversible protein complex”, if the complex is formed with a high complexing efficiency and dissolves with a high dissociation efficiency.

Further, a protein complex is defined to be a “reversible protein complex”, if the protein retains its original size, structure and/or function after dissociation of the protein complex. In particular, a protein complex is determined to be a reversible protein complex, if at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% of the proteins in a sample retain their original size, structure and/or function after they have been released from the protein complex in the dissociation step.

Preferably, a protein complex is determined to be a reversible protein complex, if at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% of the proteins in a sample retain their original size, structure and/or function after dissociation of the protein complex in 10 – 100 mM PBS (pH 7.4).

More preferably, a protein complex is determined to be a reversible protein complex, if at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% of the proteins in a sample retain their original size, structure and/or function after dissociation of the protein complex under physiological conditions.

The skilled person is aware of methods to determine complexing efficiency and dissociation efficiency. For example, methods to determine complexing efficiency are disclosed in Example 1.2.3 and methods to determine dissociation efficiency are disclosed in Example 1.2.4, respectively. Further, the skilled person is aware of methods to determine if a protein retains its original size, structure and/or function after dissociation of the protein complex.

Changes in the size of a protein mainly result from degradation of the protein, which commonly results in reduced protein size, or aggregation of two or more protein, which commonly results

in increased protein size. For example, the size of a protein may be determined before formation of the complex and after dissociation of the complex by any method known in the art, such as size exclusion chromatography (SEC) or ion exchange chromatography (IEC) (see Example 1.2.8).

That is, in certain embodiments, a protein is said to be stable if, after dissociation of RPCs in a sample, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% of the proteins are in monomeric form and not degraded, preferably when measured by size exclusion chromatography (SEC).

Alternatively, a protein is said to be stable, if the main peak percentage of the protein differs by not more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20% when analyzed by ion exchange chromatography (IEC) and compared before the formation of the RPC and after dissociation of the RPC.

The structure of a protein may be determined before formation of the complex and after dissociation of the complex by any method known in the art, such as X-ray crystallography, NMR or circular dichroism.

The function of a protein may be determined before formation of the complex and after dissociation of the complex. For example, the function of an antigen-binding molecule, such as an antibody, may be determined in binding studies. Common methods known in the art to determine the binding of a protein to a target are isothermal titration calorimetry or surface plasmon resonance. Preferably, the antigen-binding molecule may have substantially the same binding characteristics after dissociation of the complex as it had before the formation of the complex. That is, the antigen-binding molecule is said to have substantially the same binding characteristics, if the binding affinity of the antigen-binding molecule for the antigen differs less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5% when measured before complex formation and after dissociation of the complex.

The term "contacting" as used in the context of the methods of the present invention is understood by the skilled person. The term relates to bringing two compounds of the present invention, i.e., a protein and a complexing agent, in physical contact with each other. The protein and the complexing agent are preferably brought into physical contact in a buffer solution. That is, both the protein and the complexing agent are dissolved in a buffer solution such that they can get into physical contact by means of diffusion. Contacting of the compounds in the buffer solution may be facilitated by shaking, mixing, vortexing or the like.

The term "electrostatic interactions" as used herein, refers to interactions that are formed between two substances that have opposite charges, namely, a positively charged substance and a negatively charged substance. Such interactions typically involve ionic bonds.

Most proteins both contain acidic and basic functional groups. Thus, a protein is said to be negatively charged if the net charge of the protein is negative under the given conditions. Accordingly, a protein is said to be positively charged if the net charge of the protein is positive under the given conditions. The net charge of a polypeptide at a given pH can be calculated on the basis of the Henderson-Hasselbalch equation (Hasselbalch, K. A., 1917 *Biochemische Zeitschrift* 78: 112-144) and known pKa values of ionisable amino acid side chains and the N- and C-termini of a polypeptide.

The term "buffer solution", as used herein, refers to a composition, wherein the composition comprises a weak acid and its conjugate base (usually as a conjugate base salt), a weak base and its conjugate acid, or mixtures thereof. Those skilled in the art would readily recognize a variety of buffer solutions that could be used in the methods and/or formulations used in the invention. Typical buffer solutions include, but are not limited to pharmaceutically acceptable weak acids, weak bases, or mixtures thereof.

The phrase "weak acid" is a chemical acid that does not fully ionize in aqueous solution; that is, if the acid is represented by the general formula HA, then in aqueous solution A^- forms, but a significant amount of undissociated HA still remains. The acid dissociation constant (K_a) of a weak acid varies between 1.8×10^{-16} and 55.5.

The phrase "weak base" is a chemical base that does not fully ionize in aqueous solution; that is, if the base was represented by the general formula B, then in aqueous solution BH^+ forms, but a significant amount of unprotonated B still remains. The acid dissociation constant (K_a) of the resultant conjugate weak acid BH^+ varies between 1.8×10^{-16} and 55.5.

The phrase "conjugate acid" is the acid member, HX^+ , of a pair of two compounds (HX^+ , X) that transform into each other by gain or loss of a proton.

The phrase "conjugate base" is the base member, X^- , of a pair of two compounds (HX , X^-) that transform into each other by gain or loss of a proton.

The phrase "conjugate base salt" is the ionic salt comprising a conjugate base, X^- , and a positively charged counter-ion.

Within the present invention, the complexing agent may be a polymer, preferably a charged polymer. That is, the present invention is preferably based on the formation of reversible protein complexes between proteins and polymers, wherein the polymers have an opposite charge compared to the net charge of the protein when comprised in the buffer solution.

In particular, it has been demonstrated within the present invention that polymeric complexing agents result in reversible protein complexes that dissolve more efficiently at physiological conditions compared to complexes that have been formed with monomeric complexing agents such as sodium dodecyl sulfate (SDS) or sodium taurocholate (ST) (see Fig.9). In particular, it has been demonstrated that monomeric complexing agents such as SDS or ST result in significant degradation of the protein after dissociation of the complex (see Table 8).

In certain embodiments, the complexing agent is dextran sulfate. It has been shown by the inventors that dextran sulfate can be used to form reversible protein complexes that efficiently dissociate at physiological conditions. Thus, in a particular embodiment, the invention relates to the method according to the invention, wherein the complexing agent is dextran sulfate.

The term “dextran sulfate” as used herein refers a polyanionic derivative of dextran, ranging in molecular weight from 7,000 to 500,000 daltons. Dextrans are polymers of glucose in which glucose residues are joined by α -1,6 linkages. Thus, in certain embodiments, the invention relates to the method according to the invention, wherein the complexing agent is dextran sulfate with an average molecular weight ranging from 7,000 to 500,000 daltons.

Dextran sulfate with an average molecular weight of 40 kDa was shown to be particularly suitable for the formation of reversible protein complexes that efficiently dissociate at physiological conditions. Thus, in a preferred embodiment, the invention relates to the method according to the invention, wherein the complexing agent is dextran sulfate with an average molecular weight ranging from 10 kDa to 200 kDa. In a more preferred embodiment, the invention relates to the method according to the invention, wherein the complexing agent is dextran sulfate with an average molecular weight ranging from 20 kDa to 100 kDa. In an even more preferred embodiment, the invention relates to the method according to the invention, wherein the complexing agent is dextran sulfate with an average molecular weight ranging from 20 kDa to 80 kDa. In an even more preferred embodiment, the invention relates to the method according to the invention, wherein the complexing agent is dextran sulfate with an average molecular weight ranging from 30 kDa to 50 kDa. In a most preferred embodiment, the invention relates to the method according to the invention, wherein the complexing agent is dextran sulfate with an average molecular weight of 40 kDa.

In other embodiments, the complexing agent is chondroitin sulfate. Chondroitin sulfate is a sulfated glycosaminoglycan (GAG) composed of a chain of alternating sugars (N-acetylgalactosamine and glucuronic acid). Within the present invention, the term “chondroitin sulfate” encompasses chondroitin sulfate A (chondroitin-4-sulfate), chondroitin sulfate C (chondroitin-6-sulfate), chondroitin sulfate D (chondroitin-2,6-sulfate) and chondroitin sulfate E (chondroitin-4,6-sulfate). In certain embodiments, the invention relates to the method according to the invention, wherein the complexing agent is chondroitin sulfate A. In other embodiments, the invention relates to the method according to the invention, wherein the complexing agent is chondroitin sulfate C. In further embodiments, the invention relates to the method according to the invention, wherein the complexing agent is chondroitin sulfate D. In further embodiments, the invention relates to the method according to the invention, wherein the complexing agent is chondroitin sulfate E. In further embodiments, the invention relates to the method according to the invention, wherein the complexing agent is at least one of the group consisting of chondroitin sulfate A, chondroitin sulfate C, chondroitin sulfate D and chondroitin sulfate E.

The term “charged polymer” refers to any compound composed of a backbone of repeating structural units linked in linear or non-linear fashion, some of which repeating units contain positively or negatively charged chemical groups. The repeating structural units may be polysaccharide, hydrocarbon, organic, or inorganic in nature. The repeating units may range from $n=2$ to $n=\text{several million}$.

The term “positively charged polymer” as used herein refers to polymers containing chemical groups which carry, can carry, or can be modified to carry a positive charge such as ammonium, alkyl ammonium, dialkylammonium, trialkyl ammonium, and quaternary ammonium.

The term “negatively charged polymer” as used herein refers to polymers containing chemical groups which carry, can carry, or can be modified to carry a negative charge such as derivatives of phosphoric and other phosphorous containing acids, sulfuric and other sulfur containing acids, nitrate and other nitrogen containing acids, formic and other carboxylic acids.

In a particular embodiment, the invention relates to the method according to the invention, wherein the pH of the buffer solution is adjusted to be lower than the isoelectric point of the protein.

Formation of reversible protein complexes is mainly driven by electrostatic interaction between the oppositely charged proteins and complexing agents that form the complex. The charge of a molecule in a solution depends, amongst others, on the pH of the solution. Within the present

invention, it is preferred that the protein and the complexing agent are comprised in a buffer solution, wherein the pH of the buffer solution is adjusted such that the protein and the complexing agent are oppositely charged.

Proteins may comprise positively and negatively charged amino acid residues when comprised in a solution. If a protein comprises more negative charges than positive charges under a specific condition, the protein is said to have a negative net charge under said condition. If a protein comprises more positive charges than negative charges under a specific condition, the protein is said to have a positive net charge under said condition.

The term "isoelectric point" as used herein means the pH value where the overall net charge of a macromolecule such as a protein is zero. In proteins there may be many charged groups, and at the isoelectric point the sum of all these charges is zero. At a pH above the isoelectric point the overall net charge of the polypeptide will be negative, whereas at pH values below the isoelectric point the overall net charge of the polypeptide will be positive.

The skilled person is aware of methods to determine the isoelectric point of a protein. Most commonly, the isoelectric point of a protein is computed based on the amino acid sequence of the protein. Numerous tools are available online that allow computing the isoelectric point of a protein, such as "ExPASy Compute pI/Mw"; see *Protein Identification and Analysis Tools on the ExPASy Server*; Gasteiger E., Hoogland C., Gattiker A., Duvaud S., Wilkins M.R., Appel R.D., Bairoch A.; (In) John M. Walker (ed): *The Proteomics Protocols Handbook*, Humana Press (2005), pp. 571-607.

The protein forming the reversible protein complex may have a positive or negative net charge. However, it is preferred that the protein has a positive net charge when comprised in the buffer solution. That is, it is preferred that the pH of the buffer solution is lower than the isoelectric point of the protein.

It is commonly observed that exposure to strongly acidic pH may result in irreversible denaturation of proteins. Thus, it is preferred that the pH of the buffer solution is adjusted to a pH that is 2 to 5 pH units below the isoelectric point of the protein. Thus, in a particular embodiment, the invention relates to the method according to the invention, wherein the pH of the buffer solution is adjusted to 2 to 5 pH units below the isoelectric point of the protein. In a preferred embodiment, the invention relates to the method according to the invention, wherein the pH of the buffer solution is adjusted to about 3 pH units below the isoelectric point of the protein.

Adjusting the pH of the buffer solution to a pH that is only slightly below the isoelectric point of the protein ensures that the protein is positively charged when comprised in the buffer solution and, at the same time, reduces the risk of protein denaturation.

Alternatively, the pH of the buffer solution may be adjusted to a fixed value. The method of the present invention is preferably used for the production of reversible protein complexes comprising therapeutic proteins, i.e. antibodies. Most antibodies have an isoelectric point ranging from 6.5 – 9. Thus, most antibodies will have a positive net charge when comprised in a buffer solution with an acidic pH value. Thus, in a particular embodiment, the invention relates to the method according to the invention, wherein the buffer solution has a pH ranging from 1 to 6. In a preferred embodiment, the invention relates to the method according to the invention, wherein the buffer solution has a pH ranging from 3 to 6. In a more preferred embodiment, the invention relates to the method according to the invention, wherein the buffer solution has a pH ranging from 4.5 to 5.5.

It has been shown by the inventors that complex formation requires a buffer solution with an ionic strength of at least 5 mM, preferably at least 20 mM (Fig.13). Thus, in a particular embodiment, the invention relates to the method according to the invention, wherein the buffer solution has an ionic strength ranging from 5 to 50 mM, preferably 20 to 50 mM. In a preferred embodiment, the invention relates to the method according to the invention, wherein the buffer solution has an ionic strength ranging from 20 to 30 mM.

The term “ionic strength” is used herein as the following function of the concentration of all ions in a solution:

$$I = \frac{1}{2} \sum_{i=1}^n c_i z_i^2$$

wherein c_i is the molar concentration of ion i (M, mol/L), z_i is the charge number of that ion and the sum is taken over all ions in the solution.

The buffer solution may comprise any buffering agent that allows efficient reversible protein complex formation. Many buffering agents are known in the art that allow to maintain the pH of a solution near a chosen pH value. Buffering agents that may be used for the method of the present invention include, without limitation, formate, citrate, succinate, acetate, propionate, malate, pyridine, piperazine, cacodylate, succinate, MES, maleate, histidine, bis-tris, phosphate, ethanolamine, ADA and carbonate.

It is to be understood that the choice of the buffering agent depends on the protein that is to be

complexed. In certain embodiments, the buffer solution is adjusted to a pH that is below the isoelectric point of the protein that is to be complexed. The skilled person is aware of methods to determine the isoelectric point of a protein and to select a buffering agent that allows to maintain a pH value in the buffer solution that is below the isoelectric point of said protein. Thus, in certain embodiments, the invention relates to the method of the invention, wherein the buffer solution comprises a buffering agent that allows to maintain the pH of the buffer solution below the isoelectric point of the protein. In a particular embodiment, the invention relates to the method according to the invention, wherein the buffer solution comprises a buffering agent that allows to maintain the pH of the buffer solution 1 to 3 pH units below the isoelectric point of the protein. In a particular embodiment, the invention relates to the method according to the invention, wherein the buffer solution comprises a buffering agent that allows to maintain the pH of the buffer solution 2 pH units below the isoelectric point of the protein.

It has been shown by the inventors that histidine is particularly suited as a buffering agent when forming reversible protein complexes comprising different types of antibodies. The conjugate acid (protonated form) of the imidazole side chain in histidine has a *pKa* of approximately 6.0. Histidine buffers are most effective in a pH range from 5.5 to 7.4. Thus, histidine is particularly suited as a buffering agent when forming reversible protein complexes of antibodies with an isoelectric point between 6 and 9. Accordingly, in a particular embodiment, the invention relates to the method according to the invention, wherein the buffer solution comprises histidine as a buffering agent.

Further, it has been demonstrated that citrate is particularly suited as a buffering agent when forming reversible protein complexes comprising different types of antibodies. Citrate is a weak tricarboxylic acid with three different *pKa* values (3.1, 4.7, and 6.4). Citrate buffers are most effective in a pH range from 2.5 to 7. Thus, citrate is particularly suited as a buffering agent when forming reversible protein complexes of antibodies with an isoelectric point between 3 and 6, but may also be used for complexing protein with a higher isoelectric point. Accordingly, in a particular embodiment, the invention relates to the method according to the invention, wherein the buffer solution comprises citrate as a buffering agent.

The term, "buffering agent," as used herein, refers to a weak acid or base used to maintain the pH of a solution near a chosen pH value after the addition of another acidic or basic compound. The function of such an agent is to prevent the change in pH when acids or bases are added to a solution. Such agents may be acids, bases, or combinations thereof.

In a particular embodiment, the invention relates to the method according to the invention, wherein the buffer solution comprising the protein and the complexing agent is obtained by

mixing a first solution comprising the protein and a second solution comprising the complexing agent.

Within the present invention, it is preferred that the protein and the complexing agent are contacted by mixing a first solution comprising the protein with a second solution comprising the complexing agent. The skilled person is aware of methods for preparing solutions comprising proteins or complexing agents and to mix these solutions.

Alternatively, the protein and the complexing agent may be contacting by (i) providing either the protein or the complexing agent in a buffer solution and (ii) adding the remaining component of the RPC to the buffer solution in solid form.

In a particular embodiment, the invention relates to the method according to the invention, wherein the first solution comprising the protein and/or the second solution comprising the complexing agent comprises a buffering agent.

Both the solution comprising the protein and/or the solution comprising the complexing agent may comprise a buffering agent. That is, in certain embodiments, the method involves a step of contacting a first solution comprising a protein and a buffering agent with a second solution comprising a complexing agent and a buffering agent. The buffering agent in the first and second solution may be identical or may be a different buffering agent. In certain embodiments, both the first and second solution comprise histidine as the buffering agent. In other embodiments, both the first and second solution comprise citrate as the buffering agent.

Alternatively, the protein and the complexing agent may be contacted by mixing a solution comprising a buffering agent with a solid. For example, a solution comprising a protein and a buffering agent may be contacted with a complexing agent by adding the complexing agent in solid form to the solution such that the complexing agent dissolves in the solution and forms a reversible protein complex with the protein. Correspondingly, a solution comprising a complexing agent and a buffering agent may be contacted with a protein by adding the protein in solid form to the solution such that the protein dissolves in the solution and forms a reversible protein complex with the complexing agent.

In certain embodiments, the complexing agent is added gradually to a protein solution. Preferably, a solution comprising the complexing agent is added gradually to a protein solution. More preferably, a buffered solution comprising the complexing agent is added gradually to a buffered protein solution. The buffered solution may comprise any of the buffering agents disclosed herein.

Complex formation is driven by electrostatic interactions between oppositely charged proteins and complexing agents. Within the present invention, the protein and the complexing agent will be contacted in the buffer solution at a specific mole-charge ratio. In certain embodiments, the protein has a positive net charge when comprised in the buffer solution. In these cases, the mole-charge ratio between the protein and the complexing agent is defined as the ratio between the total number of positive charges on all proteins comprised in the buffer solution and the total number of negative charges on all complexing agents comprised in the buffer solution. Thus, at a mole-charge ratio of 1:1, the positive charges of the proteins comprised in the buffer solution should theoretically completely be neutralized by the negative charges of the complexing agents comprised in the buffer solution.

Within the present invention, it has been surprisingly found that a mole-charge ratio between the protein and the complexing agent of 1:1 or even higher than 1:1 (excess of protein) is sufficient for obtaining nearly complete complex formation, thereby reducing the demand for the complexing agent when producing the RPCs of the invention (Table 7). For example, it has been shown that for the complexing agent dextran sulfate a mole-charge ratio of 1:0.6 is sufficient to achieve complete complexation of proteins. For the complexing agent chondroitin sulfate, even a mole-charge ratio of 1:0.2 was sufficient to achieve complete complexation of proteins. Thus, in a particular embodiment, the invention relates to the method according to the invention, wherein the protein and the complexing agent are contacted at a mole-charge ratio ranging from 1:0.1 to 1:6, in particular wherein the protein and the complexing agent are contacted at a mole-charge ratio ranging from 1:0.2 to 1:1.

It is to be understood that the mole-charge ratio that is required to achieve complete complexation of the protein varies between complexing agents.

Thus, in certain embodiments, the invention relates to the method according to the invention, wherein the protein and the complexing agent dextran sulfate are contacted at a mole-charge ratio ranging from 1:0.2 to 1:2, preferably wherein the protein and the complexing agent dextran sulfate are contacted at a mole-charge ratio ranging from 1:0.5 to 1:2, more preferably wherein the protein and the complexing agent dextran sulfate are contacted at a mole-charge ratio ranging from 1:0.5 to 1:1, most preferably wherein the protein and the complexing agent dextran sulfate are contacted at a mole-charge ratio of 1:0.6.

In other embodiments, the invention relates to the method according to the invention, wherein the protein and the complexing agent chondroitin sulfate are contacted at a mole-charge ratio ranging from 1:0.2 to 1:2, preferably wherein the protein and the complexing agent chondroitin sulfate are contacted at a mole-charge ratio ranging from 1:0.2 to 1:1.

It further has been shown by the inventors that the complexing efficiency depends on the concentration of the protein in the buffer solution. In particular, it has been shown that complex formation can be achieved at protein concentrations ranging from 1 – 40 mg/mL (Fig.10). Thus, in a particular embodiment, the invention relates to the method according to the invention, wherein the protein is contacted with the complexing agent in the buffer solution at a protein concentration ranging from 1 - 40 mg/mL.

It has been shown by the inventors that complexes formed at protein concentrations above 5 mg/mL have a larger particle size, which may have reduced the injectability of the complexes (Fig.11). Further, complexes that have been formed at protein concentrations above 5 mg/mL do not dissociate as efficiently as protein complexes that have been formed at lower protein concentrations (Fig.10). Thus, in a preferred embodiment, the invention relates to the method according to the invention, wherein the protein is contacted with the complexing agent in the buffer solution at a protein concentration ranging from 1 - 5 mg/mL.

The method of the present invention is not restricted to a specific type of protein. That is, the method of the present invention may be used for the production of reversible protein complexes comprising any type of protein. Since the skilled person is aware of methods to determine the isoelectric point of any protein based on its amino acid sequence, the skilled person is able to select buffer conditions that are suitable for the formation of reversible protein complexes with dextran sulfate or chondroitin sulfate.

Preferably, the method of the present invention is used for the production of reversible protein complexes comprising therapeutic proteins. That is, the method of the invention allows the production of pharmaceutical compositions with high protein concentrations that are suitable for subcutaneous, intramuscular or transdermal application.

The term "therapeutic protein," as used herein, refers to any peptide or protein that is known to be useful for the prevention, treatment, or amelioration of a disease or disorder, e.g., an antibody, growth factor, cell surface receptor, cytokine, hormone, toxin, or fragments and/or fusion proteins of any of the foregoing. Thus, in a particular embodiment, the invention relates to the method according to the invention, wherein the protein is an antibody, a growth factor, a hormone, a cytokine, an enzyme, or a fragment and/or fusion protein of any of the foregoing.

The present invention is directed to the production of reversible protein complexes. However, it is to be noted that the present invention also encompasses the production of reversible complexes comprising peptides. Thus, the term protein is used herein interchangeably with the term peptide or polypeptide.

The term "peptide" or "polypeptide" as used herein refers to a compound made up of a single unbranched chain of amino acid residues linked by peptide bonds. The number of amino acid residues in such compounds varies widely.

The term "protein" as used herein may be used synonymously with the term "peptide" or "polypeptide" or may refer to, in addition, a complex of two or more peptides which may be linked by bonds other than peptide bonds, for example, such peptides making up the protein may be linked by disulfide bonds. The term "protein" may also comprehend peptide(s) or a family of peptides having identical amino acid sequence(s) but different post-translational modification(s), such as phosphorylation(s), acylation(s), glycosylation(s), and the like, particularly as may be added when such proteins are expressed in eukaryotic hosts.

Examples of proteins encompassed within the definition herein include mammalian proteins, such as, e.g., growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; α -1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIc, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator (t-PA, e.g., Activase®, TNKase®, Retevase®); bombazine; thrombin; tumor necrosis factor- α and - β ; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1- α); serum albumin such as human serum albumin; mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; DNase; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; an integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT5, or NT-6), or a nerve growth factor such as NGF- β ; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF- α and TGF- β , including TGF- β 1, TGF- β 2, TGF- β 3, TGF- β 4, or TGF- β 5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I); insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin (EPO); thrombopoietin (TPO); osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon- α , - β , and - γ ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor (DAF); a viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; immunoadhesins; antibodies; and

biologically active fragments or variants of any of the above-listed polypeptides.

The term "growth factor", as used herein, refers to a polypeptide molecule that is capable of effectuating differentiation of cells. Examples of growth factors as contemplated for use in accord with the teachings herein include an epidermal growth factor (EGF), transforming growth factor-alpha (TGF-alpha), transforming growth factor-beta (TGF-beta), human endothelial cell growth factor (ECGF), granulocyte macrophage colony stimulating factor (GM-CSF), bone morphogenetic protein (BMP), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and/or platelet derived growth factor (PDGF).

The term "hormone" as used herein refers to a substance normally formed by one organ that stimulates the function of another organ. Polypeptide hormones include, but are not limited to, insulin, growth hormone, gastric inhibitory polypeptide, and cholecystokinin.

The term "cytokine," as used herein, means any secreted polypeptide that affects the functions of other cells, and that modulates interactions between cells in the immune or inflammatory response. Cytokines include, but are not limited to monokines, lymphokines, and chemokines regardless of which cells produce them. For instance, a monokine is generally referred to as being produced and secreted by a monocyte, however, many other cells produce monokines, such as natural killer cells, fibroblasts, basophils, neutrophils, endothelial cells, brain astrocytes, bone marrow stromal cells, epidermal keratinocytes, and B-lymphocytes. Lymphokines are generally referred to as being produced by lymphocyte cells. Examples of cytokines include, but are not limited to, interleukin-1 (IL-1), interleukin-6 (IL-6), Tumor Necrosis Factor alpha (TNF α), and Tumor Necrosis Factor beta (TNF β).

The term "enzyme" as used herein refers to a macromolecular compound mainly comprised of a protein and catalyzing a chemical reaction.

Preferably, the method of the present invention is used for the production of reversible protein complexes comprising antibodies. Thus, in a preferred embodiment, the invention relates to the method according to the invention, wherein the protein is an antibody, or a fragment thereof.

In a particular embodiment, the invention relates to the method according to the invention, wherein the antibody is a monoclonal antibody, a polyclonal antibody, a chimeric antibody, a multispecific antibody, an antibody fusion protein, an antibody-drug-conjugate or an antibody fragment.

The term “antibody” herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments, so long as they exhibit the desired biological activity (Miller et al (2003) *Jour. of Immunology* 170:4854-4861). Antibodies may be murine, human, humanized, chimeric, or derived from other species. An antibody is a protein generated by the immune system that is capable of recognizing and binding to a specific antigen. (Janeway, C., Travers, P., Walport, M., Shlomchik (2001) *Immuno Biology*, 5th Ed., Garland Publishing, New York). A target antigen generally has numerous binding sites, also called epitopes, recognized by CDRs (complementary determining regions) on multiple antibodies. Each antibody that specifically binds to a different epitope has a different structure. Thus, one antigen may have more than one corresponding antibody. An antibody includes a full-length immunoglobulin molecule or an immunologically active portion of a full-length immunoglobulin molecule, i.e., a molecule that contains an antigen binding site that immunospecifically binds an antigen of a target of interest or part thereof, such targets including but not limited to, cancer cell or cells that produce autoimmune antibodies associated with an autoimmune disease. The immunoglobulin disclosed herein can be of any type (e.g., IgG, IgE, IgM, IgD, and IgA), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. The immunoglobulins can be derived from any species. In one embodiment, however, the immunoglobulin is of human, murine, or rabbit origin.

The antibody may be an intact antibody. The term “intact antibody” as used herein is one comprising a VL and VH domains, as well as a light chain constant domain (CL) and heavy chain constant domains, CH1, CH2 and CH3. The constant domains may be native sequence constant domains (e.g., human native sequence constant domains) or amino acid sequence variant thereof. The intact antibody may have one or more “effector functions” which refer to those biological activities attributable to the Fc constant region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody. Examples of antibody effector functions include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; and down regulation of cell surface receptors such as B cell receptor and BCR.

The term “Fc region” as used herein means a C-terminal region of an immunoglobulin heavy chain that contains at least a portion of the constant region. The term includes native sequence Fc regions and variant Fc regions. In one embodiment, a human IgG heavy chain Fc region extends from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, the C-terminal lysine (Lys447) of the Fc region may or may not be present. Unless otherwise specified herein, numbering of amino acid residues in the Fc region or constant region is according to the EU numbering system, also called the EU index, as described in Kabat et al.,

Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991.

The term “framework” or “FR” as used herein refers to variable domain residues other than hypervariable region (HVR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences generally appear in the following sequence in VH (or VL): FR1-H1(L1)-FR2- H2(L2)-FR3-H3(L3)-FR4.

The terms “full length antibody”, “intact antibody,” and “whole antibody” are used herein interchangeably to refer to an antibody having a structure substantially similar to a native antibody structure or having heavy chains that contain an Fc region as defined herein.

The term "antibody fragment(s)" as used herein comprises a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies; minibodies (Olafsen et al (2004) Protein Eng. Design & Sel.17(4):315-323), fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, CDR (complementary determining region), and epitope-binding fragments of any of the above which immunospecifically bind to cancer cell antigens, viral antigens or microbial antigens, single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

In certain embodiments, the antibody may be a monoclonal antibody. The term “monoclonal antibody” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations which include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies. The modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the subject matter described herein may be made by the hybridoma method first described by Kohler et al (1975) Nature, 256:495, or may be made by recombinant DNA methods (see for example: US 4816567; US 5807715). The monoclonal antibodies may also be isolated from phage antibody libraries using the techniques described in Clackson et al (1991) Nature, 352:624- 628; Marks et al (1991) J. Mol. Biol., 222:581-597; for

example.

In certain embodiments, the antibody may be a native antibody. "Native antibodies" refer to naturally occurring immunoglobulin molecules with varying structures. For example, native IgG antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light chains and two identical heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant domains (CH1, CH2, and CH3). Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain. The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

In certain embodiments, the antibody is an engineered antibody. An "engineered antibody" may be any antibody in which one or more amino acid residues have been introduced, deleted or substituted by means of genetic engineering. The term "engineered antibody" further encompasses "glycoengineered antibodies". "Glycoengineered antibodies" are antibodies in which the composition of the attached glycans has been modified. Modification of the glycans may be achieved, without limitation, chemically or enzymatically. Further, genetically modified host cells are known in the art that may be used for the synthesis of glycoengineered antibodies.

In certain embodiments, the antibody is a human antibody. A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (US 4816567; and Morrison et al (1984) Proc. Natl. Acad. Sci. USA, 81:6851-6855). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g., Old World Monkey, Ape, etc.) and human constant region sequences.

The term “chimeric” antibody refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

A “humanized” antibody refers to a chimeric antibody comprising amino acid residues from non-human HVRs and amino acid residues from human FRs. In certain embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the HVRs (e.g., CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region derived from a human antibody. A “humanized form” of an antibody, e.g., a non-human antibody, refers to an antibody that has undergone humanization.

In other embodiments, the antibody is a polyclonal antibody. The term “polyclonal antibody” as used herein refers to a heterogeneous mixture of antibodies that recognize and bind to different epitopes on the same antigen. Polyclonal antibodies may be obtained from crude serum preparations or may be purified using, for example, antigen affinity chromatography, Protein A/Protein G affinity chromatography, and the like.

In certain embodiments, an antibody provided herein is a multispecific antibody, e.g. a bispecific antibody. The term “multispecific antibody” as used herein refers to an antibody comprising an antigen-binding domain that has polyepitopic specificity (i.e., is capable of binding to two, or more, different epitopes on one molecule or is capable of binding to epitopes on two, or more, different molecules).

In some embodiments, multispecific antibodies are monoclonal antibodies that have binding specificities for at least two different antigen binding sites (such as a bispecific antibody). In some embodiments, the first antigen-binding domain and the second antigen-binding domain of the multispecific antibody may bind the two epitopes within one and the same molecule (intramolecular binding). For example, the first antigen-binding domain and the second antigen-binding domain of the multispecific antibody may bind to two different epitopes on the same protein molecule. In certain embodiments, the two different epitopes that a multispecific antibody binds are epitopes that are not normally bound at the same time by one monospecific antibody, such as e.g. a conventional antibody or one immunoglobulin single variable domain. In some embodiments, the first antigen-binding domain and the second antigen-binding domain of the multispecific antibody may bind epitopes located within two distinct molecules (intermolecular binding). For example, the first antigen-binding domain of the multispecific antibody may bind to one epitope on one protein molecule, whereas the second antigen-binding

domain of the multispecific antibody may bind to another epitope on a different protein molecule, thereby cross-linking the two molecules.

In some embodiments, the antigen-binding domain of a multispecific antibody (such as a bispecific antibody) comprises two VH/VL units, wherein a first VH/VL unit binds to a first epitope and a second VH/VL unit binds to a second epitope, wherein each VH/VL unit comprises a heavy chain variable domain (VH) and a light chain variable domain (VL). Such multispecific antibodies include, but are not limited to, full length antibodies, antibodies having two or more VL and VH domains, and antibody fragments (such as Fab, Fv, dsFv, scFv, diabodies, bispecific diabodies and triabodies, antibody fragments that have been linked covalently or non-covalently). A VH/VL unit that further comprises at least a portion of a heavy chain variable region and/or at least a portion of a light chain variable region may also be referred to as an “arm” or “hemimer” or “half antibody.” In some embodiments, a hemimer comprises a sufficient portion of a heavy chain variable region to allow intramolecular disulfide bonds to be formed with a second hemimer. In some embodiments, a hemimer comprises a knob mutation or a hole mutation, for example, to allow heterodimerization with a second hemimer or half antibody that comprises a complementary hole mutation or knob mutation.

In certain embodiments, a multispecific antibody provided herein may be a bispecific antibody. The term “bispecific antibody” as used herein refers to a multispecific antibody comprising an antigen-binding domain that is capable of binding to two different epitopes on one molecule or is capable of binding to epitopes on two different molecules. A bispecific antibody may also be referred to herein as having “dual specificity” or as being “dual specific.” Exemplary bispecific antibodies may bind both protein and any other antigen. In certain embodiments, bispecific antibodies may bind to two different epitopes of the same protein molecule. In certain embodiments, bispecific antibodies may bind to two different epitopes on two different protein molecules. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express protein.

Bispecific antibodies can be prepared as full-length antibodies or antibody fragments. In certain embodiments, the bispecific antibody fragment is a dutafab, as disclosed by Beckmann et al. (DutaFabs are engineered therapeutic Fab fragments that can bind two targets simultaneously; Nature Communications; 2021; vol.2:708). In certain embodiments, the dutafab may specifically bind to human vascular endothelial growth factor (VEGF/VEGF-A) and human angiopoietin-2 (Ang-2). In certain embodiments, the dutafab may specifically bind to human vascular endothelial growth factor (VEGF/VEGF-A) and to a human platelet-derived growth factor (PDGF).

Engineered antibodies with three or more functional antigen binding sites, including “Octopus antibodies” or “dual-variable domain immunoglobulins” (DVDs) are also included herein (see, e.g., US 2006/0025576A1, and Wu et al. Nature Biotechnology (2007)).). The antibody or fragment herein also includes a “Dual Acting FAb” or “DAF” comprising an antigen binding site that binds to a target protein as well as another, different antigen (see, US 2008/0069820, for example).

The antibody of the present invention may be a naked antibody, a fusion antibody or comprised in an antibody-drug conjugate.

A “naked antibody” refers to an antibody that is not conjugated to a heterologous moiety (e.g., a cytotoxic moiety) or radiolabel.

The term “immunoconjugate” or “antibody drug conjugate” as used herein refers to the linkage of an antibody or an antigen binding fragment thereof with another agent, such as a chemotherapeutic agent, a toxin, an immunotherapeutic agent, an imaging probe, and the like. The linkage can be covalent bonds, or non-covalent interactions such as through electrostatic forces. Various linkers, known in the art, can be employed in order to form the immunoconjugate. Additionally, the immunoconjugate can be provided in the form of a fusion protein that may be expressed from a polynucleotide encoding the immunoconjugate.

By the term “antibody fusion protein” as used herein, is meant a polypeptide molecule having an amino acid sequence which comprises the amino acid sequence of a portion of an antigen-binding protein. The portion of the antigen-binding protein may, for example, be an entire antibody or a fragment thereof. In particular, the antibody fusion protein may comprise a first amino acid sequence of an antibody or a fragment thereof, and a second amino acid sequence of another polypeptide or protein. The second amino acid sequence may for example be an amino acid sequence of a cytokine. The antibody fusion protein may be created through the joining of two or more polynucleotides which originally coded for separate proteins (including peptides and polypeptides). Translation of the fusion gene results in a single protein with functional properties derived from each of the original proteins.

In certain embodiments, the protein is an antibody. Exemplary molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the HER receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mol, p150,95, VLA-4, ICAM-1, VCAM and α v/ β 3 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3

receptor; obesity (OB) receptor; protein C etc.

Exemplary antibodies that may be utilized include, but are not limited to, hRI (anti-IGF-1R, U.S. Patent Application Serial No. 12/722,645, filed 3/12/10), hPAM4 (anti-mucin, U.S. Patent No. 7,282,567), hA20 (anti-CD20, U.S. Patent No. 7,251,164), hA19 (anti-CD19, U.S. Patent No. 7,109,304), WMMU31 (anti-AFP, U.S. Patent No. 7,300,655), hLL1 (anti-CD74, U.S. Patent No. 7,312,318), hLL2 (anti-CD22, U.S. Patent No. 7,074,403), hMu-9 (anti-CSAp, U.S. Patent No. 7,387,773), hL243 (anti-HLA-DR, U.S. Patent No. 7,612,180), hMN-14 (anti-CEACAM5, U.S. Patent No. 6,676,924), hMN-15 (anti-CEACAM6, U.S. Patent No. 7,541,440), hRS7 (anti-EGP-1, U.S. Patent No. 7,238,785), hMN-3 (anti-CEACAM6, U.S. Patent No. 7,541,440), Abl24 and Abl 25 (anti-CXCR4, U.S. Patent No. 7,138,496).

Alternative antibodies of use include, but are not limited to, abciximab (anti-glycoprotein IIb/IIIa), alemtuzumab (anti-CD52), bevacizumab (anti-VEGF), cetuximab (anti-EGFR), gemtuzumab (anti-CD33), ibritumomab tiuxetan (anti-CD20), panitumumab (anti-EGFR), rituximab (anti-CD20), tositumomab (anti-CD20), trastuzumab (anti-ErbB2), abagovomab (anti-CA-125), adecatumumab (anti-EpCAM), atlizumab (anti-IL-6 receptor), benralizumab (anti-CD125), CC49 (anti-TAG-72), AB-PG1-XG1-026 (anti-PSMA, U.S. Patent Application 11/983,372, deposited as ATCC PTA-4405 and PTA-4406), D2/B (anti-PSMA, WO 2009/130575), tocilizumab (anti-IL-6 receptor), basiliximab (anti-CD25), daclizumab (anti-CD25), efalizumab (anti-CD11a), GA101 (anti-CD20; Glycart Roche), muromonab-CD3 (anti-CD3 receptor), natalizumab (anti- α 4 integrin), omalizumab (anti-IgE); anti-TNF- α antibodies such as CDP571 (Ofei et al., 2011, Diabetes 45:881-85), MTNFAL, M2TNFAL, M3TNFAL, M3TNFABI, M302B, M303 (Thermo Scientific, Rockford, IL), infliximab (Centocor, Malvern, PA), certolizumab pegol (UCB, Brussels, Belgium), anti-CD40L (UCB, Brussels, Belgium), adalimumab (Abbott, Abbott Park, IL), Benlysta (Human Genome Sciences); antibodies for therapy of Alzheimer's disease such as Alz 50 (Ksiezak-Reding et al., 1987, J Biol Chem 263:7943-47), gantenerumab, solanezumab and infliximab; anti-fibrin antibodies like 59D8, T2G1s, MH1; anti-HIV antibodies such as P4/D10 (U.S. Patent Application Serial No. 11/745,692), Ab 75, Ab 76, Ab 77 (Paulik et al., 1999, Biochem Pharmacol 58: 1781-90); and antibodies against pathogens such as CR6261 (anti-influenza), exbivirumab (anti-hepatitis B), felvizumab (anti-respiratory syncytial virus), foravirumab (anti-rabies virus), motavizumab (anti-respiratory syncytial virus), palivizumab (anti-respiratory syncytial virus), panobacumab (anti-Pseudomonas), rafivirumab (anti-rabies virus), regavirumab (anti-cytomegalovirus), sevirumab (anti-cytomegalovirus), tivirumab (anti-hepatitis B), and urtoxazumab (anti-E. coli).

In certain embodiments, the antibody may be a bispecific anti-VEGF/anti-angiopoietin-2 (Ang-2) antibody, an anti-alpha synuclein (aSyn) antibody, a bispecific anti-FAP/anti-OX40

antibody, a bispecific anti-VEGF/anti-PDGF antibody (dutafab), Bevacizumab, Pertuzumab or Gantenerumab.

That is, in a certain embodiment, the antibody may be a bispecific anti-VEGF/anti-angiopoietin-2 (Ang-2) antibody. In a certain embodiment, the bispecific anti-VEGF/anti-angiopoietin-2 (Ang-2) antibody may be faricimab, as disclosed in WO2014/009465 as “VEGFang2-0016”. In a certain embodiment, the bispecific anti-VEGF/anti-angiopoietin-2 (Ang-2) antibody may be a dutafab.

In a certain embodiment, the antibody may be an anti-alpha synuclein (aSyn) antibody. In a certain embodiment, the antibody may be a bispecific anti-FAP/anti-OX40 antibody. In a certain embodiment, the antibody may be a dutafab. In a certain embodiment, the dutafab may be a bispecific anti-VEGF/anti-PDGF dutafab. In a certain embodiment, the antibody may be Bevacizumab. In a certain embodiment, the antibody may be Pertuzumab. In a certain embodiment, the antibody may be Gantenerumab.

In another embodiment, the antibody is human or humanized. In one aspect, the antibody is selected from alemtuzumab (LEMTRADA®), atezolizumab (TECENTRIQ®), bevacizumab (AVASTIN®), cetuximab (ERBITUX®), panitumumab (VECTIBIX®), pertuzumab (OMNITARG®, 2C4), trastuzumab (HERCEPTIN®), tositumomab (Bexxar®), abciximab (REOPRO®), adalimumab (HUMIRA®), apolizumab, aselizumab, atlizumab, bapineuzumab, basiliximab (SIMULECT®), bavituximab, belimumab (BENLYSTA®) briankinumab, canakinumab (ILARIS®), cedelizumab, certolizumab pegol (CIMZIA®), cidfusituzumab, cidtuzumab, cixutumumab, clazakizumab, crenezumab, daclizumab (ZENAPAX®), dalotuzumab, denosumab (PROLIA®, XGEVA®), eculizumab (SOLIRIS®), efalizumab, epratuzumab, erlizumab, emicizumab (HEMLIBRA®), felvizumab, fontolizumab, golimumab (SIMPONI®), ipilimumab, imgatuzumab, infliximab (REMICADE®), labetuzumab, lebrikizumab, lexatumumab, lintuzumab, lucatumumab, lulizumab pegol, lumretuzumab, mapatumumab, matuzumab, mepolizumab, mogamulizumab, motavizumab, motovizumab, muronomab, natalizumab (TYSABRI®), necitumumab (PORTRAZZA®), nimotuzumab (THERACIM®), nolovizumab, numavizumab, olokizumab, omalizumab (XOLAIR®), onartuzumab (also known as MetMab), palivizumab (SYNAGIS®), pascolizumab, pecfusituzumab, pectuzumab, pembrolizumab (KEYTRUDA®), pexelizumab, priliximab, ralivizumab, ranibizumab (LUCENTIS®), reslivizumab, reslizumab, resyvizumab, robatumumab, rontalizumab, rovelizumab, ruplizumab, sarilumab, secukinumab, seribantumab, sifalimumab, sibrotuzumab, siltuximab (SYLVANT®) siplizumab, sontuzumab, tadocizumab, talizumab, tefibazumab, tocilizumab (ACTEMRA®), toralizumab, tucusituzumab, umavizumab, urtoxazumab, ustekinumab (STELARA®), vedolizumab

(ENTYVIO®), visilizumab, zanolimumab, zalutumumab.

In a particular embodiment, the invention relates to the method according to the invention, wherein the complexing agent has a negative net charge when comprised in the buffer solution.

Within the present invention, it is preferred that the pH of the buffer solution is adjusted to be lower than the isoelectric point of the protein. At the same time, it is preferred that the complexing agent has a negative charge when comprised in the buffer solution. The complexing agents dextran sulfate and chondroitin sulfate comprise a sulfate group which may be negatively charged when comprised in the buffer solution. In particular, the sulfate group will be negatively charged when the pH of the buffer solution is adjusted to a pH value that is higher than the *pKa* value of the sulfate group of dextran sulfate and/or chondroitin sulfate.

Thus, in a particular embodiment, the invention relates to the method according to the invention, wherein the pH of the buffer solution is adjusted to be lower than the isoelectric point of the protein and higher than the *pKa* value of the buffering agent, in particular wherein the buffering agent is dextran sulfate and/or chondroitin sulfate.

In a particular embodiment, the invention relates to the method according to the invention, wherein the composition comprising the RPCs comprises at least one excipient.

It has been shown by the inventors that the presence of excipients in the buffering solution does not significantly interfere with the formation of reversible protein complexes or the dissolution of these complexes. For example, it has been shown that the commonly used excipients sucrose, polysorbate 20 and poloxamer 188 either alone or in combination do not interfere with reversible protein complex formation and dissolution (Fig. 14 and 15). Thus, the excipient(s) may be added to the buffer solution before the formation of the RPCs, such that the RPCs form in the presence of the excipient(s). Accordingly, in a particular embodiment, the invention relates to the method according to the invention, wherein the at least one excipient is added to the composition before the formation of the RPCs.

Alternatively, the excipient(s) may be added to the suspension after the formation of the RPCs. That is, the RPCs may be formed in the absence of any excipient and the excipients are added to the suspensions subsequently. Thus, in another embodiment, the invention relates to the method according to the invention, wherein the at least one excipient is added to the composition after the formation of the RPCs.

In further embodiments, excipient(s) may be added to the composition of the invention before

and after formation of the RPCs.

In a particular embodiment, the invention relates to the method according to the invention, wherein the at least one excipient is a stabilizer and/or a surfactant.

The term "stabilizer", as used herein, denotes a pharmaceutically acceptable excipient, which protects the protein and/or the composition from chemical and/or physical degradation during manufacturing, storage and application. Stabilizers include but are not limited to saccharides, amino acids, polyols, e.g. mannitol, sorbitol, xylitol, dextran, glycerol, arabitol, propylene glycol, polyethylene glycol, cyclodextrines, e.g. hydroxypropyl- β -cyclodextrine, sulfobutylethyl- β -cyclodextrine, β -cyclodextrine, polyethyleneglycols, e.g. PEG 3000, PEG 3350, PEG 4000, PEG 6000, albumines, e.g. human serum albumin (HSA), bovine serum albumin (BSA), salts, e.g. sodium chloride, magnesium chloride, calcium chloride, chelators, e.g. EDTA as hereafter defined. More than one stabilizer, selected from the same or from different groups, can be present in the composition.

The term "saccharide" as used herein includes monosaccharides and oligosaccharides. A monosaccharide is a monomeric carbohydrate which is not hydrolysable by acids, including simple sugars and their derivatives, e.g. aminosugars. Saccharides are usually in their D conformation. Examples of monosaccharides include glucose, fructose, galactose, mannose, sorbose, ribose, deoxyribose, neuraminic acid. An oligosaccharide is a carbohydrate consisting of more than one monomeric saccharide unit connected via glycosidic bond(s) either branched or in a linear chain. The monomeric saccharide units within an oligosaccharide can be identical or different. Depending on the number of monomeric saccharide units the oligosaccharide is a di-, tri-, tetra- penta- and so forth saccharide. In contrast to polysaccharides the monosaccharides and oligosaccharides are water soluble. Examples of oligosaccharides include sucrose, trehalose, lactose, maltose and raffinose. Preferred saccharides are sucrose and trehalose (i.e. α,α -D-trehalose), most preferred is sucrose.

The term "amino acid" as used herein denotes a pharmaceutically acceptable organic molecule possessing an amino moiety located at α -position to a carboxylic group. Examples of amino acids include but are not limited to arginine, glycine, ornithine, lysine, histidine, glutamic acid, asparagic acid, isoleucine, leucine, alanine, phenylalanine, tyrosine, tryptophane, methionine, serine, proline. The amino acid employed is preferably in each case the L-form. Basic amino acids, such as arginine, histidine, or lysine, are preferably employed in the form of their inorganic salts (advantageously in the form of the hydrochloric acid salts, i.e. as amino acid hydrochlorides).

A subgroup within the stabilizers are lyoprotectants. The term "lyoprotectant" denotes pharmaceutically acceptable excipients, which protect the labile active ingredient (e.g. a protein) against destabilizing conditions during the lyophilisation process, subsequent storage and reconstitution. Lyoprotectants comprise but are not limited to the group consisting of saccharides, polyols (such as e.g. sugar alcohols) and amino acids. Preferred lyoprotectants can be selected from the group consisting of saccharides such as sucrose, trehalose, lactose, glucose, mannose, maltose, galactose, fructose, sorbose, raffinose, neuraminic acid, amino sugars such as glucosamine, galactosamine, N-methylglucosamine ("Meglumine"), polyols such as mannitol and sorbitol, and amino acids such as arginine and glycine or mixtures thereof.

A subgroup within the stabilizers are antioxidants. The term "antioxidant" denotes pharmaceutically acceptable excipients, which prevent oxidation of the active pharmaceutical ingredient. Antioxidants comprise but are not limited to ascorbic acid, glutathione, cysteine, methionine, citric acid, EDTA.

The composition according to the invention may also comprise one or more tonicity agents. The term "tonicity agents" denotes pharmaceutically acceptable excipients used to modulate the tonicity of the composition. The composition may be hypotonic, isotonic or hypertonic. Isotonicity in general relates to the osmotic pressure of a solution, usually relative to that of human blood serum (around 250-350 mOsmol/kg). The composition according to the invention may be hypotonic, isotonic or hypertonic but will preferably be isotonic. An isotonic composition is liquid or liquid reconstituted from a solid form, e.g. from a lyophilized form, and denotes a solution having the same tonicity as some other solution with which it is compared, such as physiologic salt solution and the blood serum. Suitable tonicity agents comprise but are not limited to sodium chloride, potassium chloride, glycerin and any component from the group of amino acids or sugars, in particular glucose.

Within the stabilizers and tonicity agents there is a group of compounds which can function in both ways, i.e. they can at the same time be a stabilizer and a tonicity agent. Examples thereof can be found in the group of sugars, amino acids, polyols, cyclodextrines, polyethyleneglycols and salts. An example for a sugar which can at the same time be a stabilizer and a tonicity agent is sucrose.

The compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, and by the inclusion of various antibacterial and antifungal agents, e.g. paraben, chlorobutanol, phenol, sorbic acid, and the like. Preservatives comprise but are not limited to ethanol, benzyl alcohol, phenol, m-cresol, p-chlor-m-cresol,

methyl or propyl parabens, benzalkonium chloride.

The term "surfactant" as used herein denotes a pharmaceutically acceptable, surface-active agent. Preferably, a non-ionic surfactant is used. Examples of pharmaceutically acceptable surfactants include, but are not limited to, polyoxyethylen-sorbitan fatty acid esters (Tween), polyoxy ethylene alkyl ethers (Brij), alkylphenylpolyoxyethylene ethers (Triton X), polyoxyethylene-polyoxypropylene copolymers (Poloxamer, Pluronic), and sodium dodecyl sulphate (SDS). Preferred polyoxyethylene-sorbitan fatty acid esters are polysorbate 20 (polyoxy ethylene sorbitan mono laureate, sold under the trademark Tween 20™) and polysorbate 80 (polyoxy ethylene sorbitan monooleate, sold under the trademark Tween 80™). Preferred polyethylene-polypropylene copolymers are those sold under the names Pluronic® F68 or Poloxamer 188™. Preferred polyoxyethylene alkyl ethers are those sold under the trademark Brij™. Preferred alkylphenylpolyoxyethylene ethers are sold under the tradename Triton X, most preferred is p-tert-octylphenoxy polyethoxyethanol (sold under the tradename Triton X- 100™).

It is to be understood that the stabilizers and surfactants listed above may be added to the compositions of the invention at a concentration that is sufficient to obtain the intended effect. The skilled person is aware of these concentrations or, alternatively, is able to determine these concentrations by routine experimentation.

A preferred stabilizer in the compositions of the present invention is sucrose. Sucrose is commonly used as a stabilizer in pharmaceutical compositions due to its protein-stabilizing properties. In certain embodiments, sucrose is added to the composition of the invention before the RPCs are formed. That is, sucrose is added to the buffer solution before the protein and the complexing agent are contacted in said buffer solution. In certain embodiments, sucrose is present in the buffer solution at a concentration ranging from 50 to 500 mM, preferably ranging from 100 to 250 mM.

In other embodiments, sucrose may be added to the composition after the formation of the RPCs. That is, in certain embodiments, sucrose may be added to a suspension before a spray drying or lyophilization step. In certain embodiments, sucrose may be added to a suspension according to the invention at a concentration ranging from 0.5 to 10 mg/mL, ranging from 0.5 to 5 mg/mL, or ranging from 1 to 3 mg/mL.

Preferred solubilizers in the compositions of the present invention are poloxamer 188 and polysorbate 20. Poloxamer 188 and polysorbate 20 are commonly used as solubilizers in pharmaceutical compositions. In certain embodiments, poloxamer 188 and/or polysorbate 20

are added to the composition of the invention before the formation of RPCs. That is, poloxamer 188 and/or polysorbate 20 may be added to the buffer solution before the contacting of the protein and the complexing agent in said buffer solution. In certain embodiments, poloxamer 188 and/or polysorbate 20 are present in the buffer solution at a concentration ranging from 0.01 to 1% (w/v), ranging from 0.01 to 0.5% (w/v), ranging from 0.01 to 0.1% (w/v), or ranging from 0.02 to 0.06% (w/v).

In other embodiments, poloxamer 188 and/or polysorbate 20 may be added to the composition, in particular any suspension of the present invention, after the formation of the RPCs. That is, in certain embodiments, poloxamer 188 and/or polysorbate 20 may be added to a composition comprising RPCs or an enriched RPC suspension before the spray drying step. In certain embodiments, poloxamer 188 and/or polysorbate 20 may be added to a suspension according to the invention at a concentration ranging from 0.1 to 2 mg/mL, ranging from 0.1 to 1 mg/mL, or ranging from 1 to 0.5 mg/mL.

In certain embodiments, the suspension comprising RPCs or the enriched RPC suspension is free of stabilizers and/or solubilizers. That is, in certain embodiments, the suspension comprising RPCs or the enriched RPC suspension comprises one or more stabilizer but is free of solubilizers. In other embodiments, the suspension comprising RPCs or the enriched RPC suspension comprises one or more solubilizers but is free of stabilizers. In further embodiments, the suspension comprising RPCs or the enriched RPC suspension is free of solubilizers and stabilizers.

In a particular embodiment, the invention relates to the method according to the invention, wherein the method comprises a further step of exchanging the liquid fraction of the suspension comprising the RPCs.

That is, the liquid fraction of the suspension comprising the RPCs may be replaced with another liquid. The skilled person is aware of methods for exchanging the liquid fraction of a suspension.

For example, the liquid fraction of the suspension comprising the RPCs may be exchanged by centrifugation. That is, the suspension comprising the RPCs may be centrifuged at an appropriate speed to facilitate sedimentation of the RPCs. Subsequently, the sedimented RPCs may be resuspended in another liquid. The centrifugation and resuspension steps may be repeated 1, 2, 3, 4, 5 or more times. Preferably, the sedimented RPCs are resuspended in a buffer solution or in water.

Thus, in a particular embodiment, the invention relates to the method according to the invention, wherein the liquid fraction of the suspension comprising the RPCs is exchanged by centrifugation of the suspension comprising the RPCs and resuspension of the sedimented RPCs in a buffer solution or water.

Alternatively, the liquid fraction of the suspension comprising the RPCs may be exchanged by dialysis. Dialysis may be performed, without limitation, in a dialysis cartridge or in a dialysis tube. The skilled person is able to select a suitable molecular weight cutoff of the dialysis cartridge or the dialysis tube based on the size of the protein comprised in the RPC, such that the RPCs are retained in the dialysis cartridge or tube. Preferably, the dialysis cartridge or tube may have a molecular weight cutoff ranging from 10 to 10,000 kDa. Dialysis of the RPCs may be performed against any liquid, preferably against a buffer solution or water. The dialysis step may be repeated 1, 2, 3, 4 or 5 times.

Thus, in a particular embodiment, the invention relates to the method according to the invention, wherein the liquid fraction of the suspension comprising the RPCs is exchanged by dialysis of the suspension comprising the RPCs against a buffer solution or water.

In certain embodiments, the liquid fraction of the suspension comprising the RPCs is exchanged with a buffer solution. In certain embodiments, the buffer solution may have a similar or identical composition as the buffer solution in which the protein and/or the complexing agent have been dissolved before the formation of the RPCs. That is, in certain embodiments, the liquid fraction of the suspension comprising the RPCs may be exchanged with a fresh buffer solution. A fresh buffer solution is a buffer solution which does not comprise a protein, a complexing agent and/or an RPC. In certain embodiments, the fresh buffer solution may be any of the buffer solutions disclosed herein. In certain embodiments, the liquid fraction of the suspension comprising the RPCs may be exchanged with 20 mM histidine buffer (pH 5).

In certain embodiments, the liquid fraction of the suspension comprising the RPCs is exchanged with water. It has been surprisingly shown by the inventors that dialysis of RPCs against ultrapure water results in very small particles with diameters in the nanometer range. Such small RPCs are particularly attractive for the administration to patients through a syringe. That is, in one preferred embodiment, RPCs are dialyzed against ultrapure water.

The term “water” refers to the chemical compound having the chemical formula H_2O . Within the meaning of the present invention, water is free or substantially free of solutes. Preferably, the water used in the method of the invention is distilled water and/or deionized water.

In certain embodiments, the water used in the method of the invention is ultrapure water. The term "ultrapure water" as used herein means water from which impurities have been removed as much as possible and which has a specific resistance of $16 \text{ M}\Omega\cdot\text{cm}$ or above. In certain embodiments, the term "ultrapure water" means water with a specific resistance of at least $17 \text{ M}\Omega\cdot\text{cm}$. In certain embodiments, the term "ultrapure water" means water with a specific resistance of at least $18 \text{ M}\Omega\cdot\text{cm}$. The term ultrapure water encompasses Ultra pure water (MilliQ water).

In a particular embodiment, the invention relates to the method according to the invention, wherein the method comprises a further step of enriching the RPCs in the suspension to obtain an enriched RPC suspension.

The method of the present invention may comprise a further step of enriching the RPCs in the suspension. That is, in a particular embodiment, the invention relates to the method according to the invention, wherein enriching the RPCs in the suspension comprises the steps of: (a) centrifuging the suspension comprising the RPCs to obtain a supernatant and a precipitate comprising an enriched RPC suspension; and (b) removing the supernatant from the precipitate to obtain an enriched RPC suspension.

The RPCs in the suspension may be enriched by any method known in the art. Preferably, the RPCs in the suspension are enriched by centrifugation. The RPCs in the suspension have a higher density than the soluble components of the suspension. Thus, centrifugation of the suspension will result in the migration of the RPCs towards the bottom of the centrifugation vessel. Exhaustive centrifugation will consequently result in the formation of a precipitate or pellet comprising the RPCs at the bottom of the vessel and of a supernatant that is substantially free of RPCs. The supernatant is said to be "substantially free" of RPC if, after the centrifugation step, less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5%, less than 4%, less than 3%, less than 2%, or less than 1% of the RPCs in a sample are comprised in the supernatant. The skilled person is aware that the concentration of RPCs in the supernatant depends on the duration and the speed of the centrifugation step.

To enrich the RPCs in the suspension, the supernatant may be partially or completely removed after the centrifugation step. That is, in certain embodiments, the supernatant is partially removed from the centrifugation vessel and the precipitate is subsequently re-suspended in the remaining supernatant to obtain an enriched suspension. In other embodiments, the supernatant may be removed completely from the centrifugation vessel and the precipitate may be re-suspended in a liquid that is added to the centrifugation vessel. In certain embodiments, this liquid has a lower volume than the supernatant that has been removed in the first step. The

liquid in which the precipitate is resuspended may be identical to the buffer solution in which the RPCs have been formed or may be different from the buffer solution in which the RPCs have been formed.

The term “centrifugation” as used herein refers to the rotation in a compartment of an apparatus, said compartment spun about an axis for the purpose of separating materials. The term “precipitate”, as used herein, refers to any solid or semisolid material capable of being physically separated from the fluid portion of the suspension. The term “supernatant,” as used herein, describes the fluid portion of the suspension, after particles, such as RPCs, have settled to the bottom of the vessel.

Besides centrifugation, other methods may be used for obtaining an enriched suspension comprising RPCs. For example, in certain embodiments, an enriched suspension may be obtained by letting the RPCs in the suspension settle by gravity and decanting the supernatant. In other embodiments, an enriched suspension comprising RPCs may be obtained by filtration and/or dialysis of the suspension comprising RPCs.

The skilled person is aware of means of centrifugation to obtain enriched RPC suspensions. Further, the skilled person is aware that the concentration of RPCs in the enriched RPC suspensions depends at least on the centrifugation speed, the centrifugation time and the volume of the liquid the RPCs are re-suspended in. Thus, the skilled person is able to adjust the centrifugation method such that an enriched RPC suspension with a desired protein concentration can be obtained.

Within the present invention, the RPCs in the suspension may be enriched to obtain a protein concentration above 50 mg/mL. Preferably, the suspension comprising RPCs may be enriched to obtain a protein concentration between 50 and 300 mg/mL. More preferably, the suspension comprising RPCs may be enriched to obtain a protein concentration between 50 and 250 mg/mL. Most preferably, the suspension comprising RPCs may be enriched to obtain a protein concentration between 100 and 250 mg/mL.

It has to be noted that the enrichment step may be performed before or after exchanging the liquid fraction of the suspension comprising the RPCs. That is, in certain embodiments, the liquid fraction of the suspension comprising the RPCs may be exchanged first and the RPCs in the obtained suspension are enriched subsequently. For example, the liquid fraction of the suspension comprising the RPCs may be dialysed against ultrapure water in a first step and the RPCs in the resulting suspension may then be enriched to a desired concentration by centrifugation in a second step.

In certain embodiments, RPCs in a suspension may first be enriched to a desired concentration and the liquid fraction of the enriched suspension may then be exchanged by centrifugation and resuspension or by dialysis in a second step.

In certain embodiments, RPCs may be enriched while the liquid fraction of the suspension comprising the RPCs is exchanged. That is, RPCs may be sedimented by centrifugation and subsequently resuspended in a smaller volume of a fresh buffer solution or ultrapure water. Optionally, the RPCs may be washed one or multiple times before the final resuspension in a smaller volume.

In a particular embodiment, the invention relates to the method according to the invention, wherein the liquid fraction of the enriched RPC suspension is at least in part replaced with a non-aqueous solvent during the enrichment step.

After the centrifugation step, the supernatant may be partially or completely removed from the centrifugation vessel and replaced with a non-aqueous solvent. That is, in certain embodiments, the supernatant may be removed completely after the centrifugation step and the precipitate comprising the RPCs may be resuspended in a non-aqueous solvent. In other embodiments, the supernatant may be removed partially after the centrifugation step and the precipitate may be resuspended in a mixture of the remaining supernatant and a non-aqueous solvent.

In a particular embodiment, the invention relates to the method according to the invention, wherein the non-aqueous solvent is any one of Table 4, but preferably triacetin, diethylene glycol monoethyl ether or ethyl oleate.

In a particular embodiment, the invention relates to the method according to the invention, wherein the method comprises a further step of lyophilizing the suspension comprising the RPCs or the enriched RPC suspension to obtain a lyophilisate.

In an additional step, the suspension comprising the RPCs or the enriched RPC suspension may be lyophilized. It has been shown by the inventors that lyophilized RPCs can be reconstituted such that they efficiently dissociate after storage at 5°C, 25°C or 40°C for at least 4 weeks (Table 12). In addition, lyophilized RPCs remained stable for at least 4 weeks without significant formation of protein aggregates or degradation of the protein (Table 13). Thus, lyophilisates that have been obtained from suspensions comprising RPCs or from enriched RPC suspensions are suitable for storing therapeutic proteins for longer time periods without the need for an intact cooling chain.

The term "lyophilization" as used herein refers to a "freeze-dry" process comprising the conversion of water from a frozen state to a gaseous state without going through a liquid state. The freeze-dry process removes moisture from a water-containing material while the material remains frozen. The basic process of lyophilization comprises the following steps: freezing, primary drying (sublimation), and secondary drying (desorption). At first, a dissolved and/or suspended substance is frozen at a low temperature (for example, -60°C). Slow freezing produces larger crystals which allow the sublimating material to escape. Some products form a glassy material and annealing may be required during the freezing process. Annealing, first lowering the temperature then raising the temperature and then lowering it again, locks the constituents in place and then allows the crystals to grow. Freezing can range from 1 hour to 24 hours, depending on the application. In step 2 (primary drying) the water or diluent is then extracted via vacuum, resulting in a porous, dry "cake". Sublimation occurs under vacuum with the product temperature below its critical temperature. This is typically the longest process. At the end of the primary drying cycle, the product will have 3 to 5 % moisture content. There is a final drying step (secondary drying) to remove residual unfrozen water molecules. This is done by heating the product. Secondary drying can result in moisture levels of about 0.5 %. The term "lyophilisate" refers to the freeze-dried product of a lyophilisation step.

In a particular embodiment, the invention relates to the method according to the invention, wherein at least one cryoprotectant is added to the suspension comprising the RPCs or the enriched RPC suspension before the lyophilisation step.

To protect the proteins comprised in the RPCs from damage during the lyophilisation step, a cryoprotectant may be added to the suspension before the lyophilisation step.

The term "cryoprotectant" is herein used analogously with the term "lyoprotectant" to describe molecules that protect freeze-dried material. Known as lyoprotectants, these molecules are typically polyhydroxy compounds such as sugars (mono-, di-, and polysaccharides), polyalcohols, and their derivatives. Trehalose and sucrose are natural lyoprotectants. The term "lyoprotectant" as used herein, includes agents that provide stability to a biologically active compound during the drying process, e.g., by providing an amorphous glassy matrix and by binding with a protein through hydrogen bonding, replacing the water molecules that are removed during the drying process. This helps to maintain a protein's conformation, minimize protein degradation during the drying cycle, and improve the long-term product stability. Non-limiting examples of lyoprotectants include sugars, such as sucrose or trehalose; an amino acid, such as monosodium glutamate, non-crystalline glycine or histidine; a methylamine such, as betaine; a lyotropic salt, such as magnesium sulfate; a polyol, such as trihydric or higher sugar alcohols, e.g., glycerin, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene

glycol; polyethylene glycol; pluronics; and combinations thereof. The amount of lyoprotectant added to a formulation is generally an amount that does not lead to an unacceptable amount of degradation/aggregation of the protein when the protein formulation is dried. Thus, in a particular embodiment, the invention relates to the method according to the invention, wherein the at least one cryoprotectant is selected from a group consisting of: sugars, amino acids, methylamines, lyotropic salts, polyols, propylene glycol, polyethylene glycol and pluronics. In certain embodiments, the cryoprotectant is a sugar, in particular sucrose.

In a particular embodiment, the invention relates to the method according to the invention, wherein the protein concentration of the suspension comprising the RPCs or the enriched RPC suspension is adjusted to 10 to 100 mg/mL, in particular to 40 to 80 mg/mL, prior to the lyophilisation step.

The lyophilization step may be performed with any suspension. It has been demonstrated by the inventors that lyophilizing enriched RPC suspensions with a protein concentration of 60 mg/mL does not significantly reduce the stability and/or the dissociation efficiency of the RPCs. Thus, it is preferred that the lyophilization step is performed with a suspension comprising RPCs in which the protein concentration is adjusted to 10 – 100 mg/mL. More preferably, the protein concentration in the suspension comprising the RPCs is adjusted to 40 – 80 mg/mL. Most preferably, the protein concentration in the suspension comprising the RPCs is adjusted to 60 mg/mL.

In a particular embodiment, the invention relates to the method according to the invention, wherein the method comprises a further step of spray drying the suspension comprising the RPCs or the enriched RPC suspension to obtain a spray dried powder.

Besides lyophilization, the suspension comprising RPCs or the enriched RPC suspension may be spray dried to obtain a solid composition, in this case a spray dried powder.

The term "spray-drying", as used herein, refers to a method of producing a dry powder comprising micron-sized particles from a solution or suspension by using a spray-dryer. Spray-drying is, in principle, a solvent extraction process. The constituents of the product to be obtained are dissolved/dispersed in a liquid and then fed, for example by using a peristaltic pump, to an atomiser of a spray-dryer. A suitable atomizer which can be used for atomization of the liquid, include nozzles or rotary discs. With nozzles, atomization occurs due to the action of the compressed gas, while in case of using rotary discs atomization occurs due to the rapid rotation of the disc. In both cases, atomization leads to disruption of the liquid into small droplets into the drying chamber, wherein the solvent is extracted from the aerosol droplets and

is discharged out, for example through an exhaust tube to a solvent trap.

Drop sizes from 1 to 500 pm may be generated by spray-drying. As the solvent (water or organic solvent) dries, the nanoparticles-containing droplets dries into a micron-sized particle, forming powder-like particles.

A number of commercially available spray drying machines can be used to prepare the composition of the invention, for example, suitable machines are manufactured by Buchi and Niro. Examples of suitable spray-driers include lab scale spray-dryers from Buchi, such as the Mini Spray Dryer 290, or a MOBILE MINOR™, or a Pharma Spray Dryer PharmaSD® from Niro, or a 4M8-TriX from Procept NV.

In a typical spray drying machine the suspension to be dried is pumped from a stirred reservoir to an atomization chamber where it is sprayed from a nozzle as fine droplets into a stream of heated air, for example, inlet temperatures in the range of 50 to 250° C (nitrogen can be used in place of air if there is a risk of undesirable oxidation of the product). The temperature of the heated air must be sufficient to evaporate the liquid and dry the microparticles to a free flowing powder but should not be so high as to degrade the product. The microparticles may be collected in a cyclone or a filter or a combination of cyclones and filters.

The suspension comprising RPCs or the enriched RPC suspension may be adjusted to a specific protein concentration before the spray-drying step. That is, in a particular embodiment, the invention relates to the method according to the invention, wherein the protein concentration of the suspension comprising the RPCs or the enriched RPC suspension is adjusted to 1 to 10 mg/mL, in particular to 1 to 5 mg/mL, prior to the spray drying step.

It has been shown by the inventors that the protein concentration in the suspension has an influence on the size of the spray dried particles. Particles with a smaller size may facilitate injectability of the spray-dried powder when resuspended in a liquid. Thus, in certain embodiments, it is preferred that the protein concentration of the suspension comprising the RPCs or the enriched RPC suspension is lower than 10 mg/mL, 9 mg/mL, 8 mg/mL, 7 mg/mL, 6 mg/mL, 5 mg/mL, 4 mg/mL, 3 mg/mL, 2 mg/mL or 1 mg/mL.

In a particular embodiment, the invention relates to the method according to the invention, wherein the liquid fraction of the suspension comprising the RPCs or the enriched RPC suspension is exchanged prior to the spray drying step.

That is, the liquid phase of the suspension comprising RPCs or the enriched RPC suspension

may be exchanged before the spray-drying step. Exchange of the liquid phase of the suspension may be achieved, without limitation, by dialysis.

The term "dialysis" as used herein refers to the diffusion of dissolved solutes across a selectively permeable membrane against a concentration gradient in an effort to achieve equilibrium. While small solutes pass through the membrane larger solutes and particles, such as RPCs, are trapped on one side. By exchanging the dialysate buffer on the outside side of the membrane, smaller solutes can be continuously removed to purify the trapped larger molecules.

Several rounds of dialysis may be used for buffer exchange. In general, dialysis will be most effective when the buffer is replaced multiple times, for example 2, or 3 times, and then preferably left overnight at room temperature on a stir plate. A standard protocol for dialysis is 16 to 24 hours. Many factors affect the dialysis rate, including: diffusion coefficients, pH, temperature, time, concentration of species, sample volume, dialysate (buffer) volume, number of dialysate changes, membrane surface area, membrane thickness, molecular charges and dialysate agitation (stirring). Several types of membranes for dialysis are commercially available and are well known in the art. Illustrative non limiting examples are Polyvinylidene Difluoride (PVDF) membranes, cellulose ester (CE) membranes and regenerated cellulose (C) membranes.

In a particular embodiment, the invention relates to the method according to the invention, wherein exchanging the liquid fraction of the suspension comprising the RPCs or the enriched RPC suspension reduces the concentration of at least one buffering agent, complexing agent, stabilizer and/or solubilizer in the suspension.

In certain embodiments, it is desirable that the protein content in the spray dried powder is as high as possible. Thus, it may be desired to reduce the non-protein components in the suspension before the spray drying step.

It has been demonstrated by the inventors in Example 2.2 that a spray dried powder wherein the integrity of the protein is not significantly compromised may be obtained in the absence of a buffering agent. Thus, in a particular embodiment, the invention relates to the method according to the invention, wherein the suspension comprising the RPCs or the enriched RPC suspension is substantially free of buffering agent after exchanging the liquid fraction of the suspension. In certain embodiments, the RPCs may be dialysed against ultrapure water before the spray drying step.

It is to be understood that due to the dilution effect during dialysis the buffering agent can never

be completely removed from the liquid fraction of the suspension. Thus, a suspension is said to be substantially free of buffering agent if the concentration of the buffering agent in the suspension before the spray-drying step is below 5 mM, below 4 mM, below 3 mM, below 2 mM, below 1 mM, below 0.5 mM, below 0.1 mM or below 0.01 mM.

In a particular embodiment, the invention relates to the method according to the invention, wherein exchanging the liquid fraction of the suspension comprising the RPCs or the enriched RPC suspension reduces the concentration the buffering agent in the suspension comprising the RPCs or the enriched RPC suspension by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90, at least 95% or at least 99%.

Further, it has been demonstrated in Example 2.2 that the protein content in the spray dried powder can be increased by reducing the concentration of the complexing agent, the surfactant and/or the stabilizer in the suspension before the spray drying step without compromising the stability of the protein.

That is, in certain embodiments, the invention relates to the method according to the invention, wherein the liquid fraction of the suspension comprising the RPCs or the enriched RPC suspension is exchanged before the spray-drying step to reduce the concentration of the complexing agent in the suspension comprising the RPCs or the enriched RPC suspension by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90, at least 95% or 100%.

In a preferred embodiment, the invention relates to the method according to the invention, wherein the liquid fraction of the suspension comprising the RPCs or the enriched RPC suspension is exchanged before the spray-drying step to reduce the concentration of the complexing agent in the suspension comprising the RPCs or the enriched RPC suspension by 20 – 50%, more preferably by 30 – 40%, most preferably by 33%.

In further embodiments, the invention relates to the method according to the invention, wherein the liquid fraction of the suspension comprising the RPCs or the enriched RPC suspension is exchanged before the spray-drying step to reduce the concentration of the stabilizer in the suspension comprising the RPCs or the enriched RPC suspension by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or 100%. In a preferred embodiment, the invention relates to the method according to the invention, wherein the liquid fraction of the suspension comprising the RPCs or the enriched RPC suspension is exchanged before the spray-drying step to reduce the concentration of the stabilizer in the suspension comprising the RPCs or the enriched RPC suspension by 30

– 70%, more preferably by 40 – 60%, most preferably by 50%.

In further embodiments, the invention relates to the method according to the invention, wherein the liquid fraction of the suspension comprising the RPCs or the enriched RPC suspension is exchanged before the spray-drying step to reduce the concentration of the solubilizer in the suspension comprising the RPCs or the enriched RPC suspension by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or 100%. In a preferred embodiment, the invention relates to the method according to the invention, wherein the liquid fraction of the suspension comprising the RPCs or the enriched RPC suspension is exchanged before the spray-drying step to reduce the concentration of the stabilizer in the suspension comprising the RPCs or the enriched RPC suspension by 30 – 70%, more preferably by 40 – 60%, most preferably by 50%.

Consequently, reducing the complexing agent in the suspension will result in a reduced mole-charge ratio between the protein and the complexing agent. Thus, in a particular embodiment, the invention relates to the method according to the invention, wherein the liquid fraction of the suspension is exchanged before the spray-drying step to obtain a mole-charge ratio between the protein and the complexing agent between 1:0.2 to 1:1, in particular between 1:0.4 to 1:0.8.

In a preferred embodiment, the invention relates to the method according to the invention, wherein the liquid fraction of the suspension is exchanged before the spray-drying step to obtain a mole-charge ratio between the protein and the complexing agent chondroitin sulfate between 1:0.2 to 1:1, more preferably between 1:0.2 to 1:0.6, even more preferably between 1:0.2 to 1:0.4, most preferably of about 1:0.2.

In another preferred embodiment, the invention relates to the method according to the invention, wherein the liquid fraction of the suspension is exchanged before the spray-drying step to obtain a mole-charge ratio between the protein and the complexing agent dextran sulfate between 1:0.2 to 1:1, more preferably between 1:0.4 to 1:0.8, even more preferably between 1:0.5 to 1:0.7, most preferably of about 1:0.6.

The spray dried powder may be obtained at any condition that does not result in aggregation or damaging of the proteins comprised in the RPCs. The skilled person is capable of optimizing spray drying conditions to prevent damaging and/or aggregation of the proteins by routine experimentation and is aware of methods to determine the stability of the proteins in the RPCs subsequent to the spray drying step (see Examples 1.2.8 and 2.2)

It has been demonstrated by the inventors in Example 2.2 that spray drying at an inlet

temperature of 115°C, an outlet temperature of 48°C and a flow rate of 17 mL/min results in the formation of a spray dried powder comprising RPCs, wherein the proteins comprised in the RPCs are stable.

Accordingly, spray drying may be performed at an inlet temperature ranging from 50 – 250°C, preferably 100 - 200°C, more preferably 100 - 150°C, even more preferably 100 – 130°C, most preferably 115°C.

Further, spray drying may be performed at an outlet temperature ranging from 40 – 150°C, preferably 40 - 100°C, more preferably 40 - 80°C, even more preferably 40 – 60°C, most preferably 48°C.

Further, spray drying may be performed at a flow rate ranging from 1 – 35 mL/min, preferably 5 – 30 mL/min, more preferably 10 – 25 mL/min, even more preferably 15 – 20 mL/min, most preferably 17 mL/min.

In a particular embodiment, the invention relates to the method according to the invention, wherein spray drying is performed at an inlet temperature ranging from 90 - 250°C, an outlet temperature ranging from 40 - 150°C and/or a flow rate ranging from 1 – 35 mL/min.

In a preferred embodiment, the invention relates to the method according to the invention, wherein spray drying is performed at an inlet temperature ranging from 100 - 200°C, an outlet temperature ranging from 40 - 100°C and/or a flow rate ranging from 5 – 30 mL/min.

In a more preferred embodiment, the invention relates to the method according to the invention, wherein spray drying is performed at an inlet temperature ranging from 100 - 150°C, an outlet temperature ranging from 40 - 80°C and/or a flow rate ranging from 10 – 25 mL/min.

In an even more preferred embodiment, the invention relates to the method according to the invention, wherein spray drying is performed at an inlet temperature ranging from 100 - 130°C, an outlet temperature ranging from 40 - 60°C and/or a flow rate ranging from 15 – 20 mL/min.

In an most preferred embodiment, the invention relates to the method according to the invention, wherein spray drying is performed at an inlet temperature of 115°C, an outlet temperature of 48°C and/or a flow rate of 17 mL/min.

In a particular embodiment, the invention relates to the method according to the invention, wherein spray drying is performed at an inlet temperature 115°C and/or an outlet temperature

of 48°C.

In a particular embodiment, the invention relates to the method according to the invention, wherein spray drying is performed at a feed rate of 17 mL/min.

In a particular embodiment, the invention relates to the method according to the invention, wherein the method comprises a further step of re-suspending the spray dried powder in a non-aqueous solvent (NAS) to obtain an RPC-NAS suspension.

That is, the spray dried powder comprising the RPCs may be resuspended in a non-aqueous solvent (NAS) to obtain an RPC-NAS suspension. It has been demonstrated by the inventors in Example 2.3 that spray dried powders comprising RPCs can be resuspended in non-aqueous solvents to obtain a suspension with high protein concentration. In particular, it has been demonstrated that the viscosity of RPC-NAS suspensions is significantly lower compared to the suspensions that have been obtained by re-suspending spray-dried RPCs in aqueous solvents.

The term “RPC-NAS suspension” refers to a heterogeneous mixture comprising solid particles, in this case RPCs, and a liquid phase. The liquid phase consists of or comprises at least one non-aqueous solvent.

In a particular embodiment, the invention relates to the method according to the invention, wherein the non-aqueous solvent is at least one selected from a group consisting of: diethylene glycol monoethyl ether, ethyl oleate, triacetin, isosorbide dimethyl ether and glycofurol.

The non-aqueous solvent in which the spray-dried RPCs are re-suspended may be any non-aqueous solvent known in the art that retains the protein in a stable and non-aggregated form and does not interfere with subsequent dissociation of the RPCs at physiological conditions. Further, it is preferred that the NAS is a NAS that is contained in the EU and/or US pharmacopeia.

Diethylene glycol monoethyl ether (CAS Number 111-90-0; $\text{C}_2\text{H}_5\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OH}$), also known under the trade name Transcutol®, is a liquid which has a long history of use in cosmetic and over-the-counter topically applied products. Transcutol® has been applied in several commercial preparations and is used in many studies as the main ingredient of formulations.

Ethyl oleate (CAS Number 111-62-6, $\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOC}_2\text{H}_5$) is a fatty acid ester

formed by the condensation of oleic acid and ethanol. Ethyl oleate is used as a solvent for pharmaceutical drug preparations involving lipophilic substances such as steroids. It also finds use as a lubricant and a plasticizer.

The triglyceride 1,2,3-triacetoxyp propane (CAS Number 102-76-1, $C_9H_{14}O_6$) is more generally known as triacetin, glycerin triacetate or 1,2,3-triacetyl glycerol. It is the triester of glycerol and acetylating agents, such as acetic acid and acetic anhydride. It is an artificial chemical compound, commonly used as a food additive, for instance as a solvent in flavourings, and for its humectant function, with E number E1518 and Australian approval code A1518. It is used as an excipient in pharmaceutical products, where it is used as a humectant, a plasticizer, and as a solvent.

Isosorbide dimethyl ether (CAS Number 5306-85-4; $C_8H_{14}O_4$) is a sustainable solvent that is widely used in various cosmetic and pharmaceutical formulation.

Glycofurol (CAS Number: 31692-85-0), also known as tetraglycol or tetrahydrofurfuryl alcohol polyethyleneglycol ether, is a non-aqueous solvent that is frequently used as a solvent in parenteral products for intravenous or intramuscular injection.

It has been demonstrated by the inventors in Example 2.3 that transcitol, ethyl oleate and triacetin are particularly suitable for the production of RPC-NAS suspensions, as they result in high protein stability and only low levels of protein aggregation. Thus, in a preferred embodiment, the invention relates to the method according to the invention, wherein the non-aqueous solvent is at least one selected from a group consisting of: diethylene glycol monoethyl ether, ethyl oleate and triacetin.

In a particular embodiment, the invention relates to the method according to the invention, wherein the spray dried powder is resuspended to obtain an RPC-NAS suspension with a protein concentration ranging from 50 to 300 mg/mL, preferably 100 – 250 mg/mL.

The skilled person is aware of methods for producing RPC-NAS suspensions with specific protein concentrations. In particular, RPC-NAS suspensions with a specific protein concentration may be produced by re-suspending a defined amount of spray dried RPCs in a defined amount of a non-aqueous solvent.

In another aspect, the invention relates to a composition comprising reversible protein complexes (RPCs), wherein the composition is obtained by the method according to the invention.

That is, the invention relates to a composition comprising RPCs that has been obtained with any of the methods described above in any combination. In particular, the invention relates to a composition comprising RPCs, wherein the RPCs comprise the complexing agent dextran sulfate and/or chondroitin sulfate.

Thus, in another aspect, the invention relates to a composition comprising reversible protein complexes (RPCs), wherein the RPCs comprise a protein and a complexing agent, and wherein the complexing agent is dextran sulfate or chondroitin sulfate. In a preferred embodiment, the invention relates to the composition according to the invention, wherein the complexing agent is dextran sulfate, in particular dextran sulfate with 40 kDa molecular weight.

That is, the complexing agent may be any one of the complexing agents that have been disclosed above.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the protein is an antibody, a growth factor, a hormone, a cytokine, an enzyme, or a fragment and/or fusion protein of any of the foregoing.

Further, the protein comprised in the RPCs may be any one of the proteins that have been disclosed above.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the antibody is a monoclonal antibody, a polyclonal antibody, a chimeric antibody, a multispecific antibody, an antibody fusion protein, an antibody-drug-conjugate or an antibody fragment.

The protein which is comprised in the composition of the invention is preferably essentially pure and desirably essentially homogeneous (i.e. free from contaminating proteins). "Essentially pure" means that at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% of the proteins in the composition of the invention have an identical amino acid sequence. Correspondingly, it is preferred that at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% of the proteins comprised in the RPCs of the composition of the invention have an identical amino acid sequence. That is, the composition of the invention may comprise more than one type of protein.

The RPCs in the composition are stabilized mainly by electrostatic interactions. Thus, it is preferred that the RPCs are formed between a positively charged protein and one or more

negatively charged complexing agents. Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the protein has a positive net charge when comprised in the RPCs. In another embodiment, the invention relates to the composition according to the invention, wherein the complexing agent has a negative charge when comprised in the RPCs.

The composition of the invention may comprise one or more excipients, in particular a stabilizer and/or a surfactant. Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the composition comprises at least one excipient. In a preferred embodiment, the invention relates to the composition according to the invention, wherein the at least one excipient is a stabilizer and/or a surfactant. Examples of excipients, stabilizers and solubilizers are given above.

It has been demonstrated by the inventors that the thermal stability of proteins is higher when the protein is comprised in an RPC. Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the protein has a higher melting temperature when comprised in the RPC compared to the uncomplexed protein.

The melting temperature of a protein, also referred to as the denaturation midpoint, is defined as the temperature (T_m) at which both the folded and unfolded states are equally populated at equilibrium (assuming two-state protein folding). T_m may be determined using a thermal shift assay. Different thermal shift assays for determining the T_m of a protein, namely nano differential scanning fluorimetry (nanoDSF) and differential scanning calorimetry (DSC) are described in more detail in Example 1.9.

A protein is said to be in an uncomplexed state if it is not part of a complex with a complexing agent, in particular, if it is not part of a complex with the complexing agents chondroitin sulfate and/or dextran sulfate.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the RPCs comprising the protein and the complexing agent dissociate at physiological pH and ionic strength.

It was surprisingly found by the inventors that RPCs comprising dextran sulfate and/or chondroitin sulfate as the complexing agents dissociate at physiological pH and ionic strength. Thus, liquid formulations comprising the RPCs of the invention may be administered to a subject such that they dissociate at the site of administration, consequently resulting in the release of the uncomplexed protein in said subject.

The term "physiological pH" as used herein refers to the normal pH in the cells of the tissues and organs of the mammalian body. For instance, the physiological pH of a human is normally approximately 7.4, but normal physiological pH in mammals may be any pH from about 7.0 to about 7.8.

The term "physiological ionic strength", as used herein, refers to the normal pH in the cells of the tissues and organs of the mammalian body. For instance, the physiological ionic strength of a human is normally approximately 0.15 mol/L.

Accordingly, a protein complex is said to dissociate at physiological pH and ionic strength, if at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% of the complexes in a sample dissociate into soluble proteins at physiological pH and ionic strength.

Within the present invention, physiological conditions have been simulated by using phosphate buffered saline (PBS) with a pH of 7.4 and an ionic strength of 0.137 mol/L. It has been demonstrated by the inventors that RPCs comprising different proteins dissociate in PBS (pH 7.4, 137 mM NaCl) with a dissociation efficiency of at least 96% (see Example 3.1). Hence, it is plausible that the reversible protein complexes of the present invention dissociate at physiological conditions and thus may be directly applied to a subject in an appropriate formulation and via an appropriate route of administration, e.g., subcutaneous administration.

Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the RPCs comprising the protein and the complexing agent dissociate in 10 mM to 100 mM PBS (pH 7.4, 137 mM NaCl).

It has been shown by the inventors that diluting a suspension comprising RPCs in 10 mM or 100 mM PBS (pH 7.4, 137 mM to 1370 mM NaCl) to a protein concentration of 1 mg/mL results in dissociation of the RPCs. Thus, in a preferred embodiment, the invention relates to the composition according to the invention, wherein the RPCs comprising the protein and the complexing agent dissociate in 10 mM PBS (pH 7.4, 137 mM NaCl) when diluted to a protein concentration ranging from 0.1 to 10 mg/mL, preferably ranging from 0.1 to 5 mg/mL, more preferably ranging from 0.1 to 2 mg/mL, most preferably 1 mg/mL.

In an alternative embodiment, the invention relates to the composition according to the invention, wherein the RPCs comprising the protein and the complexing agent dissociate in 100 mM PBS (pH 7.4, 137 mM NaCl) when diluted to a protein concentration ranging from 0.1 to 10 mg/mL, preferably ranging from 0.1 to 5 mg/mL, more preferably ranging from 0.1 to 2

mg/mL, most preferably 1 mg/mL.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the composition is a suspension.

That is, in certain embodiments, the RPCs of the invention may be formulated as a suspension. In a particular embodiment, the invention relates to the composition according to the invention, wherein the suspension is obtained with the method according to the invention.

The suspension may have any protein concentration. However, it is preferred that the suspension of the invention is suitable for subcutaneous administration to a subject, which ideally requires protein a concentration in the suspension of at least 100 mg/mL. Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the protein concentration in the suspension ranges from 50 to 250 mg/mL. In a preferred embodiment, the invention relates to the composition according to the invention, wherein the protein concentration in the suspension ranges from 100 to 200 mg/mL.

Methods for obtaining suspensions with the claimed protein concentrations and methods for determining the protein concentration in a suspension are disclosed herein. Methods for determining the protein concentration in a suspension are described herein. For example, the protein concentration in a suspension may be determined by preparing serial dilutions of the suspension in 10 mM PBS (pH 7.4, 137 mM NaCl) and by measuring the protein concentration in the dilutions by any methods known in the art, such as UV-Vis spectrophotometry (NanoDrop) or Bradford assay. Suspensions with the desired protein concentration may be obtained by enriching or diluting the suspensions as disclosed herein.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the suspension comprises uncomplexed complexing agent.

That is, in certain embodiments, the suspension of the invention may comprise an excess of complexing agent, preferably wherein the uncomplexed complexing agent is dissolved in the liquid fraction of the suspension. In certain embodiments, the uncomplexed complexing agent is the same complexing agent that is comprised in the RPCs. In certain embodiments, the complexing agent is comprised in the liquid fraction of the suspension at a concentration ranging from 0.1 – 100 mM, preferably ranging from 0.1 – 10 mM, more preferably ranging from 0.1 – 1 mM, most preferably ranging from 0.2 – 0.6 mM.

Preferably, the complexing agent is dextran sulfate or chondroitin sulfate. That is, in certain

embodiments, the liquid fraction of the suspension comprises dextran sulfate at a concentration ranging from 0.1 – 100 mM, preferably ranging from 0.1 – 10 mM, more preferably ranging from 0.1 – 1 mM, most preferably ranging from 0.2 – 0.6 mM. In other embodiments, the liquid fraction of the suspension comprises chondroitin sulfate at a concentration ranging from 0.1 – 100 mM, preferably ranging from 0.1 – 10 mM, more preferably ranging from 0.1 – 1 mM, most preferably ranging from 0.2 – 0.6 mM.

The additional complexing agent may be added to the suspension after the formation of the RPCs by adding the complexing to the suspension or when exchanging the liquid fraction of the suspension.

The RPCs in the suspension of the invention may have any particle size. However, it is assumed that suspensions comprising smaller RPCs have a higher injectability compared to suspension comprising larger RPCs.

Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the RPCs comprised in the suspension have a mean particle size ranging from 5 to 20 μm . In a preferred embodiment, the invention relates to the composition according to the invention, wherein the RPCs comprised in the suspension have a mean particle size ranging from 6 to 12 μm .

It has been surprisingly found by the inventors that dialysis of RPCs against ultrapure water results in particularly small RPCs (see e.g. FIG. 20). That is, in a particular embodiment, the invention relates to the composition according to the invention, wherein the RPCs comprised in the suspension have a mean particle size ranging from 100 to 4000 nm, in particular wherein the RPCs comprised in the suspension have a mean particle size ranging from 150 to 2000 nm.

In certain embodiments, the invention relates to the composition according to the invention, wherein the RPCs comprised in the suspension have a mean particle size ranging from 0.1 to 20 μm , in particular wherein the RPCs comprised in the suspension have a mean particle size ranging from 0.1 to 12 μm .

The term “mean particle size”, as used herein, generally refers to the statistical mean particle size (diameter) of the particles in the composition. The diameter of an essentially spherical particle may be referred to as the physical or hydrodynamic diameter. The diameter of a non-spherical particle may refer preferentially to the hydrodynamic diameter. As used herein, the diameter of a non-spherical particle may refer to the largest linear distance between two points on the surface of the particle. Mean particle size can be measured using methods known in the

art, such as dynamic light scattering, laser diffraction analysis (see Example 1.2.5) or scanning electron microscopy (SEM).

It is preferred that the suspension comprising the RPCs is directly applied to a subject by means of injection. That is, the suspension of the invention is preferably injectable through needles commonly used in the art. Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the suspension is injectable through a 26G needle.

The term “26G needle” as used herein refers to a 26 gauge needle. The term “gauge”, as used herein, is meant to provide referrals to inner and outer diameters of the common numerical gauge system used for syringe needles. Advantageously, compositions of the present invention can be injected through small gauge needles having a gauge that is at least 26. A 26G needle has an outer diameter of 0.4636 mm.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the suspension is stable for at least 4 weeks at 4°C and/or 25°C.

Within the present invention, a suspension comprising RPCs is said to be stable, if the protein that is comprised in the RPCs, after dissociation of the RPCs, retains its original size, structure and/or function.

It has been demonstrated by the inventors that the suspension remains stable for at least 4 weeks at 4°C and/or 25°C (see Example 5.2). For example, only low levels of protein degradation and/or aggregation were observed after storage of the suspension for at least 4 weeks at 4°C or 25°C (Table 13).

In a preferred embodiment, a suspension is said to be stable if at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% of the proteins in the suspension are in monomeric form after dissociation of the RPCs in the suspension, preferably when measured by size exclusion chromatography (SEC).

Alternatively, a suspension is said to be stable, if the main peak percentage for the protein differs by not more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20% when analyzed by ion exchange chromatography (IEC) and compared before the formation of the RPC and after dissociation of the RPC.

In a particular embodiment, the invention relates to the composition according to the invention,

wherein the suspension has a viscosity ranging from 2 to 20 centipoise (cP), in particular ranging from 3 to 15 cP, when measured at 20°C.

Within the present invention, it is preferred that the suspension has a low viscosity to facilitate injectability of the suspension. It has been shown by the inventors that suspensions with a protein concentration as high as 120 mg/mL have a viscosity of less than 20 cP and can thus be injected through a 26G needle.

A centipoise is one millipascal-second (mPa·s) in SI units ($1 \text{ cP} = 10^{-2} \text{ P} = 10^{-3} \text{ Pa} \cdot \text{s}$). Centipoise is properly abbreviated cP. Water has a viscosity of 0.0089 poise at 25°C, or 1 centipoise at 20°C. The viscosity of a fluid may be measured with a rheometer, as described in Example 1.2.7.

To ensure the integrity of the RPCs in the suspension, it is preferred that the suspension has a pH that is lower than the isoelectric point of the protein and higher than the *pKa* of the complexing agent.

Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the pH of the suspension is lower than the isoelectric point of the protein.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the pH of the suspension is 2 to 5 pH units lower than the isoelectric point of the protein, in particular wherein the pH of the suspension is 3 pH units lower than the isoelectric point of the protein.

That is, in certain embodiments, the pH of the suspension is adjusted to be lower than the isoelectric point of the protein that is comprised in the RPCs. A pH below the isoelectric point of the protein results in positively charged proteins and thus prevents dissociation of the RPCs in the suspension.

In certain embodiments the protein is an antibody. Antibodies have an isoelectric point ranging from 6 to 9. To maintain the antibody in a positive charge, it is preferred that the suspension has a pH ranging from 1 to 6, preferably from 4.5 to 5.5. Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the pH of the suspension ranges from 1 to 6, in particular wherein the pH of the suspension ranges from 4.5 to 5.5.

In certain embodiments, the suspension comprises a buffering agent. The suspension may

comprise any buffering agent that allows to maintain the RPCs in suspension without compromising the stability of the RPCs or the proteins comprised therein. Buffering agents that may be used in the composition of the present invention include, without limitation, formate, citrate, succinate, acetate, propionate, malate, pyridine, piperazine, cacodylate, succinate, MES, maleate, histidine, bis-tris, phosphate, ethanolamine, ADA and carbonate.

It has been demonstrated that suspensions comprising the buffering agent histidine remain stable for long periods and have a low viscosity, which allows injection of the suspension through a 26G needle. Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the suspension comprises a buffering agent. In a preferred embodiment, the invention relates to the composition according to the invention, wherein the buffering agent is histidine or citrate.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the suspension has an ionic strength ranging from 20 to 50 mM, in particular wherein the suspension has an ionic strength ranging from 20 to 30 mM.

In certain embodiments, the suspension comprises 20 to 50 mM histidine or citrate as buffering agent. Preferably, the suspension comprises 20 to 30 mM histidine or citrate as buffering agent.

In other embodiments, the suspension is free of a buffering agent. It has been demonstrated by the inventors that RPCs suspensions comprising only RPCs, a complexing agent and ultrapure (MilliQ) water can be prepared. Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the suspension is substantially free of buffering agents.

A suspension is said to be substantially free of buffering agent if the concentration of the buffering agent in the suspension before the spray-drying step is below 5 mM, below 4 mM, below 3 mM, below 2 mM, below 1 mM, below 0.5 mM, below 0.1 mM or below 0.01 mM.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the liquid fraction of the suspension consists of ultrapure (MilliQ) water. Ultrapure (MilliQ) water, when exposed to air, has a pH of approximately 5.5. Thus, suspensions, wherein the liquid fraction consists of ultrapure (MilliQ) water, preferably comprise RPCs comprising proteins with an isoelectric point of 6 or higher.

The skilled person is aware of methods to remove a buffering agent from a suspension comprising RPCs. For example, a suspension comprising RPCs may be dialyzed against a liquid

that is free of buffering agents. The dialysis step may be repeated until the suspension is substantially free of buffering agents. A suspension is said to be substantially free of buffering agents, if the concentration of buffering agents in the suspension is below 1 mM, 0.5 mM, 0.2 mM, 0.1 mM, 0.01 mM.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the suspension further comprises a non-aqueous solvent.

In certain embodiments, the liquid fraction of the suspension may be a mixture of aqueous solvents and non-aqueous solvents. That is, the ratio between aqueous solvents and non-aqueous solvent may be 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90.

The suspension may comprise any non-aqueous solvent that allows to maintain the RPCs in the suspension without comprising the stability of the RPCs or the proteins comprised therein. In a particular embodiment, the invention relates to the composition according to the invention, wherein the non-aqueous solvent is any one of Table 4, but preferably triacetin, diethylene glycol monoethyl ether or ethyl oleate.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the composition is a lyophilisate.

That is, in certain embodiments, the invention relates to a lyophilisate comprising the RPCs of the invention. In a particular embodiment, the invention relates to the composition according to the invention, wherein the lyophilisate is obtained with the method according to the invention.

The term "lyophilisate" as used herein in connection with the composition according to the invention denotes a formulation which is manufactured by freeze-drying methods known in the art per se. The solvent (e.g., water) is removed by freezing following sublimation under vacuum and desorption of residual water at elevated temperature. In the pharmaceutical field, the lyophilisate has usually residual moisture of about 0.1 to 5% (w/w) and is present as a powder or a physical stable cake. The lyophilisate is characterized by a fast dissolution after addition of a reconstitution medium.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the lyophilisate comprises a buffering agent.

That is, the lyophilisate may comprise a buffering agent. Preferably, the lyophilisate is obtained by freeze-drying the suspension of the invention. Thus, in a preferred embodiment, the

lyophilisate comprises the same buffering agent as the suspension of the invention. More preferably, the buffering agent is histidine or citrate. Thus, in a preferred embodiment, the invention relates to the composition according to the invention, wherein the buffering agent is histidine or citrate.

In certain embodiments, the lyophilisate may be free or substantially free of buffering agents. In particular, the suspension comprising the RPCs may be dialysed against water before the lyophilization step.

Freeze-drying of a suspension may confer damage to the protein comprised in the RPCs. To prevent damage to the proteins during lyophilization, a cryoprotectant may be added to the suspension before the freeze-drying step. Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the lyophilisate comprises at least one cryoprotectant. In a preferred embodiment, the invention relates to the composition according to the invention, wherein the at least one cryoprotectant is selected from a group consisting of: sugars, amino acids, methylamines, lyotropic salts, polyols, propylene glycol, polyethylene glycol and pluronics.

It has been shown by the inventors that the lyophilisate of the invention is stable for a prolonged time at elevated temperatures. For example, the lyophilisate of the invention has been stored for 4 weeks at 40° C. To the surprise of the inventors, it was shown that the RPCs comprised in the lyophilisate of the invention can be efficiently dissolved after 4 weeks at 40°C and that the proteins comprised in the lyophilisate can be completely recovered (Table 12). Further, it was shown by the inventors that the protein comprised in the lyophilisate remains stable after storing the lyophilisate for 4 weeks at 40°C. Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the lyophilisate is stable for at least 4 weeks at 40°C. In an alternative embodiment, the invention relates to the composition according to the invention, wherein the lyophilisate is stable for at least 4 weeks at 5°C and/or 25°C.

Within the present invention, a lyophilisate comprising RPCs is said to be stable, if the protein that is comprised in the RPCs, after dissociation of the RPCs, retains its original size, structure and/or function.

Thus, in a preferred embodiment, a lyophilisate is said to be stable if at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% of the proteins in the lyophilisate are in monomeric form after reconstitution of the lyophilisate and dissociation of the RPCs in the reconstituted lyophilisate, preferably when measured by size exclusion chromatography (SEC). When analyzing if a protein is in

monomeric form, the RPCs are preferably reconstituted and dissociated in PBS (pH 7.4, 137 mM NaCl).

Alternatively, a lyophilisate is said to be stable, if the main peak percentage for the protein differs by not more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20% when analyzed by ion exchange chromatography (IEC) and compared before the formation of the RPCs and after dissociation of the RPCs, preferably when the lyophilisate is reconstituted and dissociated in PBS (pH 7.4, 137 mM NaCl).

In a particular embodiment, the invention relates to the composition according to the invention, wherein the lyophilisate is reconstituted in a liquid to a protein concentration ranging from 50 to 250 mg/mL, in particular wherein the lyophilisate is reconstituted in a liquid to a protein concentration ranging from 100 to 200 mg/mL.

The lyophilisate of the invention may be reconstituted in a liquid before administration to a subject. Thus, in certain embodiments, the composition of the invention may be a reconstituted lyophilisate comprising the RPCs of the invention.

The term "reconstitute" as used herein, unless otherwise specified, refers to a process by which the lyophilisate is mixed with a liquid to form a liquid product. Once reconstituted in the liquid, the ingredients of the lyophilisate may be any combination of one or more of dissolved, dispersed, suspended, colloiddally suspended, emulsified, or otherwise blended within the matrix of the liquid product. Therefore, the resulting reconstituted liquid product, may be characterized as any combination of a solution, a dispersion, a suspension, a colloidal suspension, an emulsion, or a homogeneous blend. A reconstituted composition may be said to be "reconstituted" even if a nominal portion (e.g., less than 10%) of the polyophilisate remains unreconstituted in the resulting liquid product.

The lyophilisate of the invention may be reconstituted in any volume. Preferably, the lyophilisate of the invention may be reconstituted in a liquid such that a protein concentration ranging from 50 to 250 mg/mL is obtained in the reconstituted lyophilisate. Even more preferably, the lyophilisate of the invention is reconstituted in a liquid such that a protein concentration ranging from 100 to 200 mg/mL is obtained in the reconstituted lyophilisate.

Methods for obtaining reconstituted lyophilisates with the claimed protein concentrations and methods for determining the protein concentration in a reconstituted lyophilisate are disclosed herein. Methods for determining the protein concentration in a reconstituted lyophilisate are described herein. For example, the protein concentration in a reconstituted lyophilisate may be

determined by preparing serial dilutions of the resuspended lyophilisate in 10 mM PBS (pH 7.4, 137 mM NaCl) and by measuring the protein concentration in the dilutions by any method known in the art, such as UV-Vis spectrophotometry (NanoDrop) or Bradford assay. Reconstituted lyophilisates with the desired protein concentration may be obtained by reconstituting the lyophilisate in a specific volume of a liquid.

Preferably, the lyophilisate of the invention is reconstituted in PBS, more preferably PBS (pH 7.4, 137 mM NaCl). Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the liquid is PBS.

It has been shown by the inventors that the lyophilisate of the invention can be reconstituted in PBS to obtain a reconstituted lyophilisate with a protein concentration of at least 130 mg/mL (Table 12). Further, reconstituting the lyophilisate in PBS (pH 7.4, 137 mM NaCl) may result in the dissociation of the RPCs comprised in the lyophilisate. Thus, in certain embodiments, the reconstituted lyophilisate is a solution, preferably a solution comprising PBS, more preferably a solution comprising PBS and a protein concentration ranging from 50 to 250 mg/mL, most preferably a solution comprising PBS and a protein concentration ranging from 100 to 200 mg/mL.

The reconstituted lyophilisate may have a viscosity ranging from 2 to 20 cP, 3 to 20 cP, 4 to 20 cP, 5 to 20 cP, 6 to 20 cP, 7 to 20 cP, 8 to 20 cP, 9 to 20 cP, or 10 to 20 cP. Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the resuspended lyophilisate has a viscosity ranging from 2 to 20 cP, in particular ranging from 10 to 20 cP.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the composition is a spray dried powder.

That is, in certain embodiments, the RPCs of the invention may be formulated as a spray dried powder. In a particular embodiment, the invention relates to the composition according to the invention, wherein the spray dried powder is obtained with the method according to the invention.

It has been surprisingly shown by the inventors that spray drying of the suspension of the invention may result in a spray dried powder with a protein content of more than 40% (see Table 3). Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the protein content of the spray dried powder is at least 40% by weight (w/w), at least 50% by weight (w/w), at least 60% by weight (w/w) .

The skilled person is aware of methods to determine the protein content of a spray dried powder. For example, the spray dried powder may be reconstituted in PBS to dissolve the RPCs comprised in the spray dried powder and the concentration of the re-solubilized protein may be determined by any method known in the art, such as Nanodrop, Bradford assay and so forth. The skilled person is able to calculate the protein content of the spray dried powder based on the protein concentration in the reconstituted spray dried powder.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the spray dried powder comprises a buffering agent.

That is, the spray dried powder may comprise a buffering agent. Preferably, the spray dried powder is obtained by spray-drying the suspension of the invention. Thus, in a preferred embodiment, the spray dried powder comprises the same buffering agent as the suspension of the invention. More preferably, the buffering agent is histidine or citrate. Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the buffering agent is histidine or citrate.

The concentration of the buffering agent in the suspension before the spray drying step was shown to have an influence on the protein content in the spray dried powder (Table 3). That is, the higher the concentration of the buffering agent in the suspension before the spray drying step, the lower the protein content in the resulting spray dried powder. To increase the protein content in the spray dried powder, the concentration of the buffering agent in the suspension may be reduced before the spray drying step. For example, the concentration of the buffering agent may be reduced by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or 100% before the spray drying step. That is, in a particular embodiment, the invention relates to the composition according to the invention, wherein the spray dried powder is substantially free of buffering agents.

It has been demonstrated by the inventors that spray drying of a suspension that is free of the buffering agent histidine results in spray dried powders with a protein content of at least 60% (see Table 3). Thus, in a preferred embodiment, the invention relates to the composition according to the invention, wherein the spray dried powder has been obtained from a suspension comprising less than 20 mM, less than 15 mM, less than 10 mM, less than 5 mM, less than 1 mM or 0 mM of a buffering agent, in particular wherein the buffering agent is histidine or citrate.

Similarly, it has been shown by the inventors that reducing the concentration of the complexing agent and/or the excipients in the suspension before the spray drying step increases the protein

content in the spray dried powder, without compromising the stability of the protein in the spray dried powder.

That is, in certain embodiments, the invention relates to the composition according to the invention, wherein the spray dried powder has been obtained from a suspension comprising less than 100 mg/mL, less than 90 mg/mL, less than 80 mg/mL, less than 70 mg/mL, or less than 60 mg/mL of a complexing agent, in particular wherein the complexing agent is dextran sulfate.

In other embodiments, the invention relates to the composition according to the invention, wherein the spray dried powder has been obtained from a suspension comprising less than 2.5 mg/mL, less than 2 mg/mL, less than 1.75 mg/mL, less than 1.5 mg/mL, or less than 1 mg/mL of a stabilizer, in particular wherein the stabilizer is sucrose.

In other embodiments, the invention relates to the composition according to the invention, wherein the spray dried powder has been obtained from a suspension comprising less than 0.5 mg/mL, less than 0.4 mg/mL, less than 0.3 mg/mL or less than 0.25 mg/mL of a solubilizer, in particular wherein the solubilizer is polysorbate 20.

In a more preferred embodiment, the invention relates to the composition according to the invention, wherein the spray dried powder has been obtained from a suspension comprising between 0 and 20 mM histidine, between 0.5 and 1 mg/mL dextrane sulphate, between 0.5 and 2.5 mg/mL sucrose, between 0.1 and 0.5 mg/mL polysorbate 20 and between 1 and 10 mg/mL RPCs.

In an even more preferred embodiment, the invention relates to the composition according to the invention, wherein the spray dried powder has been obtained from a suspension comprising between 0 and 10 mM histidine, between 0.5 and 0.7 mg/mL dextrane sulphate, between 0.5 and 1.5 mg/mL sucrose, between 0.1 and 0.3 mg/mL polysorbate 20 and between 1 and 5 mg/mL RPCs.

The particles in the spray dried powder may have any size. However, it is preferred that the particles in the spray dried powder have a small particle size to facilitate injection of these particles. Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the RPCs comprised in the spray dried powder have a mean particle size ranging from 5 to 50 μm . In a preferred embodiment, the invention relates to the composition according to the invention, wherein the RPCs comprised in the spray dried powder have a mean particle size ranging from 10 to 40 μm . In more preferred embodiment, the invention relates to the composition according to the invention, wherein the RPCs comprised

in the spray dried powder have a mean particle size ranging from 20 to 35 μm .

In an alternative embodiment, the invention relates to the composition according to the invention, wherein the RPCs comprised in the spray dried powder have a mean particle size ranging from 10 to 25 μm .

Within the present invention, a spray dried powder comprising RPCs is said to be stable, if the proteins comprised in the RPCs, after re-suspension and/or dissociation of the RPCs, retain their original size, structure and/or function.

Thus, in a preferred embodiment, a spray dried powder is said to be stable if at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% of the proteins in the spray dried powder are in monomeric form after re-suspension and/or dissociation of the RPCs, preferably when measured by size exclusion chromatography (SEC). For analyzing if a protein is in monomeric state, the RPCs are preferably dissolved in PBS (pH 7.4, 137 mM NaCl).

Alternatively, a spray dried powder is said to be stable, if the main peak percentage for the protein differs by not more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20% when analyzed by ion exchange chromatography (IEC) and compared before the formation of the RPCs and after re-suspension and/or dissociation of the RPCs, preferably when the RPCs are dissolved in PBS (pH 7.4, 137 mM NaCl).

In a particular embodiment, the invention relates to the composition according to the invention, wherein the spray dried powder is reconstituted in a liquid.

The spray dried powder of the invention may be re-suspended in a liquid before administration to the subject. Thus, in certain embodiments, the composition of the invention may be a re-suspended spray dried powder comprising RPCs.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the spray dried powder is re-suspended in a liquid to a protein concentration ranging from 50 to 300 mg/mL, in particular wherein the spray dried powder is reconstituted in a liquid to a protein concentration ranging from 100 to 250 mg/mL.

The spray dried powder of the invention may be re-suspended in any volume. Preferably, the spray dried powder of the invention is re-suspended in a liquid such that a protein concentration

ranging from 50 to 300 mg/mL is obtained in the re-suspended spray dried powder. Even more preferably, the spray dried powder of the invention is re-suspended in a liquid such that a protein concentration ranging from 100 to 250 mg/mL is obtained in the re-suspended spray dried powder.

The spray dried powder may be resuspended in any liquid. However, it is preferred that the spray dried powder is re-suspended in a non-aqueous solvent such that an RPC-NAS suspension is obtained. Thus, in a preferred embodiment, the invention relates to the composition according to the invention, wherein the liquid is a non-aqueous solvent.

In a particular embodiment, the invention relates to the composition according to the invention, wherein the non-aqueous solvent is at least one selected from the solvents of Table 4, a group consisting of: diethylene glycol monoethyl ether, ethyl oleate, triacetin, isosorbide dimethyl ester and glycofurol, preferably triacetin, diethylene glycol monoethyl ether or ethyl oleate.

It has been shown by the inventors that re-suspending the RPCs in the non-aqueous solvents transcitol, ethyl oleate or triacetin results does not significantly compromise the stability of the protein comprised in the RPCs. Thus, in a preferred embodiment, the invention relates to the composition according to the invention, wherein the non-aqueous solvent is at least one selected from the solvents of Table 4, or a group consisting of: diethylene glycol monoethyl ether, ethyl oleate, triacetin, isosorbide dimethyl ester and glycofurol, preferably triacetin, diethylene glycol monoethyl ether or ethyl oleate.

Within the present invention, a liquid is suitable for re-suspending the spray dried powders of the invention, if the proteins comprised in the spray dried powder, after re-suspension of the RPCs, retain their original size, structure and/or function.

Thus, in a preferred embodiment, a liquid is said to be suitable for the re-suspension of the spray dried powder of the invention, if at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% of the proteins in the resuspended spray dried powder are in monomeric form after dissociation of the RPCs, preferably when measured by size exclusion chromatography (SEC). For analyzing if a protein is in monomeric state, the RPCs are preferably dissolved in PBS (pH 7.4, 137 mM NaCl).

Alternatively, a liquid is said to be suitable for the re-suspension of the spray dried powder of the invention, if the main peak percentage for the protein differs by not more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20% when analyzed by ion exchange chromatography (IEC) and compared before the formation of

the RPCs and after re-suspension and dissociation of the RPCs in said liquid, preferably when the RPCs are dissolved in PBS (pH 7.4, 137 mM NaCl).

Further, it has been shown that re-suspending the RPCs in a non-aqueous solvent results in lower viscosity when compared to a suspension comprising the same concentration of RPCs in an aqueous solvent (see Table 4). Thus, in a particular embodiment, the invention relates to the composition according to the invention, wherein the reconstituted spray dried powder has a viscosity ranging from 10 to 300 cP, preferably 20 to 80 cP, preferably 20 to 50 cP. In another embodiment, the invention relates to the composition according to the invention, wherein the reconstituted spray dried powder has a viscosity ranging from 50 to 300 cP. In another embodiment, the invention relates to the composition according to the invention, wherein the reconstituted spray dried powder has a viscosity ranging from 80 to 280 cP. In another embodiment, the invention relates to the composition according to the invention, wherein the reconstituted spray dried powder has a viscosity ranging from 100 to 250 cP.

In another aspect, the invention relates to a pharmaceutical formulation comprising the composition according to the invention.

In certain embodiments, the composition as such is a pharmaceutical formulation. In particular, the suspension comprising RPCs, the enriched RPC suspension, the reconstituted lyophilisate, or the RPC-NAS suspension may be used as pharmaceutical formulations.

In other embodiments, a pharmaceutical formulation may be obtained by bringing a solid composition into liquid form. That is, in certain embodiments, a pharmaceutical formulation may be obtained by reconstituting the lyophilisate of the invention in a suitable liquid, preferably in PBS (pH 7.4, 137 mM NaCl). In other embodiments, a pharmaceutical formulation may be obtained by re-suspending the spray dried powder of the invention in a suitable liquid, preferably a non-aqueous solvent.

The term "pharmaceutical formulation", as used herein, refers to a dosage form comprising the reversible protein complex of the invention and at least one pharmaceutically accepted excipient. The term "pharmaceutically acceptable", as used herein, means suited for normal pharmaceutical applications, i.e. giving rise to no adverse events in patients. The term "excipient" or "pharmaceutically acceptable excipient" as used herein means a nontoxic material that is compatible with the physical and chemical characteristics of the active pharmaceutical ingredient and does not interfere with the effectiveness of the biological activity of the active pharmaceutical ingredient, which is generally safe, non-toxic and neither biologically nor otherwise undesirable, and acceptable for veterinary use as well as human

pharmaceutical use. An “excipient” or “pharmaceutically acceptable excipient” as used in the specification includes both one and more than one such excipient.

In certain embodiments, the pharmaceutical formulation is a liquid that may be administered to a subject via injection. Preferably, the pharmaceutical formulation may be administered subcutaneously, intramuscularly or transdermally. In certain embodiments, the pharmaceutical formulation may be administered ocularly.

In another aspect, the invention relates to the pharmaceutical formulation according to the invention for use as a medicament.

That is, the pharmaceutical formulation of the invention, in particular the suspension, the reconstituted lyophilisate or the re-suspended spray dried powder, may be used as a medicament. Thus, in a preferred embodiment, the pharmaceutical formulation of the invention is the suspension comprising RPCs of the invention, the enriched RPC suspension of the invention, the reconstituted lyophilisate of the invention or the RPC-NAS suspension of the invention.

The term “medicament” as used herein means a pharmaceutical formulation suitable for administration of a pharmaceutically active compounds, i.e., a therapeutic protein to a patient in need thereof.

In a preferred embodiment, the pharmaceutical formulation according to the invention is used as a medicament for the treatment of a disease selected from the group consisting of autoimmune disease, immune dysregulation disease, carcinoma, sarcoma, glioma, melanoma, lymphoma, leukemia, chronic lymphocytic leukemia, follicular lymphoma, diffuse large B cell lymphoma, multiple myeloma, non-Hodgkin's lymphoma, Alzheimer's disease, type 1 or type 2 diabetes, amyloidosis, and atherosclerosis. Thus, in a particular embodiment, the invention relates to the pharmaceutical composition according to the invention for use in the treatment of an autoimmune disease, an immune dysregulation disease, carcinoma, sarcoma, glioma, melanoma, lymphoma, leukemia, chronic lymphocytic leukemia, follicular lymphoma, diffuse large B cell lymphoma, multiple myeloma, non-Hodgkin's lymphoma, Alzheimer's disease, type 1 or type 2 diabetes, amyloidosis, or atherosclerosis

In certain embodiments, the pharmaceutical formulation according to the invention is used for the treatment of cancer. The term “cancer”, as used herein, refers to diseases in which abnormal cells divide without control and can invade nearby tissues, and includes, but is not restricted to, acute lymphoblastic leukemia, acute myelogenous leukemia, bladder cancer, bone sarcoma,

breast cancer, cervical cancer, choriadenoma destruens, choriocarcinoma, gastric cancer, Hodgkin lymphoma, hydatidiform mole, lung cancer, malignant mesothelioma, mycosis fungoides (a type of cutaneous T-cell lymphoma), neuroblastoma, non-Hodgkin lymphoma, non-small cell lung cancer, osteosarcoma, ovarian cancer, small cell lung cancer, soft tissue sarcoma, squamous cell carcinoma of the head and neck, testicular cancer, thyroid cancer, transitional cell bladder cancer, Wilms tumor and the like.

The pharmaceutically active ingredient in the pharmaceutical formulation of the invention is a protein. Preferably, the pharmaceutical formulation for use according to the invention comprises an antibody. That is, the RPCs comprised in the pharmaceutical formulation for use according to the invention may comprise an antibody and a complexing agent, in particular the complexing agent dextran sulfate or chondroitin sulfate. The skilled person is able to identify antibodies known in the art that can be used in the treatment of a specific disease.

However, in certain embodiments, the antibody may bind to any of the herein disclosed target molecules, for example CD proteins such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the HER receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mol, p150,95, VLA-4, ICAM-1, VCAM and $\alpha v/\beta 3$ integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; protein C etc.

Exemplary antibodies that may be comprised in the pharmaceutical formulation of the invention include, but are not limited to, hR1 (anti- IGF-1R, U.S. Patent Application Serial No. 12/722,645, filed 3/12/10), hPAM4 (anti-mucin, U.S. Patent No. 7,282,567), hA20 (anti-CD20, U.S. Patent No. 7,251,164), hA19 (anti-CD19, U.S. Patent No. 7,109,304), WMMU31 (anti-AFP, U.S. Patent No. 7,300,655), hLL1 (anti- CD74, U.S. Patent No. 7,312,318), hLL2 (anti-CD22, U.S. Patent No. 7,074,403), hMu-9 (anti-CSAp, U.S. Patent No. 7,387,773), hL243 (anti-HLA-DR, U.S. Patent No. 7,612,180), hMN-14 (anti-CEACAM5, U.S. Patent No. 6,676,924), hMN-15 (anti-CEACAM6, U.S. Patent No. 7,541,440), hRS7 (anti-EGP-1, U.S. Patent No. 7,238,785), hMN-3 (anti- CEACAM6, U.S. Patent No. 7,541,440), Abl24 and Abl 25 (anti-CXCR4, U.S. Patent No. 7,138,496).

Alternative antibodies that may be comprised in the pharmaceutical formulation of the invention include, but are not limited to, abciximab (anti- glycoprotein IIb/IIIa), alemtuzumab (anti-CD52), bevacizumab (anti-VEGF), cetuximab (anti-EGFR), gemtuzumab (anti-CD33), ibritumomab tiuxetan (anti-CD20), panitumumab (anti-EGFR), rituximab (anti-CD20), tositumomab (anti-CD20), trastuzumab (anti-ErbB2), abagovomab (anti-CA-125),

adecatumumab (anti-EpCAM), atlizumab (anti-IL-6 receptor), benralizumab (anti-CD125), CC49 (anti-TAG-72), AB-PG1 -XG1-026 (anti-PSMA, U.S. Patent Application 11/983,372, deposited as ATCC PTA-4405 and PTA-4406), D2/B (anti- PSMA, WO 2009/130575), tocilizumab (anti-IL-6 receptor), basiliximab (anti-CD25), daclizumab (anti-CD25), efalizumab (anti-CD11a), GA101 (anti-CD20; Glycart Roche), muromonab-CD3 (anti-CD3 receptor), natalizumab (anti- α 4 integrin), omalizumab (anti- IgE); anti-TNF- α antibodies such as CDP571 (Ofei et al., 2011 , Diabetes 45:881 -85), MTNFAI, M2TNFAI, M3TNFAI, M3TNFABI, M302B, M303 (Thermo Scientific, Rockford, IL), infliximab (Centocor, Malvern, PA), certolizumab pegol (UCB, Brussels, Belgium), anti-CD40L (UCB, Brussels, Belgium), adalimumab (Abbott, Abbott Park, IL), Benlysta (Human Genome Sciences); antibodies for therapy of Alzheimer's disease such as Alz 50 (Ksiezak-Reding et al., 1987, J Biol Chem 263:7943-47), gantenerumab, solanezumab and infliximab; anti-fibrin antibodies like 59D8, T2G1 s, MH1 ; anti-HIV antibodies such as P4/D10 (U.S. Patent Application Serial No. 11/745,692), Ab 75, Ab 76, Ab 77 (Paulik et al., 1999, Biochem Pharmacol 58: 1781-90); and antibodies against pathogens such as CR6261 (anti-influenza), exbivirumab (anti-hepatitis B), felvizumab (anti-respiratory syncytial virus), foravirumab (anti-rabies virus), motavizumab (anti-respiratory syncytial virus), palivizumab (anti-respiratory syncytial virus), panobacumab (anti-Pseudomonas), rafivirumab (anti-rabies virus), regavirumab (anti-cytomegalovirus), sevirumab (anti-cytomegalovirus), tivirumab (anti-hepatitis B), and urtoxazumab (anti-E. coli).

In certain embodiments, the antibody may be a bispecific anti-VEGF/anti-angiopoietin-2 (Ang-2) antibody, an anti-alpha synuclein (aSyn) antibody, a bispecific anti-FAP/anti-OX40 antibody, a bispecific anti-VEGF/anti-PDGF antibody (dutafab), Bevacizumab, Pertuzumab or Gantenerumab.

Preferably, the pharmaceutical formulation of the invention may be used for subcutaneous, intramuscular or transdermal application to a subject. Thus, in a particular embodiment, the invention relates to the pharmaceutical formulation for use according to the invention, wherein the pharmaceutical formulation is administered subcutaneously, intramuscularly or transdermally. In a preferred embodiment, the invention relates to the pharmaceutical formulation for use according to the invention, wherein the pharmaceutical formulation is administered subcutaneously.

Subcutaneous, intramuscular and transdermal administration of the pharmaceutical formulation of the invention has the advantage that the pharmaceutical formulation may be self-administered by a subject. Further, the pharmaceutical formulation according to the invention may be used for the administration of subjects in which intravenous application is difficult or impossible.

In certain embodiments, the invention relates to pharmaceutical formulation for use according to the invention, wherein the pharmaceutical formulation is the enriched RPC suspension of the invention, and wherein the enriched RPC suspension is administered subcutaneously, intramuscularly, transdermally, ocularly, such as subconjunctivally, intracamerally, intravitreally, subretinally, or suprachoroidally, to the brain, such as intralumbarily, intrathecally, or intraventricularly, intra-articularly, or by inhalation.

In other embodiments, the invention relates to pharmaceutical formulation for use according to the invention, wherein the pharmaceutical formulation is the reconstituted lyophilisate of the invention, and wherein the reconstituted lyophilisate is administered subcutaneously, intramuscularly or transdermally.

In further embodiments, the invention relates to pharmaceutical formulation for use according to the invention, wherein the pharmaceutical formulation is the re-suspended spray dried powder of the invention, and wherein the re-suspended spray dried powder is administered subcutaneously, intramuscularly, transdermally, ocularly, such as subconjunctivally, intracamerally, intravitreally, subretinally, or suprachoroidally, to the brain, such as intralumbarily, intrathecally, or intraventricularly, intra-articularly, or by inhalation.

In certain embodiments, the invention relates to the pharmaceutical formulation according to the invention for use in the treatment of ophthalmic diseases. In certain embodiments, the ophthalmic disease may be an ocular vascular disease.

The RPCs in the pharmaceutical formulation for use in the treatment of ocular vascular diseases may comprise an antibody or an antibody fragment. In certain embodiments, the antibody or antibody fragment may specifically bind to human vascular endothelial growth factor (VEGF/VEGF-A). In certain embodiments, the antibody or antibody fragment may specifically bind to human angiopoietin-2 (Ang-2). In certain embodiments, the antibody or antibody fragment may specifically bind to VEGF/VEGF-A and Ang-2.

That is, in certain embodiments, the antibody or the antibody fragment comprised in the RPCs of the pharmaceutical formulation may be a bispecific antibody or a bispecific antibody fragment. In certain embodiments, the bispecific antibody may bind specifically to VEGF/VEGF-A and Ang-2. In certain embodiments, the bispecific anti-VEGF/anti-angiopoietin-2 (Ang-2) antibody may be faricimab, as disclosed in WO2014/009465 as "VEGFang2-0016".

In certain embodiments, the bispecific antibody fragment may be a dutafab. In certain

embodiments, the dutafab may bind specifically to VEGF/VEGF-A and/or Ang-2.

The term "ocular vascular disease" includes, but is not limited to intraocular neovascular syndromes such as diabetic retinopathy, diabetic macular edema, retinopathy of prematurity, neovascular glaucoma, retinal vein occlusions, central retinal vein occlusions, macular degeneration, age-related macular degeneration, retinitis pigmentosa, retinal angiomatous proliferation, macular telangiectasia, ischemic retinopathy, iris neovascularization, intraocular neovascularization, corneal neovascularization, retinal neovascularization, choroidal neovascularization, and retinal degeneration. (Garner, A., Vascular diseases, In: Pathobiology of ocular disease, A dynamic approach, Garner, A., and Klintworth, G.K., (eds.), 2nd edition, Marcel Dekker, New York (1994), pp. 1625-1710). As used herein, ocular vascular disorder refers to any pathological conditions characterized by altered or unregulated proliferation and invasion of new blood vessels into the structures of ocular tissues such as the retina or cornea. In one embodiment the ocular vascular disease is selected from the group consisting of: wet age-related macular degeneration (wet AMD), dry age-related macular degeneration (dry AMD), diabetic macular edema (DME), cystoid macular edema (CME), non-proliferative diabetic retinopathy (NPDR), proliferative diabetic retinopathy (PDR), cystoid macular edema, vasculitis (e.g. central retinal vein occlusion), papilledema, retinitis, conjunctivitis, uveitis, choroiditis, multifocal choroiditis, ocular histoplasmosis, blepharitis, dry eye (Sjogren's disease) and other ophthalmic diseases wherein the eye disease or disorder is associated with ocular neovascularization, vascular leakage, and/or retinal edema. So the pharmaceutical formulation according to the invention may be useful in the prevention and treatment of wet AMD, dry AMD, CME, DME, NPDR, PDR, blepharitis, dry eye and uveitis, also preferably wet AMD, dry AMD, blepharitis, and dry eye, also preferably CME, DME, NPDR and PDR, also preferably blepharitis, and dry eye, in particular wet AMD and dry AMD, and also particularly wet AMD. In some embodiments, the ocular disease is selected from the group consisting of wet age-related macular degeneration (wet AMD), macular edema, retinal vein occlusions, retinopathy of prematurity, and diabetic retinopathy. Other diseases associated with corneal neovascularization include, but are not limited to, epidemic keratoconjunctivitis, Vitamin A deficiency, contact lens overwear, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, sjogrens, acne rosacea, phlyctenulosis, syphilis, Mycobacteria infections, lipid degeneration, chemical burns, bacterial ulcers, fungal ulcers, Herpes simplex infections, Herpes zoster infections, protozoan infections, Kaposi sarcoma, Mooren ulcer, Terrien's marginal degeneration, marginal keratolysis, rheumatoid arthritis, systemic lupus, polyarteritis, trauma, Wegeners sarcoidosis, Scleritis, Steven's Johnson disease, periphigoid radial keratotomy, and corneal graft rejection. Diseases associated with retinal/choroidal neovascularization include, but are not limited to, diabetic retinopathy, macular degeneration, sickle cell anemia, sarcoid, syphilis, pseudoxanthoma elasticum, Pagets disease, vein occlusion,

artery occlusion, carotid obstructive disease, chronic uveitis/vitritis, mycobacterial infections, Lyme's disease, systemic lupus erythematosus, retinopathy of prematurity, retinitis pigmentosa, retina edema (including macular edema), Eales disease, Bechets disease, infections causing a retinitis or choroiditis, presumed ocular histoplasmosis, Bests disease, myopia, optic pits, Stargarts disease, pars planitis, chronic retinal detachment, hyperviscosity syndromes, toxoplasmosis, trauma and post-laser complications. Other diseases include, but are not limited to, diseases associated with rubeosis (neovascularization of the angle) and diseases caused by the abnormal proliferation of fibrovascular or fibrous tissue including all forms of proliferative vitreoretinopathy.

In certain embodiments, the pharmaceutical formulation of the invention is used for the treatment of AMD, in particular wet AMD. In certain embodiments, the pharmaceutical formulation of the invention is used for the treatment of diabetic macular edema. In certain embodiments, the RPCs comprised in the pharmaceutical formulation for the treatment of AMD and/or diabetic macular edema comprise an antibody or an antibody fragment that binds specifically to VEGF/VEGF-A and/or Ang-2. In certain embodiments, the RPCs comprised in the pharmaceutical formulation for the treatment of AMD and/or diabetic macular edema comprise a bispecific antibody that binds specifically to VEGF/VEGF-A and Ang-2. In certain embodiments, said bispecific antibody is faricimab. In certain embodiments, the RPCs comprised in the pharmaceutical formulation for the treatment of AMD and/or diabetic macular edema comprise a dutafab that binds specifically to VEGF/VEGF-A and Ang-2.

Retinopathy of prematurity (ROP) is a disease of the eye that affects prematurely born babies. It is thought to be caused by disorganized growth of retinal blood vessels which may result in scarring and retinal detachment. ROP can be mild and may resolve spontaneously, but may lead to blindness in serious cases. As such, all preterm babies are at risk for ROP, and very low birth weight is an additional risk factor. Both oxygen toxicity and relative hypoxia can contribute to the development of ROP. Macular degeneration is a medical condition predominantly found in elderly adults in which the center of the inner lining of the eye, known as the macula area of the retina, suffers thinning, atrophy, and in some cases, bleeding. This can result in loss of central vision, which entails inability to see fine details, to read, or to recognize faces. According to the American Academy of Ophthalmology, it is the leading cause of central vision loss (blindness) in the United States today for those over the age of fifty years. Although some macular dystrophies that affect younger individuals are sometimes referred to as macular degeneration, the term generally refers to age-related macular degeneration (AMD or ARMD). Age-related macular degeneration begins with characteristic yellow deposits in the macula (central area of the retina which provides detailed central vision, called fovea) called drusen between the retinal pigment epithelium and the underlying choroid. Most people with these

early changes (referred to as age-related maculopathy) have good vision. People with drusen can go on to develop advanced AMD. The risk is considerably higher when the drusen are large and numerous and associated with disturbance in the pigmented cell layer under the macula. Large and soft drusen are related to elevated cholesterol deposits and may respond to cholesterol lowering agents or the Rheo Procedure.

Advanced AMD, which is responsible for profound vision loss, has two forms: dry and wet. Central geographic atrophy, the dry form of advanced AMD, results from atrophy to the retinal pigment epithelial layer below the retina, which causes vision loss through loss of photoreceptors (rods and cones) in the central part of the eye. While no treatment is available for this condition, vitamin supplements with high doses of antioxidants, lutein and zeaxanthin, have been demonstrated by the National Eye Institute and others to slow the progression of dry macular degeneration and in some patients, improve visual acuity.

Retinitis pigmentosa (RP) is a group of genetic eye conditions. In the progression of symptoms for RP, night blindness generally precedes tunnel vision by years or even decades. Many people with RP do not become legally blind until their 40s or 50s and retain some sight all their life. Others go completely blind from RP, in some cases as early as childhood. Progression of RP is different in each case. RP is a type of hereditary retinal dystrophy, a group of inherited disorders in which abnormalities of the photoreceptors (rods and cones) or the retinal pigment epithelium (RPE) of the retina lead to progressive visual loss. Affected individuals first experience defective dark adaptation or nyctalopia (night blindness), followed by reduction of the peripheral visual field (known as tunnel vision) and, sometimes, loss of central vision late in the course of the disease.

Macular edema occurs when fluid and protein deposits collect on or under the macula of the eye, a yellow central area of the retina, causing it to thicken and swell. The swelling may distort a person's central vision, as the macula is near the center of the retina at the back of the eyeball. This area holds tightly packed cones that provide sharp, clear central vision to enable a person to see form, color, and detail that is directly in the line of sight. Cystoid macular edema is a type of macular edema that includes cyst formation.

In certain embodiments the pharmaceutical formulation according to the invention is administered alone (without an additional therapeutic agent) for the treatment of one or more ocular vascular diseases described herein.

In other embodiments the pharmaceutical formulation according to the invention may be administered in combination with one or more additional therapeutic agents or methods for the

treatment of one or more ocular vascular diseases described herein.

The additional therapeutic agents may include, but are not limited to, Tryptophanyl-tRNA synthetase (TrpRS), Eye001 (Anti-VEGF Pegylated Aptamer), squalamine, RETAANE(TM) (anecortave acetate for depot suspension; Alcon, Inc.), Combretastatin A4 Prodrug (CA4P), MACUGEN(TM), MIFEPREX(TM) (mifepristone-ru486), subtenon triamcinolone acetonide, intravitreal crystalline triamcinolone acetonide, Prinomastat (AG3340-synthetic matrix metalloproteinase inhibitor, Pfizer), fluocinolone acetonide (including fluocinolone intraocular implant, Bausch & Lomb/Control Delivery Systems), VEGFR inhibitors (Sugen), VEGF-Trap (Regeneron/Aventis), VEGF receptor tyrosine kinase inhibitors such as 4-(4-bromo-2-fluoroanilino)-6-methoxy-7-(1-methylpiperidin-4-ylmethoxy)quinazoline (ZD6474), 4-(4-fluoro-2-methylindol-5-yloxy)-6-methoxy-7-(3-pyrrolidin-1-ylpropoxy)quinazoline (AZD2171), vatalanib (PTK787) and SU1 1248 (sunitinib), linomide, and inhibitors of integrin $\alpha_v\beta_3$ function and angiostatin.

Other pharmaceutical therapies that may be used in combination with the pharmaceutical formulation according to the invention include, but are not limited to, VISUDYNE(TM) with use of a non-thermal laser, PKC 412, Endovion (NeuroSearch A/S), neurotrophic factors, including by way of example Glial Derived Neurotrophic Factor and Ciliary Neurotrophic Factor, diatazem, dorzolamide, Phototrop, 9-cis-retinal, eye medication (including Echo Therapy) including phospholine iodide or echthiophate or carbonic anhydrase inhibitors, AE-941 (AEterna Laboratories, Inc.), Sirna-027 (Sima Therapeutics, Inc.), pegaptanib (NeXstar Pharmaceuticals/Gilead Sciences), neurotrophins (including, by way of example only, NT-4/5, Genentech), Cand5 (Acuity Pharmaceuticals), INS-37217 (Inspire Pharmaceuticals), integrin antagonists (including those from Jerini AG and Abbott Laboratories), EG-3306 (Ark Therapeutics Ltd.), BDM-E (BioDiem Ltd.), thalidomide (as used, for example, by EntreMed, Inc.), cardiotrophin-1 (Genentech), 2-methoxyestradiol (Allergan/Oculex), DL-8234 (Toray Industries), NTC-200 (Neurotech), tetrathiomolybdate (University of Michigan), LYN-002 (Lynkeus Biotech), microalgal compound (Aquasearch/ Albany, Mera Pharmaceuticals), D-9120 (Celltech Group plc), ATX-S10 (Hamamatsu Photonics), TGF-beta 2 (Genzyme/Celtrix), tyrosine kinase inhibitors (Allergan, SUGEN, Pfizer), NX-278- L (NeXstar Pharmaceuticals/Gilead Sciences), Opt-24 (OPTIS France SA), retinal cell ganglion neuroprotectants (Cogent Neurosciences), N- nitropyrazole derivatives (Texas A&M University System), KP-102 (Krenitsky Pharmaceuticals), cyclosporin A, "limited retinal translocation", photodynamic therapy, (including, by way of example only, receptor-targeted PDT, Bristol-Myers Squibb, Co.; porfimer sodium for injection with PDT; verteporfin, QLT Inc.; rostoporfm with PDT, Miravent Medical Technologies; talaporfm sodium with PDT, Nippon Petroleum; motexafm lutetium, Pharmacyclics, Inc.), antisense oligonucleotides

(including, by way of example, products tested by Novagali Pharma SA and ISIS- 13650, Isis Pharmaceuticals), laser photocoagulation, drusen lasering, macular hole surgery, macular translocation surgery, implantable miniature telescopes, Phi-Motion Angiography (also known as Micro-Laser Therapy and Feeder Vessel Treatment), Proton Beam Therapy, microstimulation therapy, Retinal Detachment and Vitreous Surgery, Scleral Buckle, Submacular Surgery, Transpupillary Thermotherapy, Photosystem I therapy, use of RNA interference (RNAi), extracorporeal rheopheresis (also known as membrane differential filtration and Rheotherapy), microchip implantation, stem cell therapy, gene replacement therapy, ribozyme gene therapy (including gene therapy for hypoxia response element, Oxford Biomedica; Lentipak, Genetix; PDEF gene therapy, GenVec), photoreceptor/retinal cells transplantation (including transplantable retinal epithelial cells, Diacrin, Inc.; retinal cell transplant, Cell Genesys, Inc.), and acupuncture.

Any anti-angiogenic agent may be used in combination with the pharmaceutical formulation according to the invention, include, but are not limited to those listed by Carmeliet and Jain, 2000, Nature 407:249-257. In certain embodiments, the anti-angiogenic agent may be a VEGF antagonist or a VEGF receptor antagonist such as VEGF variants, soluble VEGF receptor fragments, aptamers capable of blocking VEGF or VEGFR, neutralizing anti- VEGFR antibodies, low molecule weight inhibitors of VEGFR tyrosine kinases and any combinations thereof and these include anti-VEGF aptamers (e.g. Pegaptanib), soluble recombinant decoy receptors (e.g. VEGF Trap). . In certain embodiments, the anti-angiogenic agent may include corticosteroids, angiostatic steroids, anecortave acetate, angiostatin, endostatin, small interfering RNA's decreasing expression of VEGFR or VEGF ligand, post- VEGFR blockade with tyrosine kinase inhibitors, MMP inhibitors, IGFBP3, SDF-1 blockers, PEDF, gamma-secretase, Delta-like ligand 4, integrin antagonists, HIF-1 alpha blockade, protein kinase CK2 blockade, and inhibition of stem cell (i.e. endothelial progenitor cell) homing to the site of neovascularization using vascular endothelial cadherin (CD- 144) and stromal derived factor (SDF)-I antibodies. Small molecule RTK inhibitors targeting VEGF receptors including PTK787 can also be used. Agents that have activity against neovascularization that are not necessarily anti-VEGF compounds can also be used and include anti-inflammatory drugs, m-Tor inhibitors, rapamycin, everolimus, temsirolimus, cyclospohne, anti-TNF agents, anti-complement agents, and nonsteroidal antiinflammatory agents. Agents that are neuroprotective and can potentially reduce the progression of dry macular degeneration can also be used, such as the class of drugs called the 'neurosteroids.' These include drugs such as dehydroepiandrosterone (DHEA)(Brand names: Prastera(R) and Fidelin(R)), dehydroepiandrosterone sulfate, and pregnenolone sulfate. Any AMD (age-related macular degeneration) therapeutic agent can be used in combination with the pharmaceutical formulation according to the invention, including but not limited to verteporfin in combination

with PDT, pegaptanib sodium, zinc, or an antioxidant(s), alone or in any combination.

In embodiments where the pharmaceutical formulation according to the invention is used for the treatment of ophthalmic diseases, such as, without limitation, ocular vascular diseases, it is preferred that the pharmaceutical formulation according to the invention is administered intraocularly or intravitreally.

The term "intraocular" as used herein refers to anywhere within the globe of the eye. The term "intravitreal" as used herein refers to inside the gel in the back of the eye. That is, the pharmaceutical formulation for the treatment of ophthalmic diseases may be administered subretinally, intracapsularly, suprachoroidally, intracamerally, intrapalpebrally, to the subtenon, to the subconjunctival area, to the cul-de-sac, to the retrobulbar space or to the peribulbar space.

The term "subretinal" as used herein refers to the area between the retina and choroid. The term "intracapsular" as used herein refers to within the lens capsule. The term "suprachoroidal" as used herein refers to the area between the choroid and sclera. The term "subtenon" as used herein refers to the area posterior to the orbital septum, outside the sclera, below tenon's capsule. The term "subconjunctival" as used herein refers to the area between the conjunctiva and sclera. The term "intracameral" as used herein refers to "into a chamber" of the eye, for e.g., into the anterior or posterior chamber of the eye. The term "intrapalpebral" as used herein refers to into the eyelid. The term "cul-de-sac" as used herein refers to the space between the eyelid and globe. The term "retrobulbar" as used herein refers to behind the orbit of the eye. The term "peribulbar" as used herein refers to within the orbit or adjacent to the eye.

In another aspect, the invention relates to the use of the pharmaceutical formulation according to the invention for the treatment of a disease selected from the group consisting of autoimmune disease, immune dysregulation disease, carcinoma, sarcoma, glioma, melanoma, lymphoma, leukemia, chronic lymphocytic leukemia, follicular lymphoma, diffuse large B cell lymphoma, multiple myeloma, non-Hodgkin's lymphoma, Alzheimer's disease, type 1 or type 2 diabetes, amyloidosis, and atherosclerosis.

In another aspect, the invention relates to the use of the pharmaceutical formulation according to the invention in the preparation of a medicament for treatment of a disease selected from the group consisting of autoimmune disease, immune dysregulation disease, carcinoma, sarcoma, glioma, melanoma, lymphoma, leukemia, chronic lymphocytic leukemia, follicular lymphoma, diffuse large B cell lymphoma, multiple myeloma, non-Hodgkin's lymphoma, Alzheimer's disease, type 1 or type 2 diabetes, amyloidosis, and atherosclerosis.

In certain embodiments, the invention relates to the use of the pharmaceutical formulation according to the invention for the treatment of ophthalmic diseases. In certain embodiments, the invention relates to the use of the pharmaceutical formulation according to the invention in the preparation of a medicament for the treatment of ophthalmic diseases. The ocular disease may be any one of the ophthalmic diseases disclosed herein.

In another embodiment, the invention relates to a method of treating a disease selected from the group consisting of autoimmune disease, immune dysregulation disease, carcinoma, sarcoma, glioma, melanoma, lymphoma, leukemia, chronic lymphocytic leukemia, follicular lymphoma, diffuse large B cell lymphoma, multiple myeloma, non-Hodgkin's lymphoma, Alzheimer's disease, type 1 or type 2 diabetes, amyloidosis, and atherosclerosis, the method comprising the steps of (a) producing a pharmaceutical formulation according to the method of the invention; and (b) administering the pharmaceutical formulation to a subject in need thereof.

In a preferred embodiment, the invention relates to a method of treating a subject suffering from a disease selected from the group consisting of: autoimmune disease, immune dysregulation disease, carcinoma, sarcoma, glioma, melanoma, lymphoma, leukemia, chronic lymphocytic leukemia, follicular lymphoma, diffuse large B cell lymphoma, multiple myeloma, non-Hodgkin's lymphoma, Alzheimer's disease, type 1 or type 2 diabetes, amyloidosis, and atherosclerosis, the method comprising the steps of (a) producing a pharmaceutical formulation according to the method of the invention; and (b) administering the pharmaceutical formulation to a subject in need thereof, wherein the pharmaceutical composition is administered subcutaneously, intramuscularly or transdermally, in particular wherein the pharmaceutical composition is administered subcutaneously.

In another embodiment, the invention relates to a method of treating an ophthalmic disease, in particular an ocular vascular disease, the method comprising the steps of (a) producing a pharmaceutical formulation according to the method of the invention; and (b) administering the pharmaceutical formulation to a subject in need thereof. In certain embodiments, the pharmaceutical formulation for use in the method of treating an ophthalmic disease may comprise an antibody or antibody fragment that specifically binds to VEGF/VEGF-A and Ang-2. In certain embodiments, the pharmaceutical formulation for use in the method of treating an ophthalmic disease may be administered intraocularly or intravitreally.

In another embodiment, the invention relates to a method of subcutaneous, intramuscular or transdermal administration of a pharmaceutical formulation, the method comprising the steps of (a) producing a pharmaceutical formulation according to the method of the invention; and

(b) administering the pharmaceutical formulation to a subject subcutaneously, intramuscularly, transdermally.

In another embodiment, the invention relates to a method of ocularly administering a pharmaceutical formulation to a subject, the method comprising the steps of (a) producing a pharmaceutical formulation according to the method of the invention; and (b) administering the pharmaceutical formulation to a subject ocularly, such as subconjunctivally, intracamerally, intravitreally, subretinally, or suprachoroidally.

In another embodiment, the invention relates to a method of administering a pharmaceutical formulation to the brain of a subject, the method comprising the steps of (a) producing a pharmaceutical formulation according to the method of the invention; and (b) administering the pharmaceutical formulation to the brain of a subject, such as intralumbally, intrathecally, or intraventricularly.

In another embodiment, the invention relates to a method of intra-articularly administering a pharmaceutical formulation to a subject, the method comprising the steps of (a) producing a pharmaceutical formulation according to the method of the invention; and (b) administering the pharmaceutical formulation to a subject intra-articularly.

The term "administration", as used herein to refer to the delivery of an inventive pharmaceutical formulation to a subject, is not limited to any particular route but rather refers to any route accepted as appropriate by the medical community. For example, the present invention contemplates routes of delivering or administering that include, but are not limited to, subcutaneously, intramuscularly, transdermally, ocularly, such as subconjunctivally, intracamerally, intravitreally, subretinally, or suprachoroidally, to the brain, such as intralumbally, intrathecally, or intraventricularly, intra-articularly, or by inhalation.. In certain embodiments of the invention, administration is subcutaneously.

The term "subcutaneous," as used herein, refers to below the skin (e.g., in the connective tissue underlying the dermis and above the fascia of the muscle tissue).

The term "intramuscular" as used herein refers to the intramuscular route (IM route). In the IM route of administration, injections are made into the striated muscle fibers that lie beneath the subcutaneous layer.

The term "transdermal" as used herein means passage into and/or through skin or mucosa for localized or systemic delivery of an active agent.

Within the present invention, it is preferred that the pharmaceutical formulation according to the invention is administered to the subject in a therapeutically effective amount.

As used herein, the term “therapeutically effective amount” means an amount that is sufficient, when administered to an individual suffering from or susceptible to a disease, disorder, and/or condition, to treat the disease, disorder, and/or condition. Those of ordinary skill in the art will appreciate that the term “therapeutically effective amount” does not in fact require successful treatment be achieved in a particular individual. Rather, a therapeutically effective amount may be that amount that provides a particular desired pharmacological response when administered or delivered to a significant number of subjects in need of such treatment. It is specifically understood that particular subjects may, in fact, be “refractory” to a “therapeutically effective amount.” It is to be understood that the therapeutically effective amount may differ between pharmaceutical formulations comprising different proteins. However, the skilled person is aware of methods to determine the amount of the composition that is required to obtain the desired therapeutic effect.

In certain embodiments, the pharmaceutical formulation according to the invention is administered to a subject subcutaneously, intramuscularly, transdermally, ocularly, such as subconjunctivally, intracamerally, intravitreally, subretinally, or suprachoroidally, to the brain, such as intralumbally, intrathecally, or intraventricularly, intra-articularly, as a liquid composition with a protein concentration of at least 50 mg/mL, 60 mg/mL, 70 mg/mL, 80 mg/mL, 90 mg/mL, 100 mg/mL, 110 mg/mL, 120 mg/mL, 130 mg/mL, 140 mg/mL, 150 mg/mL, 160 mg/mL, 170 mg/mL, 180 mg/mL, 190 mg/mL or 200 mg/mL. The volume of the pharmaceutical formulation administered as a single dose may range from 0.1 to 10 mL, 0.1 to 9 mL, 0.1 to 8 mL, 0.1 to 7 mL, 0.1 to 6 mL, 0.1 to 5 mL, 0.1 to 4 mL, 0.1 to 3 mL, 0.1 to 2 mL, 0.1 to 1 mL, 1 to 3 mL, or 1 to 2 mL.

The term “subject” or “patient,” as used herein, refers to any animal to which the pharmaceutical formulation according to the invention may be delivered or administered. For example, a subject may be a human, dog, cat, cow, pig, horse, mouse, rat, gerbil, hamster etc. In many embodiments of the present invention, the subject is a human.

As used herein, the term “treatment” (also “treat” or “treating”) refers to any administration of a biologically active agent that partially or completely alleviates, ameliorates, relieves, inhibits, delays onset of, reduces severity of and/or reduces incidence of one or more symptoms or features of a particular disease, disorder, and/or condition. Such treatment may be of a subject who does not exhibit signs of the relevant disease, disorder and/or condition and/or of a subject who exhibits only early signs of the disease, disorder, and/or condition. Alternatively or

additionally, such treatment may be of a subject who exhibits one or more established signs of the relevant disease, disorder and/or condition.

While aspects of the invention are illustrated and described in detail in the Figures, Tables and in the foregoing description, such Figures, tables and description are to be considered illustrative or exemplary and not restrictive. Also reference signs in the claims should not be construed as limiting the scope.

It will also be understood that changes and modifications may be made by those of ordinary skill within the scope and spirit of the claims. In particular, the present invention covers further embodiments with any combination of features from different embodiments described above. It is also to be noted in this context that the invention covers all further features shown in the figures individually, although they may not have been described in the previous or following description. Also, single alternatives of the embodiments described in the figures and the description and single alternatives of features thereof can be disclaimed from the subject matter according to aspects of the invention.

Whenever the word "comprising" is used in the claims, it should not be construed to exclude other elements or steps. It should also be understood that the terms "essentially", "substantially", "about", "approximately" and the like used in connection with an attribute or a value may define the attribute or the value in an exact manner in the context of the present disclosure. The terms "essentially", "substantially", "about", "approximately" and the like could thus also be omitted when referring to the respective attribute or value. The terms "essentially", "substantially", "about", "approximately" when used with a value may mean the value $\pm 10\%$, preferably $\pm 5\%$.

A number of documents including patent applications, manufacturer's manuals and scientific publications are cited herein. The disclosure of these documents, while not considered relevant for the patentability of this invention, is herewith incorporated by reference in its entirety. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

BRIEF DISCRIPTION OF FIGURES

Figure 1: Visual aspect of RPC suspension obtained after mixing VEGF-Ang2 and dextran sulfate solutions at 1:1 mole-charge ratio in histidine buffer 20 mM pH 5.0.

Figure 2: Protein melting temperature before (naked protein) and after complexation (RPC) and dissociation.

Figure 3: DSC thermogram showing the melting temperature of a, VA2 in a complexed form ($T_m=144.2^{\circ}\text{C}$) and b, dextran sulfate sodium salt ($T_m=171.9^{\circ}\text{C}$) present in RPC formulations.

Figure 4: Visual aspect of RPC suspension at 200 mg/mL showing the paste-like aspect of the formulation. a, spatula kept face up; b, spatula turned face down.

Figure 5: Visual aspect of spray dried VA2 RPC.

Figure 6: SEM images of VA2 RPC particles after spray drying of the suspensions in histidine buffer and in Ultra pure water (MilliQ water).

Figure 7: VA2 RPC spray dried powder suspended in NAS solvents. EO, ethyl oleate; IDME, isosorbide dimethyl ether.

Figure 8: Percentages of complexation and dissociation of the different protein formats with dextran sulfate (DS) at 1:1 mole-charge ratio.

Figure 9 : Percentage complexation and dissociation of RPC using VEGF-Ang2 as a protein model and different complexing agents.* Complexation performed at pH 4.0. ** Dissociation performed with PBS 100 mM.

Figure 10: Percentages of complexation and dissociation of VA2 and DS at different protein concentrations.

Figure 11: RPC particle size obtained after complexation with DS at different protein concentrations.

Figure 12: Percentage complexation of VA2 and DS in histidine buffer 20 mM at different pH; and their corresponding percentage dissociation in PBS.

Figure 13: Percentage complexation of VA2 and DS in histidine buffer pH 5.0 at different ionic strengths; and their corresponding percentage dissociation in PBS.

Figure 14: Percentage complexation and dissociation of VA2 with DS in presence of different additives.

Figure 15: Visual aspect of the VA2 RPC in a, histidine buffer (control); b, in presence of different additives (sucrose, polysorbate 20, poloxamer 188 or mixture of those); c, in ultra pure water (MilliQ water) (no complexation).

Figure 16: Visual aspect of RPC formulations (F1-F4) and VA2 control DP solution after 4 weeks storage at different temperatures. Note hard-cake formation, gel-aspect and shrinkage of RPC suspensions after 4 weeks at 25 or 40°C. A, front view; B, bottom view.

Figure 17: Visual aspect of VA2 RPC 60 mg/mL a, before lyophilization; b and c, after lyophilization (b, front view; c, bottom view); d, after reconstitution of the lyophilized cake in PBS (120 mg/mL) stored 4-weeks at 5°C.

Figure 18: Visual aspect of RPC formulation after 4 weeks storage at different temperatures. Ctrl – control (in histidine buffer, no buffer exchange), C – centrifuged (in MilliQ, buffer exchange by centrifugation), D – dialyzed (in MilliQ, buffer exchange by dialysis), A – front view, B – bottom view before vortexing, C – bottom view after vortexing.

Figure 19: Comparison of visual aspect of the samples – “hard-cake” has formed in control sample (left), whereas particles in dialysed sample remained dispersed (right).

Figure 20: Comparison of particle size for RPC that had buffer exchanged to ultrapure water by means of centrifugation (A) or dialysis (B). The first results were obtained by laser diffraction measuring techniques (A), whereas the second by dynamic light scattering (B).

EXAMPLES

Aspects of the present invention are additionally described by way of the following illustrative non-limiting examples that provide a better understanding of embodiments of the present invention and of its many advantages. The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques used in the present invention to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should appreciate, in light of the present disclosure that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1: Development of reversible protein complex (RPC) formulations

1.1 Materials

Therapeutic proteins including monoclonal antibodies (mAbs), bispecific crossmAb, cytokine fusion mAb and DutaFab, were provided by F.Hoffmann-La Roche AG (Basel, Switzerland). Histidine-HCl and L-Histidine base were obtained from Ajinomoto (Osaka, Japan), citric acid and Trisodium citrate from Merck (Darmstadt, Germany), polysorbate 20 from Croda (East Yorkshire, UK), poloxamer 188 from BASF (Ludwigshafen, Germany) and sucrose from Pfanstiehl (Zug, Switzerland). Dextran sulfate sodium salt (DS), Sodium dodecyl sulfate (SDS), Chondroitin sulfate (CS), Sodium taurocholate hydrate (ST), Triacetin, Diethylene glycol monoethyl ether (Transcutol®), Isosorbide dimethyl ether, Tetraglycol (Glycofurol), Ethyl oleate, PBS tablets and PVDF filters were purchased from Sigma-Aldrich (Buchs, Switzerland). Slide-A-Lyzer Dialysis Cassettes (MWCO 10K) were obtained from Thermo Scientific. MilliQ water (resistivity > 18 MΩ cm) was prepared using a Merck Millipore MilliQ water purification system (Darmstadt, Germany). All solvents used were from an analytical grade.

1.2 Methods

1.2.1 Preparation of protein solutions and complexing agent solutions

The different protein solutions in histidine buffer (20 mM, pH 5.3-5.8), containing mainly sucrose, surfactants and/or sodium chloride; were dialyzed prior complexation to exchange the buffer with fresh histidine buffer (20 mM, pH 5.0). Dialysis was run during 2h at room

temperature then over night at 5 ± 3 °C. The dialyzed protein was further diluted to 5 mg/mL in histidine buffer (20 mM, pH 5.0).

Complexing agent solutions (Dextran sulfate sodium salt, Sodium dodecyl sulfate, Chondroitin sulfate, Sodium taurocholate hydrate) were prepared at 50 mg/mL in histidine buffer (20 mM, pH 5.0).

1.2.2 Protein content

Protein concentration was measured by UV absorbance at 280 nm using a spectrophotometer (NanoDrop One^c, ThermoFisher Scientific). In order to determine the protein concentration, RPC suspension was first dissociated using PBS 10 mM, then 4 µL were placed on the instrument pedestal for quantification.

1.2.3 Formation of reversible protein complexes

Proteins charge was calculated from their amino acid sequences, then the corresponding charge per mole was determined for each protein. The mole-charge was also determined for each of the complexing agents. Reversible protein complexes were prepared by mixing the protein solution (5 mg/mL) with the complexing agent solution (50 mg/mL) at 1:1 mole-charge ratio, aiming for a 100% charge neutralization. Total neutralization of the protein charge by the complexing agent leads to precipitation of the protein and formation of a whitish protein-particulate suspension.

Percentage complexation was determined after centrifugation of 1 mL sample of the RPC suspension (10 000 rpm, 5 min) and quantifying the amount of protein remaining in the supernatant using UV spectrometry (nanoDrop One^c, Thermo Scientific) according to the following equation:

$$\text{Complexation (\%)} = \frac{\text{Initial prot. conc} - \text{Prot. conc. in supernatant}}{\text{Initial protein concentration}}$$

1.2.4 Dissociation of reversible protein complexes

Reversible protein complexes were dissociated following pH increase by diluting the RPC suspension to 1 mg/mL final protein concentration in PBS 10 mM, pH 7.4. Proteins being uncharged at pH 7.4, charge interactions between the proteins and the complexing agents decrease leading to dissociation of the complexes.

Protein concentration following dissociation was determined by UV and the percentage dissociation was calculated according the following equation:

$$\text{Dissociation (\%)} = \frac{\text{conc. protein dissociated}}{\text{conc. protein complexed}}$$

1.2.5 Particle size

RPC particle size distribution was measured using a laser diffraction analyzer (Partica LA-960, HORIBA). RPC suspension was loaded in the sample bath of the instrument containing Ultra pure water (MilliQ water) to a concentration that allows 70-95% transmittance then the measurement was performed under circulation mode. Mean particle size values are reported. Particle size distribution of the spray dried RPC powder was also evaluated using SEM.

1.2.6 Zeta-potential

Particle surface charge was evaluated using Malvern Zetasizer Nano ZS. Particle surface charge of RPC was evaluated in both histidine buffer and Ultra pure water (MilliQ water) media. Since complexation only occurs under specific conditions, complexation was first performed in histidine buffer then dialysis was run against Ultra pure water (MilliQ water) to remove buffer ions. RPC suspensions in both media were diluted to 0.1 mg/mL in their corresponding media then samples (~0.8 mL) were loaded into the zeta potential cells for analysis.

1.2.7 Viscosity

Viscosity measurements were performed using a rheometer (Physica MCR 301, Anton Paar) equipped with a cone-plate geometry. The viscosity of RPC formulations was determined by placing 80 µL of the sample in the center of the plate. The method used consisted of 3 steps; 120 s for sample equilibration to 20°C in the first step, followed by a second step where 1000 s⁻¹ shear rate was applied for 10 s, then a last step where 1000 s⁻¹ shear rate was applied for 5 s.

1.2.8 Protein stability

Protein stability was evaluated before complexation and after complexation and dissociation. Protein purity was monitored using size exclusion chromatography (SEC) and protein charge was monitored using ion exchange chromatography (IEC).

1.2.9 Protein melting temperature

Melting temperature of reversible protein complexes was measured in both liquid form (suspension) using nanoDSF and solid form (spray-dried powder) by DSC.

1.2.9.1 Melting temperature by nanoDSF

Protein melting temperature was measured before complexation as naked protein, after complexation as RPC suspension, and after dissociation of RPC in PBS 10 mM. 30 μ L of each solution ($\sim$ 1.2 mg/mL) were placed in a 384 well microplate, transferred into a 48 capillary array ($\sim$ 10 μ L) then introduced in the instrument (Prometheus, nanoTemper). The method consists on applying a temperature ramp from 25 to 95°C with a 0.5°C/min heating rate. Excitation power was set at 28%, protein fluorescence intensities were recorded (ratio 350/330 nm) and melting temperature (transition midpoint T_m , 50% unfolded protein) was recorded.

1.2.9.2 Melting temperature by DSC

Protein melting temperature as RPC powder was measured after complexation and spray drying using DSC (Q2000, TA instruments). The sample was placed in the instrument ($\sim$ 50 mg) then the method consisted of an equilibration step at 25°C followed by a ramp of 5°C/min to -5°C and an isothermal step for 5 min, then a ramp of 2°C/min to 250°C and an isothermal step for 5 min and finally a ramp of 5°C/min to 25°C.

1.3 Results

Mixing the protein (VEGF-Ang2) solution with the complexing agent (dextran sulfate) solution in histidine buffer (20 mM pH 5.5) at 1:1 mole-charge ratio resulted in the formation of a whitish suspension of protein particles (**Fig.1**)

Mean particle size of the RPC suspension measured by laser diffraction was $10.8 \pm 0.8 \mu\text{m}$. Particles sedimentation was observed when formulation was left to stand, however, particles were easily resuspended after simple agitation.

Surface charge of the RPC particles in histidine buffer was $-13.9 \pm 0.6 \text{ mV}$; after buffer exchange (wash) with Ultra pure water (MilliQ water) using dialysis, the surface charge was $-43.6 \pm 0.5 \text{ mV}$.

Stability of the protein evaluated by SEC showed no significant difference before and after complexation and dissociation in PBS with percentages monomer of 95.2% and 95.3%,

respectively. Same observation using IEC with percentages main peak of 67.2% and 66.7%, respectively before and after complexation and dissociation in PBS.

Protein melting temperature (T_m) was determined in the control naked form (uncomplexed), after complexation as a suspension form and after dissociation of RPC in PBS. Results showed no significant difference in the T_m of the naked control protein (67.5°C) and after complexation and dissociation of the RPC (68.4°C) confirming the results observed by SEC and IEC concluding that complexation-dissociation process does not affect the stability of the protein (**Fig.2**). Interestingly, the complexed protein (RPC) does not show a clear inflection point suggesting that the T_m of the protein in the complexed form shifted to temperatures higher than 95°C (limit of the nanoDSF temperature range), which indicates a higher stability compared to the naked protein.

DSC was further used to determine the exact T_m of the protein in the complexed form (**Fig.3**). Results showed a shift of the protein T_m from 67.5°C to 144.2°C when it is complexed with DS, demonstrating a higher stability of the protein in the RPC form.

Example 2: Post-processing steps of reversible protein complex formulations

RPC concept was used to develop highly concentrated protein formulations. Two approaches were evaluated; the first consists on up-concentration of the RPC suspension in histidine buffer to a series of concentrations ranging from 60 mg/mL up to 200 mg/mL. The second approach consists on re-suspending the spray dried RPC particles in a non-aqueous solvent.

2.1 Up-concentration

RPC suspensions were up-concentrated by centrifugation (Eppendorf Centrifuge 5810 R). RPC suspension (5 mg/mL) was filled into falcon tubes (50 mL) containing magnetic stirrers then centrifuged (3900 rpm, 15 min, 5°C) for up-concentration. Supernatant was discarded (100% complexation) then depot were homogenized and pooled into one falcon tube. Intermediate concentration was determined by dissociating 10 μ L suspension in 990 μ L PBS 10 mM pH 7.4. Concentration was adjusted to 120 mg/mL by further centrifugation or dilution with histidine buffer.

For higher concentrations (up to 200 mg/mL), high-speed centrifuge (Beckman Coulter Optima L-90K Prep Ultracentrifuge) was used for up-concentration. RPC suspension was placed in a high-speed centrifuge tube (Beckman Coulter, 70 mL) containing magnetic stirrer then centrifuged at 10 000 rpm during 10 min at 5°C. Supernatant was removed then depot was

homogenized by vortex, 10 μ L were dissociated in 990 μ L PBS to evaluate the intermediate concentration. Centrifugation was carried on until reaching final target concentration of 200 mg/mL.

Up-concentration of RPC suspension up to 200 mg/mL resulted in a very dense white suspension with a paste-like aspect (**Fig.4**). Nevertheless, this formulation was injectable through a 26G needle. RPC suspensions at 180 and 160 mg/mL had a similar paste-like aspect, whereas RPC suspensions become more liquid at around 120 mg/mL, and RPC suspensions at 90 and 60 mg/mL were liquid.

Viscosity measurement of the different up-concentrated suspensions showed a shear thinning effect (viscosity decrease upon application of a constant shear rate). This feature renders the injectability of the formulation easier compared to a Newtonian solution. However, the viscosity measurement method used was developed for liquid solutions and was not appropriate for the paste-like RPC formulation.

2.2 Spray drying

Spray drying was performed using Büchi Mini Spray Dryer B-290. Inlet temperature was set at 115°C (outlet $\sim$ 48°C) and nitrogen aspiration was set at 100%. The feed rate (peristaltic pump) was set at 17 mL/min. RPC formulation was kept under stirring to avoid particle sedimentation during spray drying process.

Spray drying of VA2 RPC formulations at 5 mg/mL (F1-F3) resulted in a fine, white powder (**Fig.5**).

In order to optimize the protein content in the spray dried RPC powder and the stability of the protein following the spray drying process of RPC suspensions, different formulation compositions (F1 to F3) were evaluated (**Table 1**). RPC formulations were prepared as mentioned in Example 1, then the corresponding amounts of sucrose and polysorbate 20 were added to F1. Since the inventors targeted the highest protein content in the final powder, the inventors used the minimal excipients possible to limit their contribution to the final powder content while still ensuring protein stability during spray drying process. Given that the components of histidine buffer highly contribute to the final solid content of the RPC powder, histidine buffer was exchanged with ultra pure water (MilliQ water) using dialysis following complexation (F2 and F3). The corresponding amounts of sucrose and polysorbate 20 were then added to F3.

Table 1: Composition of RPC solutions at 5 mg/mL to be spray dried.

Excipient	Medium	Sucrose (mg/mL)	Polysorbate 20 (mg/mL)
F1	Histidine buffer	2.05	0.40
F2	Ultra pure water (MilliQ water)	-	-
F3	Ultra pure water (MilliQ water)	1.00	0.20

Following spray drying, protein content in the RPC powder was determined by UV after dissociation of a specific amount in PBS, and using the following the equation:

$$\text{Protein content (\%)} = \frac{\text{Actual VA2 conc.}}{\text{Theoretical VA2 conc.}}$$

Protein stability was evaluated by SEC and IEC and protein melting temperature was determined by DSC in RPC as powder form and by nanoDSF after solubilisation in PBS.

Scanning electron microscope (SEM) analysis of F1 and F3 particles obtained after spray drying showed loose, round and dispersed particles in VA2 RPC washed with Ultra pure water (MilliQ water) (F3, **Fig.6**), while particles in VA2 RPC in histidine buffer were more agglomerated forming clusters and fused-like particles (F1, **Fig.6**). Whether this difference in particle shape is due to the different media used or to the spray drying process itself is to be further investigated.

Analysis of VA2 RPC particles in histidine buffer versus Ultra pure water (MilliQ water) before and after spray drying showed an increase in the particle size after spray drying, from ~8 µm to ~30 µm (**Table 2**), probably due to the adsorption of the excipients added (sucrose and PS20) on the RPC particles and possible cluster formation during the spray drying process.

Table 2: VA2 RPC particle size in histidine buffer versus Ultra pure water (MilliQ water) before and after spray drying.

Formulation	Mean (µm)	Median (µm)
F1 – Before SD	7.2	6.4
F1 – After SD	30.6	20.1

F3 – Before SD	8.7	7.3
F3 – After SD	33.5	8.2

As expected, protein content in the spray dried powder varied in the formulations (F1-F3) according to the amount of the buffer salts and the excipients added (**Table 3**).

Table 3: Protein content and stability in VA2 RPC spray dried powder.

	VA2:DS	Medium	Excipients	VA2 cont. (%)	SEC (% mono)	IEC (% main peak)
F1	1:1	Histidine buffer 20 mM pH 5.0	DS 0.84 mg/mL Sucrose 2.1 mg/mL PS20 0.4 mg/mL	43.1	97.1	65.2
F2	1:1	Ultra pure water (MilliQ water)	DS 0.84 mg/mL	70.2	94.6	66.8
F3	1:0.6	Ultra pure water (MilliQ water)	DS 0.56 mg/mL Sucrose 1 mg/mL PS20 0.2 mg/mL	63.0	95.5	66.1

Protein content in F1 was 43.1%, with a high contribution of histidine buffer salts to the solid content of the spray dried powder. Removal of the excipient and washing out histidine buffer with MQ water in F2 increased the protein content up to 70.2%. Decreasing the protein:DS mole-charge ratio to 1:0.6, followed by a wash out of the histidine buffer salts and a decrease of the amount of excipients added in F3 led to a final protein content of 63.0%.

Protein stability during spray drying process was assessed by SEC and IEC. No significant change was observed in the chemical stability (IEC) in all formulations. However, an increase in the high molecular weight species (HMWS) was observed by SEC, especially in F2, probably due to the absence of histidine buffer and excipients to protect the RPC during the spray drying process. Indeed, in F3, adding half excipients could decrease the HMW species. Hence, F3 seems to be a good compromise to optimize the protein content in the final spray dried powder and ensure the protein stability during the spray drying process.

2.3 Resuspension of spray dried RPCs

A series of apolar solvents were tested as resuspension media including ethyl oleate, triacetin, Transcutol (diethylene glycol monoethyl ether), Glycofurol, and Isosorbide dimethyl ether. RPC spray dried powder was incorporated in the corresponding volume of the solvent to reach 100, 150, 200 and 250 mg/mL then homogenized.

RPC dissociation after up-concentration or resuspension in non-aqueous media was evaluated after dilution in PBS. Melting temperature of the spray dried RPC powder was evaluated by DSC and using nanoDSF after dissociation in PBS. Protein stability after up-concentration or resuspension in non-aqueous media was evaluated by SEC and IEC. Particle size and viscosity of the high-concentration formulations were evaluated as mentioned in Example 1.

Suspension of VA2 RPC spray dried powder in NAS resulted in white suspensions (**Fig. 7**).

After dissolving 110 mg of RPC spray dried powder in 0.25 mL PBS, the final volume was 0.29 mL. The contribution of the solid content is 0.04 mL (~14% of the total volume). This volume was taken into account when preparing the VA2 RPC-NAS suspensions at different concentrations (ex. spray dried powder was dissolved in 0.26 mL NAS for a total volume of 0.3 mL). The target and actual concentrations of VA2 in NAS are listed in **Table 4**.

Table 4: Target and actual protein concentrations in NAS, their corresponding viscosity and stability (SEC and IEC).

NAS	Target Conc (mg/mL)	Actual Conc (mg/mL)	SD (mg/mL)	Viscosity (cP)	SEC (% Mono)	IEC (% Main p.)
Diethylene glycol monoethyl ether	180	196.7	11.8	20		
	230	238.3	6.2	-	93.4	64.7
	280	258.3	2.4	30		
Isosorbide dimethyl ether	180	186.7	8.5	-		
	230	220.0	4.1	28	89.6	61.9
	280	231.7	4.7	-		
Glycofurol	180	188.3	4.7	-		
	230	210.0	7.1	60	88.1	61.7
	280	241.7	4.7	-		

Ethyl oleate	180	157.3	6.8	-		
	230	223.3	20.5	50	94.9	64.2
	280	237.3	1.9	-		
Triacetin	180	183.3	6.2	-		
	230	193.3	6.2	70	95.0	64.2
	280	283.3	8.5	100		
PBS	200	213	3.2	275	95.5	66.2

Dissociation of VA2 RPC SD powder in PBS 10 mM at 213 mg/mL resulted in very viscous solution (275 cP, **Table 4**). Suspension of VA2 RPC SD powder in NAS resulted in lower viscosity compared to PBS ranging from 20 to 70 cP for concentrations ranging from 193 to 223 mg/mL (**Table 4**). Protein stability evaluation after a short incubation time with the NAS showed an increase in the HMWS within Glycofurol and IDME. Protein aggregation was also observed within Triacetin, Transcutol and EO but at a lower extent (**Table 4**). Results from IEC also showed higher stability within Triacetin, Transcutol and EO compared to Glycofurol and IDME. Even though there is a reduction in the viscosity, injectability of the solutions was not possible through a 26G needle due to a large particle size.

Example 3: Universality of the concept

3.1 Universality of the concept using different proteins

Feasibility of RPC was evaluated using different protein formats (mAbs, bispecific crossmAb, cytokine fusion mAb, DutaFab) and dextran sulfate as the complexing agent at 1:1 mole-charge ratio.

The inventors have used dextran sulfate with 40 kDa molecular weight as the complexing agent. It is reported that DS has an average of two negative charges per monomer, corresponding to 240 negative charges per mole polymer. The number of positive charges of each protein was calculated from the amino acid sequence and are summarized in **Table 5**. Accordingly, the determined protein:DS complexation weight ratios corresponding to a 1:1 mole-charge ratio between each protein and DS are summarized in **Table 5**.

Table 5: Weight ratios between proteins and the complexing agent, dextran sulfate (DS), corresponding to 1:1 mole-charge ratio, used to prepare reversible protein complexes.

Proteins	MW (kDa)	Total (+) charges	Weight ratio	
			Protein	DS
VEGF-Ang 2	146	146	1	0.167
aSyn-mAb	145	152	1	0.175
FAP-OX40	195	188	1	0.160
VEGF-PDGF DutaFab	48	52	1	0.181
Bevacizumab	149	150	1	0.168
Pertuzumab	148	148	1	0.167
Gantenerumab	146	156	1	0.178

The study showed that RPC formation was possible with the different proteins tested corresponding to different protein formats (mAbs, bispecific crossmAbs, fusion mAbs, DutaFabs) with percentage complexation ranging from $96.1 \pm 1.4\%$ to $99.5 \pm 0.3\%$ (**Fig. 8**). The reversibility of the concept was also confirmed with regards to the different protein formats with percentage dissociation ranging from $96.5 \pm 1.6\%$ to $105.4 \pm 0.8\%$ in PBS 10 mM. Both complexation and dissociation were instantaneous at room temperature. RPC concept is hence applicable to a range a wide range of biologics.

Protein complexation with DS followed by dissociation in PBS 10 mM did not affect the stability of the protein. SEC and IEC results showed no significant difference between the percentages of monomers and main peaks obtained with the different proteins tested before and after complexation and dissociation (**Table 6**).

Table 6: Percentage monomer by SEC and main peak by IEC obtained with the different proteins before complexation and after complexation-dissociation. * Analytical methods not available.

Proteins	% Monomer by SEC		% Main Peak by IEC	
	Before complexation	After dissociation	Before complexation	After dissociation
VEGF-Ang 2	95.2	95.3	67.2	66.7
aSyn-mAb	99.0	98.6	*	*
FAP-OX40	*	*	*	*
VEGF-PDGF DutaFab	99.1	98.8	88.7	86.9
Bevacizumab	97.2	97.5	71.3	69.8
Pertuzumab	99.4	98.6	62.1	61.5

Gantenerumab	97.7	98.4	49.0	49.1
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3.2 Universality of the concept using different complexing agents

Feasibility of the RPC was also evaluated using different complexing agents (CA) including dextran sulfate (DS), chondroitin sulfate (CS), SDS and sodium taurocholate (ST), with a bispecific cross mAb (VEGF-Ang2) as the protein model. Protein solution was prepared at 5 mg/mL and complexing agents' solutions were prepared at 50 mg/mL in histidine buffer 20 mM pH 5.0, complexation was performed at different prot:CA mole-charge ratios.

The optimized Protein (VEGF-Ang2) to the complexing agent's mole-charge ratios and the corresponding volume ratios are summarized in **Table 7**. Typically, with VEGF-Ang2 and DS, the inventors previously assessed 100 % complexation and dissociation at 1:1 mole-charge ratio. The ratio optimization showed that the same complexation-dissociation efficiency could be reached even at 1:0.6 ratio, corresponding to the minimal DS to be added for a total complexation of the protein. This suggests that the theoretical charge calculation method based on the protein sequence overestimated the number of positive charges available for complexation by DS, thus the actual amount of DS needed to provide 100% charge neutralization is lower than calculated. It is important to note that the inventors were considering the total number of positive charges distributed over the protein and not the protein net charge, as the protein net charge would underestimate the number of positive charges by charge addition.

Table 7: Optimized mole-charge and weight ratio between VEGF-Ang2 and different complexing agents (CA) used to prepare reversible protein complexes (RPC).

Complexing agent (CA)	Mole-charge ratio		Weight ratio	
	Protein	CA	Protein	CA
Chondroitin sulfate (CS)	1	0.2	1	0.1
Dextran sulfate (DS)	1	0.6	1	0.1
Sodium dodecyl sulfate (SDS)	1	0.7	1	0.2
Sodium taurocholate (ST)	1	4.0	1	2.0

When adding DS, SDS and CS to the protein solution, complexation occurs at pH 5.0 (~ 100%). When using ST, complexation occurs only after adjusting the pH of the buffer solution to 4.0; moreover, 3 hours incubation time are needed to reach 100% complexation (**Fig. 9**).

Dissociation of the RPC formed with DS or CS is instant and complete. Dissociation of RPC formed using SDS and ST require more time; the use of PBS with a higher ionic strength (100 mM) accelerated the dissociation process (96.1% and 89.4%, respectively for SDS and ST, Fig.5).

SEC analysis showed a good stability of protein after complexation and dissociation when using DS and CS. However, ST and even more, SDS, significantly degraded the protein. These results were also confirmed by IEC (Table 8).

Table 8: Percentage monomer by SEC and main peak by IEC of a bispecific mAb obtained before complexation and after complexation-dissociation using different complexing agents.

Complexing agent (CA)	% Monomer by SEC		% Main Peak by IEC	
	Before complexation	After dissociation	Before complexation	After dissociation
Chondroitin sulfate	97.3	96.7	66.1	65.2
Dextran sulfate		95.2		66.7
Sodium dodecyl sulfate		21.1		61.1
Sodium taurocholate		90.1		62.9

Example 4: Robustness of the concept

Robustness of the RPC formation was evaluated along a range of protein concentration, buffer type, pH and ionic strength to determine the optimal conditions for RPC formation.

4.1 Effect of protein concentration

RPC formation was evaluated using protein concentrations ranging from 1 to 100 mg/mL in histidine buffer. VEGF-Ang2 dialyzed stock solution (130 mg/mL) was diluted to 100, 50, 40, 30, 25, 20, 5 and 1 mg/mL in histidine buffer 20 mM pH 5.0. The corresponding volume of DS (50 mg/mL) was added to 1 mL protein solution of every concentration at 1:1 mole charge ratio (1:0.167 weight ratio). Percentage complexation was evaluated after centrifugation and percentage dissociation was evaluated in PBS 10 mM.

Percentages of complexation and dissociation of VA2 and DS were determined at different protein concentrations (Fig.10). Results showed that complexation occurs at any protein concentration, from 1 mg/mL to at least 100 mg/mL (highest concentration evaluated). Although, percentage complexation ranged from 96.7 ± 2.4 % to 99.5 ± 0.0 % at protein

concentration ranging from 1 mg/mL to 40 mg/mL; complexation at concentrations higher than 40 mg/mL are limited because of the high viscosity associated with the high protein concentration, hindering the complexing agent from spreading over the solution and reaching every protein molecule to achieve a homogenous protein complexation.

Percentages of dissociation of the complexes formed at protein concentration from 1 mg/mL to 40 mg/mL ranged from 98.1 ± 2.3 % to 82.3 ± 5.4 %, with lower dissociation percentages at higher concentrations. Thus, the optimal protein concentration range for complete complexation and dissociation is defined to be from 1 mg/mL to 5 mg/mL (**Fig.10**).

Initial protein concentration used for complexation was also found to have an effect on the final RPC particle size distribution (**Table 9** and **Fig. 11**).

Table 9: RPC particle size after complexation with DS at different protein concentrations.

Protein concentration	Mean (μm)	Median (μm)	SD (μm)
1 mg/mL	7.8	7.5	2.1
5 mg/mL	11.1	8.0	13.8
20 mg/mL	21.6	19.4	10.3

4.2 Effect of buffer strength and pH on complexation

RPC formation was evaluated using a range of histidine buffers with ionic strengths ranging from 5 to 50 mM and pH ranging from 1 to 7; and within citrate buffer 10-20 mM pH 5.0 at 5 mg/mL protein concentration and 1:1 mole-charge ratio with DS. RPC formation was also evaluated using ultra pure water (MilliQ water) as medium. Protein stock solution was dialyzed against ultra pure water (MilliQ water) then diluted to 5 mg/mL in ultra pure water (MilliQ water). The complexation was prepared by mixing the protein solution with the DS (50 mg/mL in ultra pure water (MilliQ water)) solution at 1:1 mole-charge ratio. Percentage complexation was evaluated after centrifugation and percentage dissociation was evaluated in PBS 10 mM.

Percentage complexation was evaluated within histidine buffer 20 mM at different pH ranging from 1 to 7 (**Fig. 12**). Their corresponding percentage dissociation were determined in PBS.

Percentages complexation ranged from 98.6 ± 1.0 % to 99.2 ± 0.9 % from pH 1 to 5.5, 93.5 ± 0.2 % at pH 6 and 1.3 ± 0.4 % at pH 7. The corresponding percentages dissociation ranged from 98.1 ± 4.6 % to 109.2 ± 3.8 % (**Fig. 12**). Thus, the optimal buffer pH range for complexation is

from 4.5 to 5.5 in order to ensure complete complexation and protein stability (strong acidic pH may degrade protein).

On the other hand, percentage complexation was evaluated in histidine buffer pH 5 at different ionic strengths ranging from 5 mM to 50 mM (**Fig. 13**). Their corresponding percentage dissociation were determined in PBS.

Percentages complexation ranged from 98.6 ± 1.0 % to 99.9 ± 0.1 % for ionic strengths ranging from 20 mM to 50 mM, 82.2 ± 0.2 % at 10 mM and 66.3 ± 0.3 % at 5 mM. The corresponding percentages dissociation ranged from 98.9 ± 1.4 % to 109.9 ± 0.9 % (**Fig. 13**). Although, RPC formulation with histidine buffer 50 mM showed a precipitation of the particles followed by the formation of a gel-like depot. Thus, the optimal buffer ionic strength range for complexation is from 20 mM to 30 mM in order to ensure complete complexation and formulation stability.

The inventors mainly used histidine buffer in this study, although, complexation also occurs using other buffers including citrate buffer 20 mM pH 5 with a percentage complexation of 97.2 ± 2.0 % and a percentage dissociation of 108.0 ± 1.1 %.

4.3 Effect of buffer strength, pH and volume on dissociation

Complex dissociation was evaluated using different media including PBS 10 mM (pH 7.4, NaCl 137 mM), phosphate buffer 10 mM (pH 7.4) and histidine buffer 20 mM containing saline (pH 5.0, NaCl 137 mM). RPC suspension was diluted in the corresponding media to 1 mg/mL, vortexed then dissociation was evaluated by visual inspection of the samples; when the solution is clear, protein content was measured by UV to determine the percentage of dissociation.

PBS (10 mM pH 7.4) volume needed for total instantaneous dissociation of RPC 5 mg/mL was evaluated at different RPC:PBS dilution ratios; 1:4, 1:2, 1:1.5 and 1:1 by mixing 100, 100, 200, 250 μ L RPC with respectively 400, 300, 300, 250 μ L PBS 10 mM. Percentage dissociation and pH of the final solutions were determined.

Different buffer ionic strengths and pH were tested to dissociate RPC particles. RPC particles dissociate instantly and completely within PBS resulting in a clear solution. RPC dilution in phosphate buffer 10 mM pH 7.4 resulted in a turbid solution due to incomplete dissociation of the RPC particles (~50% dissociation); addition of NaCl enabled total dissociation of the RPC particles. Dissociation of RPC particles in histidine buffer 20 mM pH 5.0 containing saline was even lower (~17%), where addition of NaCl also enabled total dissociation of the RPC particles. Thus, the best buffer for complete RPC dissociation is PBS 10 mM pH 7.4 (100 mM can also

be used in some cases to accelerate the dissociation). In terms of PBS volume, a ratio PBS:RPC of at least 2:1 is required for total dissociation of RPC particles, resulting in pH increases to at least pH 6.5 in the formulation for total dissociation.

4.4 Effect of excipients

The effect of generic excipients used in protein formulations on the RPC formation was evaluated by adding sucrose and/or surfactants to the formulation buffer. Sucrose, polysorbate 20 and/or poloxamer 188 were added to the protein solution diluted to 5 mg/mL in histidine buffer 20 mM pH 5.0 (formulations F1 to F6, **Table 10**) prior complexation with DS (1:1 mole-charge ratio) then the corresponding percentages of complexation and dissociation were determined.

Table 10: Additional excipients to RPC formulations in histidine buffer.

Excipient	F1	F2	F3	F4	F5	F6
Sucrose	-	240 mM	-	-	240 mM	240 mM
Polysorbate 20	-	-	0.05%	-	0.05%	-
Poloxamer 188	-	-	-	0.05%	-	0.05%

The presence of the additives (sucrose, polysorbate 20 and/or poloxamer 188) did not affect the RPC formation. Percentage complexation ranged from 99.2 to 99.6%. Additives did not affect RPC dissociation in PBS neither, with percentages ranging from 92.3 % to 97.5% (**Fig. 14, Fig. 15**).

Complexation of VA2 with DS in ultra pure water (MilliQ water) instead of histidine buffer was not possible. Buffer is necessary for RPC formation (**Fig. 15**).

Example 5: Short-term stability study

A short-term study was conducted to evaluate the stability of the protein in RPC formulation (120 mg/mL) at different temperatures, in presence or absence of additives (sucrose, Poloxamer 188, polysorbate 20). RPC formulations (F1-F4) were formulated in histidine buffer and compared to formulation F5 where histidine buffer was exchanged with ultra pure water (MilliQ water) by dialysis.

Lyophilization of RPC suspension at 60 mg/mL (F6) was successful and resulted in a homogenous cake that was easily reconstituted (and dissociated) to a final concentration of 120 mg/mL in PBS resulting in a transparent solution (Fig. 17).

In order to evaluate the feasibility of freeze drying and reconstitution of RPC suspensions, F6 was up concentrated to 60 mg/mL, 2 mL were filled into 6-mL vials then samples were placed in the lyophilizer for freeze drying. F6 is meant to be reconstituted in 0.85 mL PBS for a final concentration of 120 mg/mL VA2, 240 mM sucrose and 0.05 % PS 20.

Stability of the protein in RPC formulations was compared to the standard protein solution (F7; drug product liquid solution 120 mg/mL).

0.5 mL of formulations F1-F5 and F7 were filled into 2-mL glass vials then stored with the lyophilized samples in the stability chambers (5, 25 and 40°C) for four weeks. Composition of the different formulations is listed in Table 11.

Stability of the formulations was monitored at different time points in terms of visual aspect, protein content (UV), physical and chemical stability (SEC, IEC) and viscosity.

Table 11: Composition of the different formulations prepared for the stability study.

Formulation	Buffer	Sucrose (mM)	NaCl (mM)	DS (mM)	Methionine (mM)	Polysorbate 20 (% w/v)	Poloxamer 188 (% w/v)
F1		-	-	0.5	-	-	-
F2	Histidine 20 mM pH 5.0	240	-	0.5	-	-	-
F3		240	-	0.5	-	0.05	-
F4		240	-	0.5	-	-	0.05
F6		120	-	0.25	-	0.025	-
F5	Ultra pure water (MilliQ water)	-	-	0.5	-	-	-
F7	Histidine acetate	160	25	-	7	0.04	-

20 mM
pH 5.8

RPC formulations up-concentrated to 120 mg/mL (F1-F4) were liquid suspensions at initial time and no change was observed after 4-weeks storage of the different formulations at 5°C. However, after 4-weeks storage at 25°C or 40°C, few visual changes were observed including the formation of a hard-cake, which in some formulations formed a shrunk pellet with or without a gel-aspect (**Fig. 16**). These hard cakes are irreversible, a magnetic stirrer and a vortex are needed to resuspend the RPC particles and reconstitute the suspension. Another interesting observation was during dissociation in PBS. After 4-weeks storage at 5°C and 25°C, RPC particles dissociated completely; however, dissociation of RPC particles in formulations stored at 40°C was partial forming a turbid solution upon dilution in PBS.

Interestingly, the hard cakes observed in F1-F4 formulations after 4-weeks storage at 25°C and 40°C were not observed in F5.

5.1 Protein content recovery after dissociation

Protein concentration measured in the different RPC suspensions (F1-F5) following dissociation in PBS showed 92.7% to 107.0% recovery of the protein content after 4-weeks storage at 5°C and 25°C. However, after 4-weeks storage at 40°C, protein content recovery ranged from 8.7% to 16.8%, resulting from incomplete dissociation of RPC particles (**Table 12**).

Total protein content was recovered following reconstitution of RPC lyophilisates (F6) in PBS after 4-weeks storage at 5, 25 and 40°C with percentage recovery ranging from 101.1±1.6 to 105.0±0.4 (**Table 12**). Lyophilisation of RPC suspension could solve the incomplete dissociation observed after storage of RPC suspension at 40°C.

Protein content in F7 remains stable along the storage period at all temperatures (**Table 12**).

Table 12: Initial protein concentration and protein content recovery after 4-weeks storage of VA2 RPC formulations (F1-F5) and VA2 control solution (F6) at different temperatures.

Initial	4 weeks 5°C		4 weeks 25°C		4 weeks 40°C	
Conc.	Conc.	Recovery	Conc.	Recovery	Conc.	Recovery
mg/mL	mg/mL	%	mg/mL	%	mg/mL	%

F1	127.0 ± 2.2	124.7±1.2	98.2±0.9	117.7±1.2	92.7±0.9	11.0±0.8	8.7±0.6
F2	121.7 ± 1.2	124.3±2.1	102.2±1.7	122.3±2.9	100.5±2.4	20.0±4.2	16.4±3.5
F3	122.7±0.9	131.3±1.7	107.0±1.4	128.7±5.8	104.9±4.7	14.7±0.5	12.0±0.5
F4	128.0±0.8	136.7±1.7	106.8±1.3	135.7±2.1	106.0±1.6	16.3±1.7	12.7±1.3
F5	115.3±0.5	113.0±0.8	98.0±0.8	108.0±2.2	93.6±1.5	19.3±3.1	16.8±2.7
F6	126.3±1.2	131.0±0.8	103.7±1.0	127.7±0.9	101.1±1.6	132.7±0.9	105.0±0.4
F7	112.7±0.5	118.0±1.4	-	118.7±1.2	-	118.0±2.4	-

5.2 Protein stability

SEC and IEC analysis of the protein in RPC formulations (F1-F4) after 4-weeks storage showed a good stability at 5°C following dissociation in PBS. After 4-weeks storage at 25°C, 1.9% to 2.5% loss in the monomer was observed by SEC and 5.1% to 5.7% loss in the main peak was observed by IEC. After 4-weeks storage at 40°C, 22.0% to 28.8% loss in the monomer was observed by SEC and 41.6% to 45.7% loss in the main peak was observed by IEC (**Table 13**).

SEC analysis clearly showed that VA2 RPC suspension is not stable in ultra pure water (MilliQ water) (F5) as 8.7% loss in the monomer was observed after 4-weeks at 25°C and 55.4% at 40°C.

No significant difference was observed before and after freeze drying process (F6) with percentages monomer obtained by SEC analysis of 96.5% and 96.8%, respectively. Follow up of the percentage monomer in RPC lyophilizate showed stable monomer after at least 4-weeks at 5°C and 25°C; 1.5% loss in the monomer was observed at 40°C. Similarly, no significant difference was observed by IEC analysis before and after freeze drying process with percentages main peak of 66.1% and 66.3%, respectively. Follow up of the percentage main peak in RPC lyophilizate showed a stable main peak after 4-weeks at 5°C, 1.7% loss at 25°C and 5.4% loss at 40°C.

Protein percentage of monomer by SEC in control formulation (F7) remains stable after 4-weeks storage at all temperatures; percentage main peak by IEC showed good stability at 5°C after 4-weeks storage, 2.4% and 22.9% loss in the main peak at 25°C and 40°C, respectively.

5.3 Viscosity

Viscosity measurements showed no significant difference between the viscosities of RPC suspensions in histidine buffer (F1-F4); the different excipients seem not to contribute significantly to the final RPC viscosity. Overall, viscosity of VA2 formulations was lower in RPC suspensions compared to the control drug product solution (F7) with 10-12 cP *versus* 20 cP, respectively. There is a clear drop in the viscosity after exchanging histidine buffer with ultra pure water (MilliQ water) (F5), from 10 cP to 4 cP. After reconstitution of the lyophilized RPC suspension in PBS (F6), viscosity increased slightly compared to RPC suspension (16 cP), however, still inferior to the control (F7) (**Table 13**).

Table 13: Initial viscosity and mean percentage monomer by SEC and percentage main peak by IEC after 4-weeks storage of VA2 RPC and control formulations (120 mg/mL) at different temperatures.

	Dosage form	Viscosity (cP)	SEC (% monomer)				IEC (% main peak)			
			Initial	4-weeks			Initial	4-weeks		
				5°C	25°C	40°C		5°C	25°C	40°C
F1	RPC	10	95.1	96.0	92.7	66.2	66.2	68.1	61.1	20.5
F2	suspension	11	95.2	96.1	93.3	73.2	66.4	68.2	61.1	24.8
F3	Histidine buffer	12	95.1	96.3	92.9	71.0	66.8	68.1	61.1	23.2
F4		11	95.4	96.4	92.9	69.8	66.5	68.3	61.1	23.7
F5	RPC suspension Ultra pure water (MilliQ water)	4	95.6	95.1	86.4	39.7	*	*	*	*
F6	RPC suspension Lyophilized	16**	96.8	96.8	96.5	95.3	66.3	66.0	64.6	60.8
F7	Control solution	20	96.0	96.7	96.4	96.0	67.9	68.2	65.5	44.9

*Analysis not performed.** After reconstitution in PBS.

Example 6: Dialysis of RPCs

In previous examples “hard-cake” formation has been observed upon storage of certain RPC samples at 25°C and 40°C. This phenomenon affects visual aspect of the samples (the sample

can appear “shrunk”) and complete resuspension of the particles is no longer possible, even if agitation is applied. Exchanging initial sample buffer (20 mM histidine buffer pH 5) with identical but fresh buffer or ultrapure water has resulted in improved sample behaviour at equal storage conditions. Buffer exchange has been performed by means of centrifugation where the protein-polymer complexes (RPC particles) had been sedimented, the supernatant was removed and the complexes in precipitate were then resuspended in fresh buffer or ultrapure water. Analyses of the samples have shown comparable particle size of RPC complexes in fresh histidine buffer and ultrapure water (i.e. in μm range) and lower physical stability of dissociated protein in ultrapure water when analyzed by size exclusion chromatography.

The aim of the subsequent experiments was to explore alternative methods for buffer exchange (e.g. dialysis) and further investigate as well as confirm improved visual aspect of samples treated in this manner.

Methods

VEGF-Ang 2 (VA2) protein stock solution was prepared in 20 mM histidine buffer (pH 5) at a concentration of 5 mg/mL. For formation of protein-polymer complexes, 50 mg/mL complexing polymer (dextran sulfate sodium salt) solution in 20 mM histidine buffer (pH 5) was gradually added to the protein at constant mixing on a magnetic stirrer.

The prepared samples were always assessed for the % of complexation which was found to be > 98%.

In the next step, complexed solution was split into 3 parts with equal volumes – one part was dialysed against ultrapure water, another against fresh histidine buffer and the latter served as control and was not further manipulated. The dialysis was carried out using dialysis cassettes or tubings with MWCO of 10-100 kDa.

After dialysis, the samples were recovered and the protein concentration was adjusted to 120 mg/mL by means of centrifugation. The precipitate containing protein-polymer complexes was collected and the supernatant discarded.

The obtained samples were filled in 2 mL glass vials, stoppered, crimped and stored at different conditions (i.e. 5°C, 25°C, 30°C or 40°C).

At predefined time points they were characterized with regard to visual aspect and particle size prior dissociation of protein-polymer complexes in 10 mM phosphate buffer saline (pH 7.4) by

means of laser diffraction or dynamic light scattering techniques (depending on the expected particle size). After dissociation, size and charge variants of the protein were determined by size exclusion and ion exchange chromatography respectively.

Results

“Hard-cake” formation has not been observed for dialysed samples (either dialysed against ultrapure water or fresh histidine buffer) when stored for 1 month at any of the storage conditions. For samples dialysed against ultrapure water, the suspension appeared more liquid and lower amounts of particles have sedimented, whereas samples dialysed against histidine buffer resulted as more “pasty” and dense. Full resuspension was possible by agitation. In contrast to that, “hard-cake” formation has been detected in control samples stored at 25°C and 40°C (**FIG. 18** and **FIG. 19**).

Surprisingly, for samples dialysed against ultrapure water, the particle size measured has been significantly lower than for samples in histidine buffer (control or dialysed against fresh buffer). For the first time, it has been in nm range, whereas for the latter (as previously measured and reported) in μm range (**FIG. 20**). Additionally, the particle size of RPC when buffer was exchanged to ultrapure water by means of centrifugation has also been in μm range, which may imply that buffer exchange by means of dialysis results in better “rinsing” of the particles and more efficient buffer exchange.

Incomplete dissociation of complexes in samples stored at 40°C was still observed, meaning full recovery of complexed protein was not possible (**Table 14**).

Stability of samples dialysed against ultrapure water was lower in comparison to the control (**Table 14**).

Table 14: Percentage monomer by SEC and main peak by IEC obtained for RPC suspension control and RPC suspension dialyzed against ultra pure water (MilliQ).

Dosage form	SEC (% monomer)				IEC (% main peak)			
	Initial	4-weeks			Initial	4-weeks		
		5°C	25°C	40°C		5°C	25°C	40°C
RPC suspension	97.5	97.6	96.5	*	73.5	73.9	66.1	*
Control								

RPC									
suspension	97.2	97.9	91.3	*	72.6	73.3	60.7	*	
MilliQ									

* Analysis not performed

CLAIMS

1. A method for producing a composition comprising reversible protein complexes (RPCs), the method comprising the steps of:
 - a) contacting a protein and a complexing agent in a buffer solution, wherein the complexing agent is dextran sulphate or chondroitin sulphate, and wherein the protein and the complexing agent have opposite net charges when comprised in the buffer solution;
 - b) formation of RPCs between the protein and the complexing agent in the buffer solution; and
 - c) obtaining a suspension comprising the RPCs formed in step (b).
2. The method according to claim 1, wherein the complexing agent is dextran sulphate, in particular dextran sulphate with 40 kDa molecular weight.
3. The method according to claim 1 or 2, wherein the pH of the buffer solution is adjusted to be lower than the isoelectric point of the protein.
4. The method according to any one of claims 1 to 3, wherein the pH of the buffer solution is adjusted to 2 to 5 pH units below the isoelectric point of the protein, in particular 3 pH units below the isoelectric point of the protein.
5. The method according to claim 1 or 2, wherein the buffer solution has a pH ranging from 1 to 6, in particular wherein the buffer solution has a pH ranging from 3 to 6, in particular wherein the buffer solution has a pH ranging from 4.5 to 5.5.
6. The method according to any one of claims 1 to 5, wherein the buffer solution has an ionic strength ranging from 20 to 50 mM, in particular wherein the buffer solution has an ionic strength ranging from 20 to 30 mM.
7. The method according to any one of claims 1 to 6, wherein the buffer solution comprises histidine or citrate as buffering agent.
8. The method according to any one of claims 1 to 7, wherein the buffer solution comprising the protein and the complexing agent is obtained by mixing a first solution comprising

the protein and a second solution comprising the complexing agent.

9. The method according to claim 8, wherein the first solution comprising the protein and/or the second solution comprising the complexing agent comprises a buffering agent.
10. The method according to any one of claims 1 to 9, wherein the protein and the complexing agent are contacted at a mole-charge ratio ranging from 1:0.2 to 1:2, in particular wherein the protein and the complexing agent are contacted at a mole-charge ratio ranging from 1:0.2 to 1:1.
11. The method according to any one of claims 1 to 10, wherein the protein is contacted with the complexing agent in the buffer solution at a protein concentration ranging from 1 - 40 mg/mL, in particular from 1 - 5 mg/mL.
12. The method according to any one of claims 1 to 11, wherein the protein is an antibody, a growth factor, a hormone, a cytokine, an enzyme, or a fragment and/or fusion protein of any of the foregoing.
13. The method according to claim 12, wherein the antibody is an antibody, in particular wherein the antibody is a monoclonal antibody, a polyclonal antibody, a chimeric antibody, a multispecific antibody, an antibody fusion protein, an antibody-drug-conjugate or an antibody fragment.
14. The method according to any one of claims 1 to 13, wherein the complexing agent has a negative net charge when comprised in the buffer solution.
15. The method according to any one of claims 1 to 14, wherein the complexing agent comprises a hydrophobic moiety.
16. The method according to any one of claims 1 to 15, wherein the composition comprising the RPCs comprises at least one excipient.
17. The method according to claim 16, wherein the at least one excipient is added to the composition before and/or after the formation of the RPCs.
18. The method according to claim 17 or 18, wherein the at least one excipient is a stabilizer and/or a surfactant.
19. The method according to any one of claims 1 to 18, wherein the method comprises a

further step of exchanging the liquid fraction of the suspension comprising the RPCs.

20. The method according to claim 19, wherein the liquid fraction of the suspension comprising the RPCs is exchanged by centrifugation of the suspension comprising the RPCs and resuspension of the sedimented RPCs in a buffer solution or water.
21. The method according to claim 19, wherein the liquid fraction of the suspension comprising the RPCs is exchanged by dialysis of the suspension comprising the RPCs against a buffer solution or water.
22. The method according to any one of claims 1 to 21, wherein the method comprises a further step of enriching the RPCs in the suspension to obtain an enriched RPC suspension.
23. The method according to claim 22, wherein enriching the RPCs in the suspension comprises the steps of:
 - a) centrifuging the suspension comprising the RPCs to obtain a supernatant and a precipitate comprising an enriched RPC suspension; and
 - b) removing the supernatant from the precipitate to obtain an enriched RPC suspension.
24. The method according to claim 22 or 23, wherein the liquid fraction of the enriched RPC suspension is at least in part replaced with a non-aqueous solvent during the enrichment step.
25. The method according to claim 24, wherein the non-aqueous solvent is triacetin, diethylene glycol monoethyl ether or ethyl oleate.
26. The method according to any one of claims 1 to 25, wherein the method comprises a further step of lyophilizing the suspension comprising the RPCs or the enriched RPC suspension to obtain a lyophilisate.
27. The method according to claim 26, wherein at least one cryoprotectant is added to the suspension comprising the RPCs or the enriched RPC suspension before the lyophilisation step.
28. The method according to claim 27, wherein the at least one cryoprotectant is selected from a group consisting of: sugars, amino acids, methylamines, lyotropic salts, polyols, propylene glycol, polyethylene glycol and pluronics.

29. The method according to any one of claims 26 to 28, wherein the protein concentration of the suspension comprising the RPCs or the enriched RPC suspension is adjusted to 10 to 100 mg/mL, in particular to 40 to 80 mg/mL, prior to the lyophilisation step.
30. The method according to any one of claims 1 to 25, wherein the method comprises a further step of spray drying the suspension comprising the RPCs or the enriched RPC suspension to obtain a spray dried powder.
31. The method according to claim 30, wherein the protein concentration of the suspension comprising the RPCs or the enriched RPC suspension is adjusted to 1 to 10 mg/mL, in particular to 1 to 5 mg/mL, prior to the spray drying step.
32. The method according to claim 30 or 31, wherein the liquid fraction of the suspension comprising the RPCs or the enriched RPC suspension is exchanged prior to the spray drying step.
33. The method according to claim 32, wherein exchanging the liquid fraction of the suspension comprising the RPCs or the enriched RPC suspension reduces the concentration of at least one buffering agent, complexing agent and/or excipient in the suspension.
34. The method according to claim 33, wherein the suspension comprising the RPCs or the enriched RPC suspension is substantially free of buffering agent after exchanging the liquid fraction of the suspension.
35. The method according to claim 33 or 34, wherein the liquid fraction of the suspension is exchanged before the spray-drying step to obtain a mole-charge ratio between the protein and the complexing agent between 1:0.2 to 1:1, in particular between 1:0.4 to 1:0.8.
36. The method according to any one of claims 30 to 35, wherein spray drying is performed at an inlet temperature 115°C and/or an outlet temperature of 48°C.
37. The method according to any one of claims 30 to 36, wherein spray drying is performed at a feed rate of 17 mL/min.
38. The method according to any one of claims 30 to 37, wherein the method comprises a further step of resuspending the spray dried powder in a non-aqueous solvent (NAS) to obtain an RPC-NAS suspension.

39. The method according to claim 38, wherein the non-aqueous solvent is at least one selected from a group consisting of: diethylene glycol monoethyl ether, ethyl oleate, triacetin, isosorbide dimethyl ether and glycofurol.
40. The method according to claim 39, wherein the spray dried powder is resuspended to obtain a RPC-NAS suspension with a protein concentration ranging from 50 to 300 mg/mL, in particular ranging from 100 – 250 mg/mL.
41. A composition comprising reversible protein complexes (RPCs), wherein the composition is obtained by the method according to any one of claims 1 to 40.
42. A composition comprising reversible protein complexes (RPCs), wherein the RPCs comprise a protein and a complexing agent, and wherein the complexing agent is dextran sulphate or chondroitin sulphate.
43. The composition according to claim 42, wherein the complexing agent is dextran sulphate, in particular dextran sulphate with 40 kDa molecular weight.
44. The composition according to claim 42 or 43, wherein the protein has a positive net charge when comprised in the RPCs.
45. The composition according to claim 44, wherein the protein is an antibody, a growth factor, a hormone, a cytokine, an enzyme, or a fragment and/or fusion protein of any of the foregoing.
46. The composition according to claim 45, wherein the antibody is a monoclonal antibody, a polyclonal antibody, a chimeric antibody, a multispecific antibody, an antibody fusion protein, an antibody-drug-conjugate or an antibody fragment.
47. The composition according to any one of claims 42 to 46, wherein the complexing agent has a negative charge when comprised in the RPCs.
48. The composition according to any one of claims 42 to 47, wherein the complexing agent comprises a hydrophobic moiety.
49. The composition according to any one of claims 42 to 48, wherein the composition comprises at least one excipient.
50. The composition according to claim 49, wherein the at least one excipient is a stabilizer

and/or a surfactant.

51. The composition according to any one of claims 42 to 50, wherein the protein has a higher melting temperature when comprised in the RPC compared to the uncomplexed protein.
52. The composition according to any one of claims 42 to 51, wherein the RPCs comprising the protein and the complexing agent dissociate at physiological pH and ionic strength.
53. The composition according to any one of claims 42 to 51, wherein the RPCs comprising the protein and the complexing agent dissociate in 10 mM to 100 mM PBS (pH 7.4, 137 mM NaCl) when diluted to a protein concentration of 0.1 to 10 mg/mL.
54. The composition according to any one of claims 42 to 53, wherein the composition is a suspension.
55. The composition according to claim 54, wherein the suspension is obtained with the method according to any one of claims 1 to 25.
56. The composition according to claims 54 or 55, wherein the protein concentration in the suspension ranges from 50 to 250 mg/mL, in particular wherein the protein concentration in the suspension ranges from 100 to 200 mg/mL.
57. The composition according to any one of claims 54 to 56, wherein the suspension comprises uncomplexed complexing agent.
58. The composition according to any one of claims 54 to 57, wherein the RPCs comprised in the suspension have a mean particle size ranging from 5 to 20 μm , in particular wherein the RPCs comprised in the suspension have a mean particle size ranging from 6 to 12 μm .
59. The composition according to any one of claims 54 to 57, wherein the RPCs comprised in the suspension have a mean particle size ranging from 100 to 4000 nm, in particular wherein the RPCs comprised in the suspension have a mean particle size ranging from 150 to 2000 nm.
60. The composition according to any one of claims 54 to 57, wherein the RPCs comprised in the suspension have a mean particle size ranging from 0.1 to 20 μm , in particular wherein the RPCs comprised in the suspension have a mean particle size ranging from 0.1 to 12 μm .

61. The composition according to any one of claims 54 to 60, wherein the suspension is injectable through a 26G needle.
62. The composition according to any one of claims 54 to 61, wherein the suspension is stable for at least 4 weeks at 4°C and/or 25°C.
63. The composition according to any one of claims 54 to 62, wherein the suspension has a viscosity ranging from 2 to 20 cP, in particular ranging from 3 to 15 cP, when measured at 20°C.
64. The composition according to any one of claims 54 to 63, wherein the pH of the suspension is lower than the isoelectric point of the protein.
65. The composition according to any one of claims 54 to 64, wherein the pH of the suspension is 1 to 3 pH units lower than the isoelectric point of the protein, in particular wherein the pH of the suspension is 2 pH units lower than the isoelectric point of the protein.
66. The composition according to any one of claims 54 to 64, wherein the pH of the suspension ranges from 1 to 6, in particular wherein the pH of the suspension ranges from 4.5 to 5.5.
67. The composition according to any one of claims 54 to 66, wherein the suspension comprises a buffering agent.
68. The composition according to claim 67, wherein the buffering agent is histidine or citrate.
69. The composition according to any one of claims 54 to 68, wherein the suspension has an ionic strength ranging from 20 to 50 mM, in particular wherein the suspension has an ionic strength ranging from 20 to 30 mM.
70. The composition according to any one of claims 54 to 66, wherein the suspension is substantially free of buffering agents.
71. The composition according to any one of claims 54 to 70, wherein the suspension further comprises a non-aqueous solvent.
72. The composition according to claim 71, wherein the non-aqueous solvent is diethylene glycol monoethyl ether, triacetin or ethyl oleate.

73. The composition according to any one of claims 42 to 53, wherein the composition is a lyophilisate.
74. The composition according to claim 73, wherein the lyophilisate is obtained with the method according to any one of claims 26 to 29.
75. The composition according to claim 73 or 74, wherein the lyophilisate comprises a buffering agent.
76. The composition according to claim 75, wherein the buffering agent is histidine or citrate.
77. The composition according to any one of claims 73 to 76 wherein the lyophilisate comprises at least one cryoprotectant.
78. The composition according to claim 77, wherein the at least one cryoprotectant is selected from a group consisting of: sugars, amino acids, methylamines, lyotropic salts, polyols, propylene glycol, polyethylene glycol and pluronics.
79. The composition according to any one of claims 73 to 78, wherein the lyophilisate is stable for at least 4 weeks at 40°C.
80. The composition according to any one of claims 73 to 79, wherein the lyophilisate is reconstituted in a liquid to a protein concentration ranging from 50 to 250 mg/mL, in particular wherein the lyophilisate is reconstituted in a liquid to a protein concentration ranging from 100 to 200 mg/mL.
81. The composition according to claim 80, wherein the liquid is PBS.
82. The composition according to claim 80 or 81, wherein the resuspended lyophilisate has a viscosity ranging from 2 to 20 cP, in particular ranging from 10 to 20 cP.
83. The composition according to any one of claims 42 to 53, wherein the composition is a spray dried powder.
84. The composition according to claim 83, wherein the protein content of the spray dried powder is at least 40% by weight (w/w), at least 50% by weight (w/w), at least 60% by weight (w/w).

85. The composition according to claim 83 or 84, wherein the spray dried powder is obtained with the method according to any one of claims 30 to 37.
86. The composition according to any one of claims 83 to 85, wherein the spray dried powder comprises a buffering agent.
87. The composition according to claim 86, wherein the buffering agent is histidine or citrate.
88. The composition according to any one of claims 83 to 85, wherein the spray dried powder is substantially free of buffering agents.
89. The composition according to any one of claims 83 to 88, wherein the RPCs comprised in the spray dried powder have a mean particle size ranging from 5 to 50 μm , in particular ranging from 10 to 40 μm , in particular ranging from 20 to 35 μm .
90. The composition according to any one of claims 83 to 89, wherein the spray dried powder is re-suspended in a liquid to a protein concentration in the suspension ranging from 50 to 300 mg/mL, in particular wherein the spray dried powder is re-suspended in a liquid to a protein concentration in the suspension ranging from 100 to 250 mg/mL.
91. The composition according to claim 90, wherein the liquid is a non-aqueous solvent.
92. The composition according to claim 91, wherein the non-aqueous solvent is at least one selected from a group consisting of: diethylene glycol monoethyl ether, ethyl oleate, triacetin, isosorbide dimethyl ester and glycofurol, preferably diethylene glycol monoethyl ether, ethyl oleate or triacetin.
93. The composition according to any one of claims 90 to 92, wherein the reconstituted spray dried powder has a viscosity ranging from 10 to 100 cP, in particular ranging from 20 to 80 cP.
94. A pharmaceutical formulation comprising the composition according to any one of claims 41 to 93.
95. The pharmaceutical formulation according to claim 94, wherein the pharmaceutical formulation comprises the suspension according to any one of claims 54 to 72, the reconstituted lyophilisate according to any one of claims 80 to 82, or the re-suspended spray dried powder according to any one of claims 90 to 93.

96. The pharmaceutical formulation according to claims 94 or 95 for use as a medicament.
97. The pharmaceutical formulation according to any one of claims 94 to 96 for use in the treatment of an autoimmune disease, an immune dysregulation disease, carcinoma, sarcoma, glioma, melanoma, lymphoma, leukemia, chronic lymphocytic leukemia, follicular lymphoma, diffuse large B cell lymphoma, multiple myeloma, non-Hodgkin's lymphoma, Alzheimer's disease, type 1 or type 2 diabetes, amyloidosis, or atherosclerosis.
98. The pharmaceutical formulation for use according to claim 97, wherein the pharmaceutical formulation is administered subcutaneously, intramuscularly, transdermally, ocularly, such as subconjunctivally, intracamerally, intravitreally, subretinally, or suprachoroidally, to the brain, such as intralumbarily, intrathecally, or intraventricularly, intra-articularly, or by inhalation.
99. Use of the pharmaceutical formulation according to claims 94 or 95 for the treatment of a disease selected from the group consisting of autoimmune disease, immune dysregulation disease, carcinoma, sarcoma, glioma, melanoma, lymphoma, leukemia, chronic lymphocytic leukemia, follicular lymphoma, diffuse large B cell lymphoma, multiple myeloma, non-Hodgkin's lymphoma, Alzheimer's disease, type 1 or type 2 diabetes, amyloidosis, and atherosclerosis.
100. Use of the pharmaceutical formulation according to claims 94 or 95 in the preparation of a medicament for the treatment of a disease selected from the group consisting of autoimmune disease, immune dysregulation disease, carcinoma, sarcoma, glioma, melanoma, lymphoma, leukemia, chronic lymphocytic leukemia, follicular lymphoma, diffuse large B cell lymphoma, multiple myeloma, non-Hodgkin's lymphoma, Alzheimer's disease, type 1 or type 2 diabetes, amyloidosis, and atherosclerosis.
101. A method of treating a subject suffering from a disease selected from the group consisting of: an autoimmune disease, an immune dysregulation disease, carcinoma, sarcoma, glioma, melanoma, lymphoma, leukemia, chronic lymphocytic leukemia, follicular lymphoma, diffuse large B cell lymphoma, multiple myeloma, non-Hodgkin's lymphoma, Alzheimer's disease, type 1 or type 2 diabetes, amyloidosis, and atherosclerosis, the method comprising the steps of (a) producing the pharmaceutical formulation according claims 94 or 95; and (b) administering the pharmaceutical formulation to a subject in need thereof.
102. The method according to claim 101, wherein the pharmaceutical composition is administered subcutaneously, intramuscularly or transdermally, in particular wherein the

pharmaceutical composition is administered subcutaneously.

103. A method of subcutaneous, intramuscular or transdermal administration of a pharmaceutical formulation, the method comprising the steps of (a) producing the pharmaceutical formulation according to claims 94 or 95; and (b) administering the pharmaceutical formulation to a subject by subcutaneous, intramuscular or transdermal delivery.

FIG.1



FIG.2

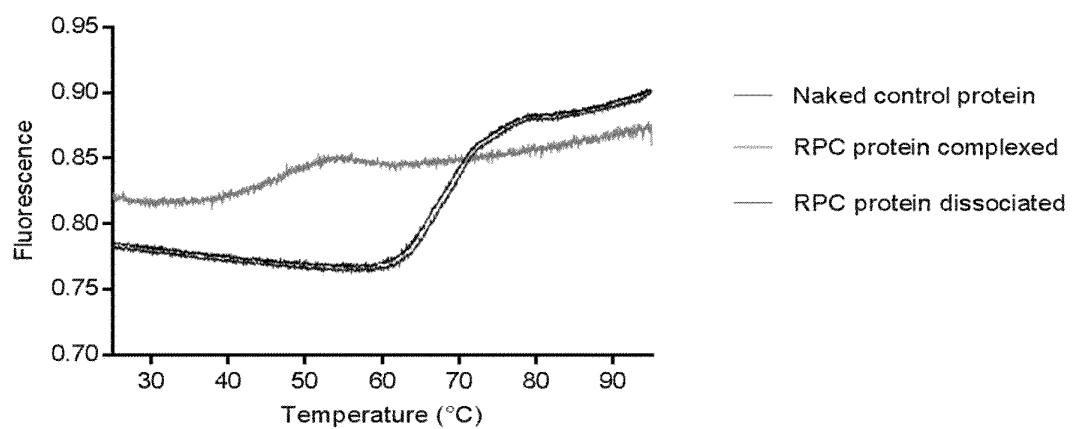


FIG.3

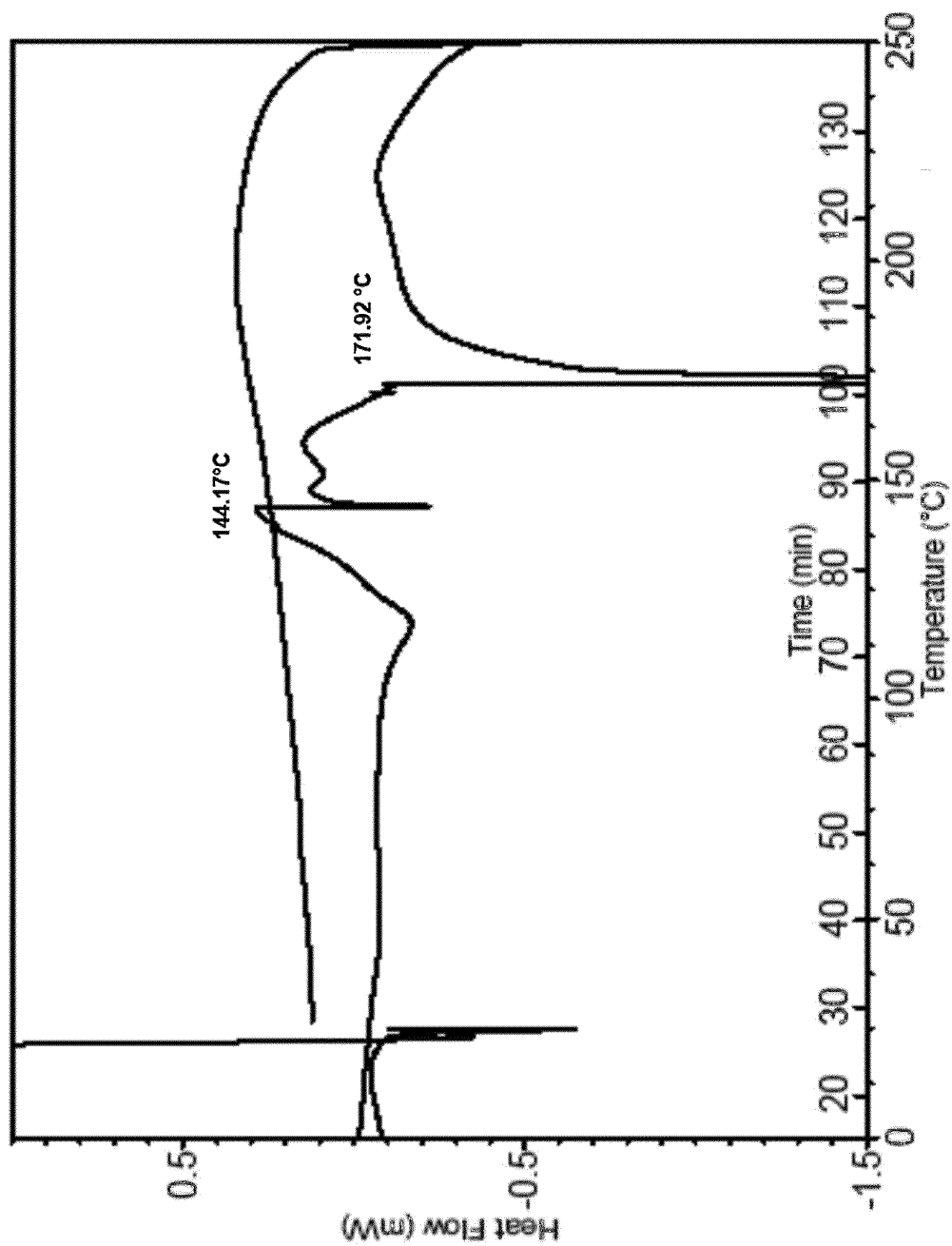


FIG.4

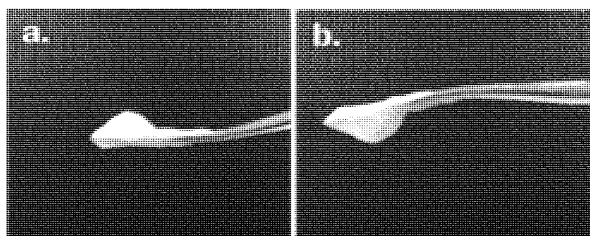


FIG.5

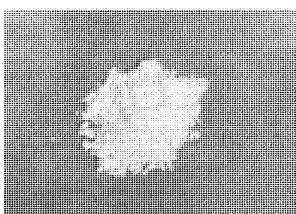


FIG.6

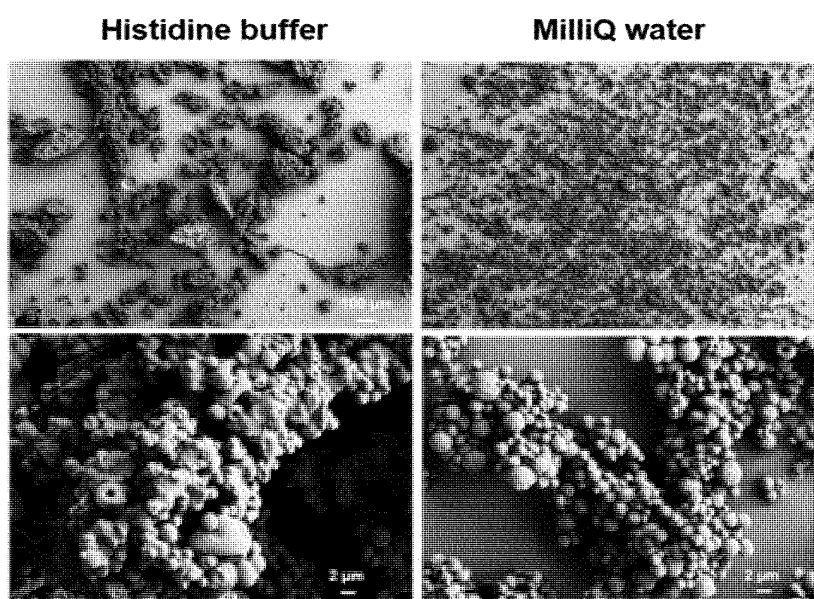


FIG.7

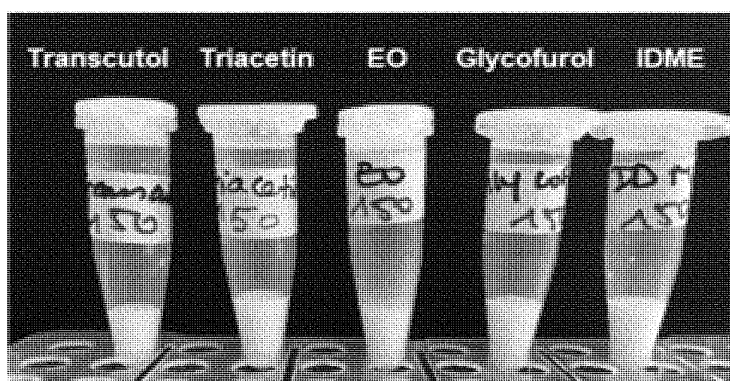


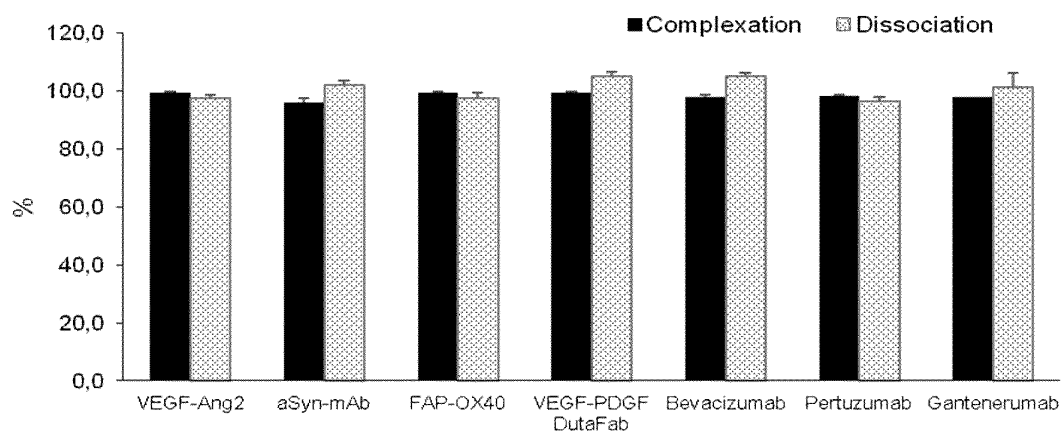
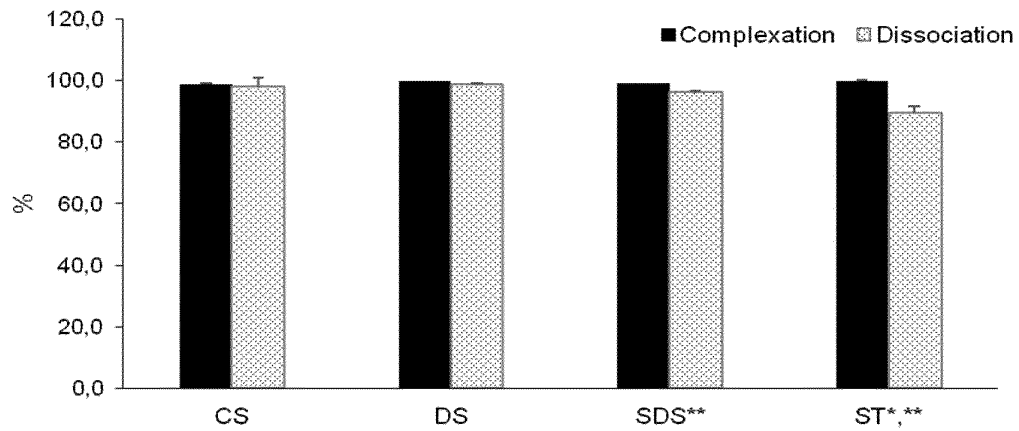
FIG.8**FIG.9**

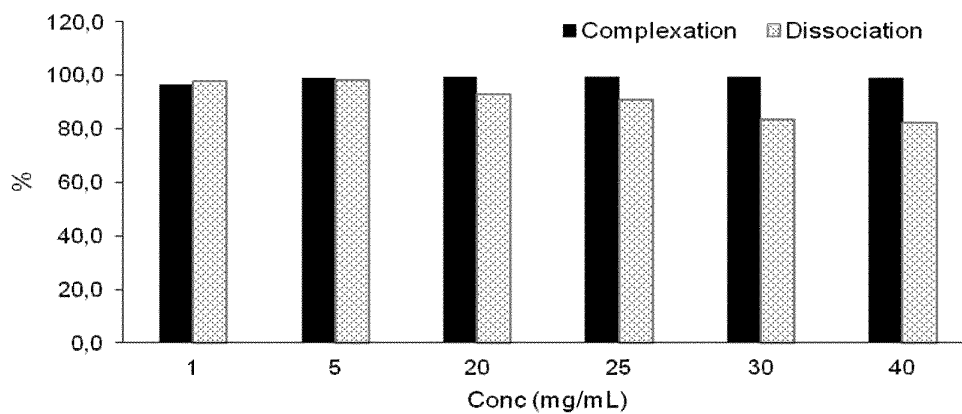
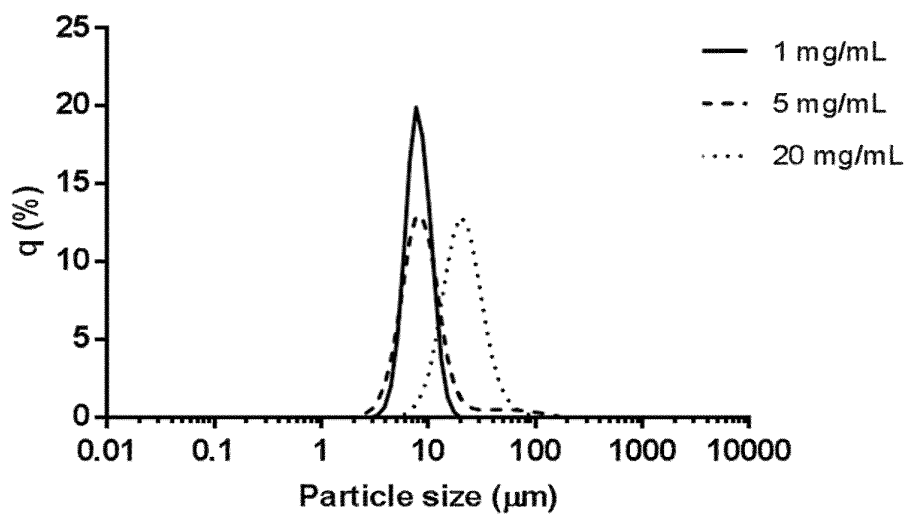
FIG.10**FIG.11**

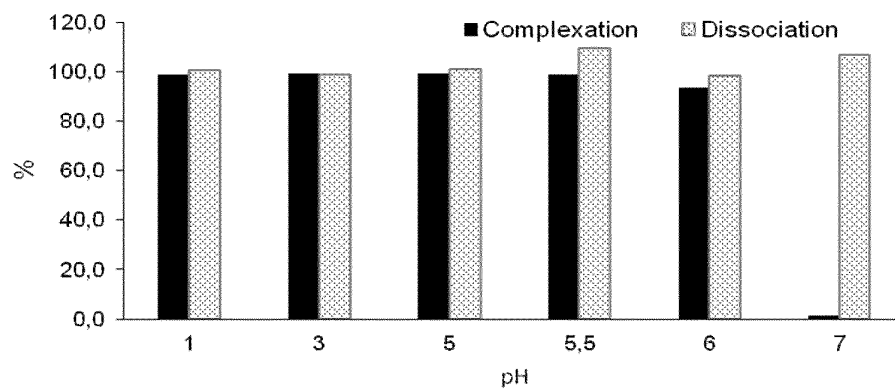
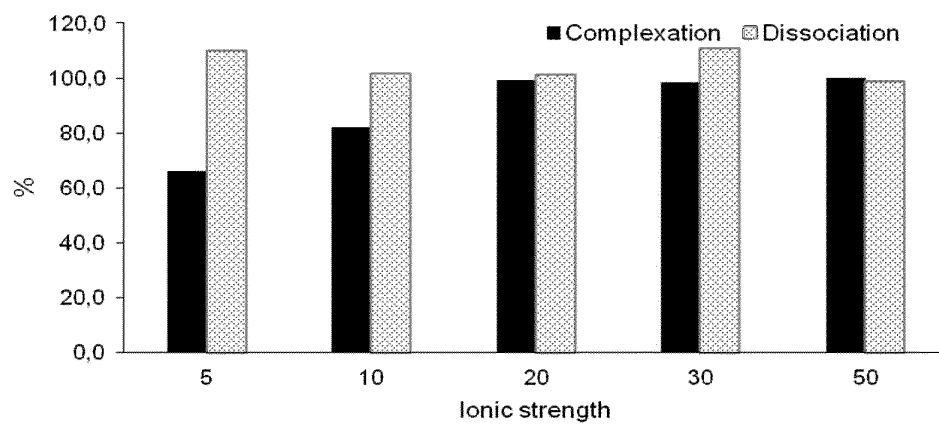
FIG.12**FIG.13**

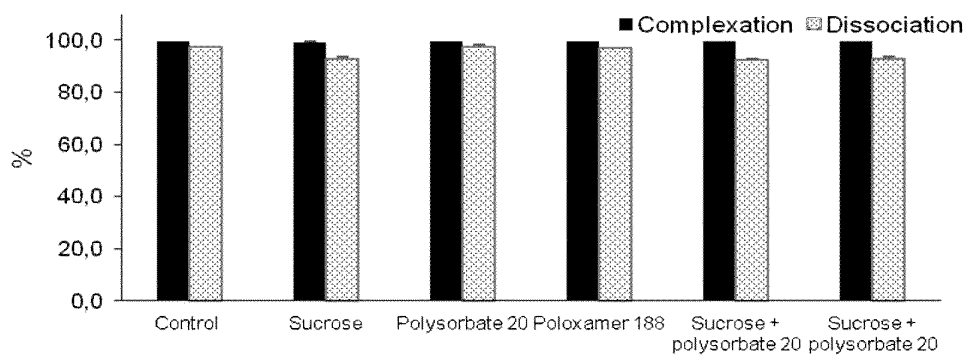
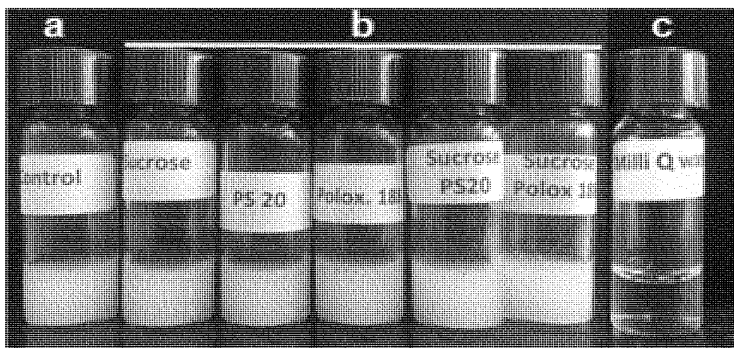
FIG.14**FIG.15**

FIG.16

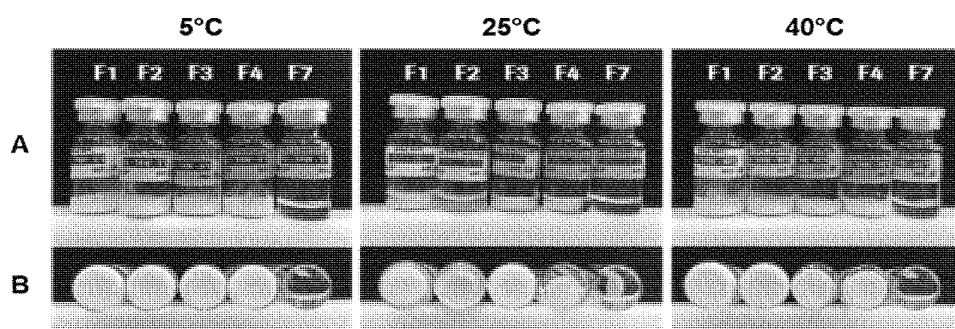


FIG.17

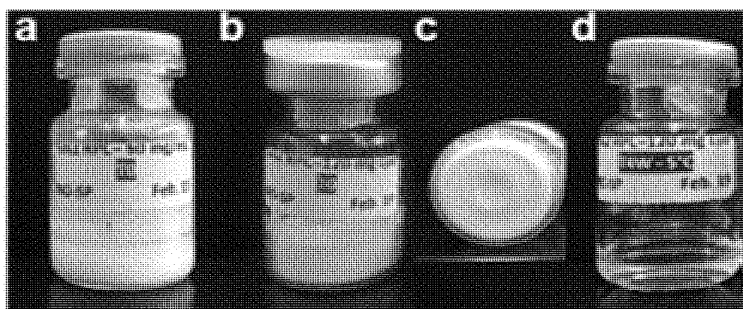


FIG.18

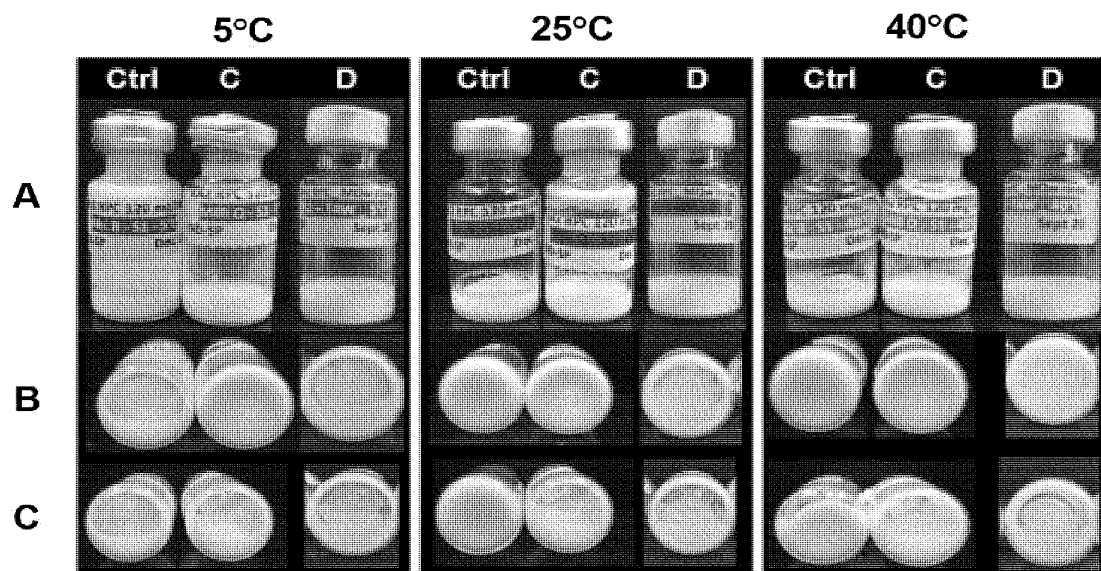


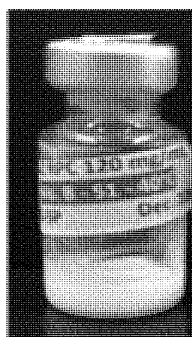
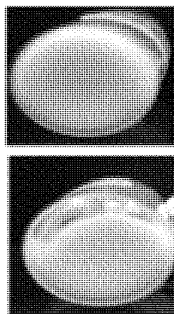
FIG.19**VA2 RPC CTRL****VA2 RPC MQ water rinse by
dialysis**

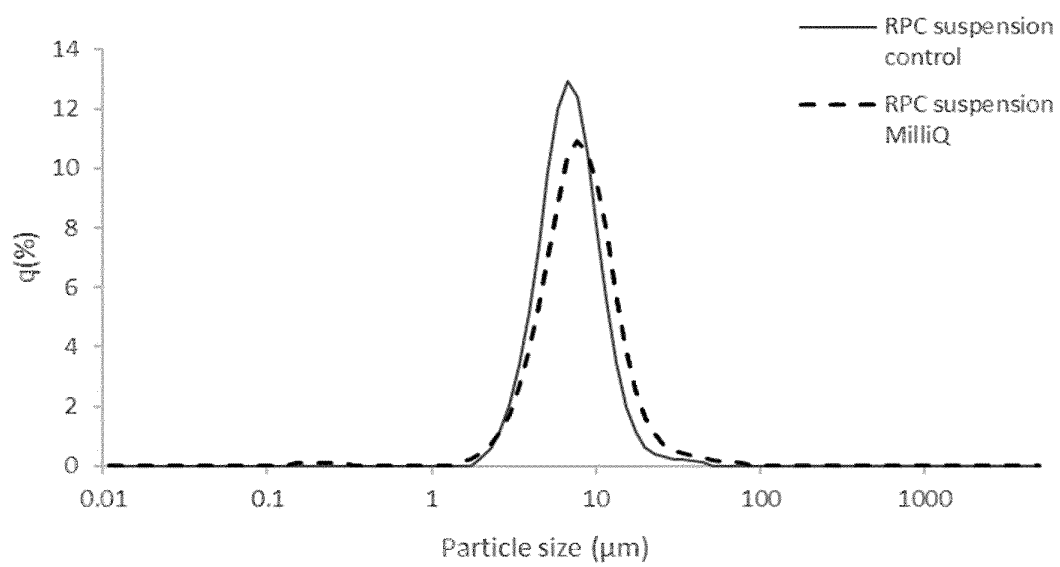
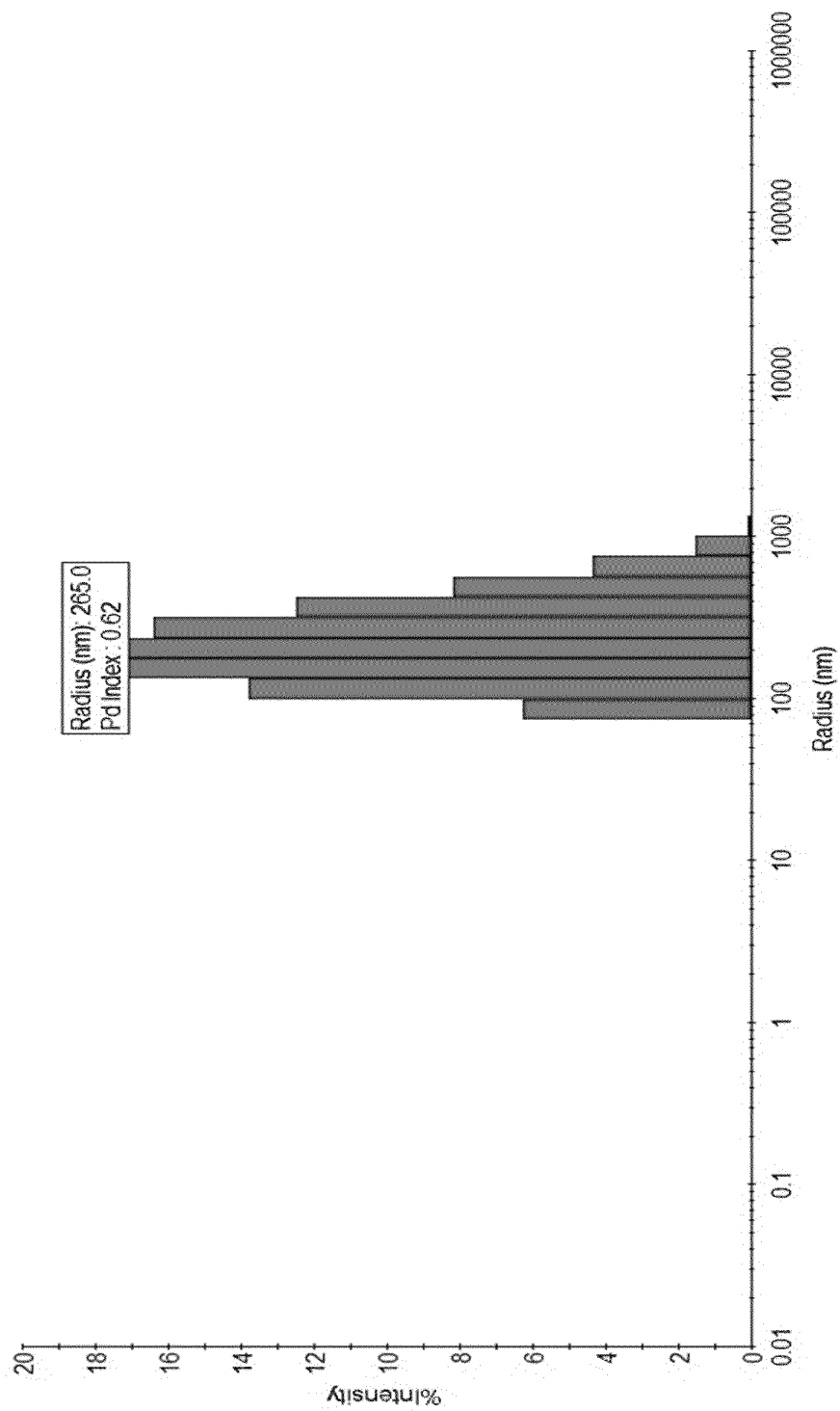
FIG.20 A

FIG.20 B



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/069090

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K9/00 A61K9/10 A61K9/16 A61K35/00 A61K47/36 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EP0-Internal, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KR 2013 0012556 A (CATHOLIC UNIV IND ACAD COOP [KR]) 4 February 2013 (2013-02-04) the whole document paragraph [0090] - paragraph [0092]; claims 1-10 -----	1-103
X	WO 03/094887 A1 (PEPTRON CO LTD [KR]; LEE HEE-YONG [KR] ET AL.) 20 November 2003 (2003-11-20) the whole document claims 1-17; examples 1-53 -----	1-103
X	WO 2005/055949 A2 (PHILADELPHIA CHILDREN HOSPITAL [US]; CHORNY MICHAEL [US] ET AL.) 23 June 2005 (2005-06-23) the whole document claims 1-47; examples 1-8 ----- -/--	1-103
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
12 October 2021		20/10/2021
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Felder, Christian

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/069090

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 060 741 A1 (EPIC THERAPEUTICS INC [US]) 20 December 2000 (2000-12-20) the whole document claims 1-20	1-103
X	----- KURT D. RISTROPH ET AL: "Hydrophobic ion pairing: encapsulating small molecules, peptides, and proteins into nanocarriers", NANOSCALE ADVANCES, vol. 1, no. 11, 1 January 2019 (2019-01-01), pages 4207-4237, XP055736481, DOI: 10.1039/C9NA00308H the whole document -----	1-103

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2021/069090

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
KR 20130012556 A	04-02-2013	NONE	
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		DE 69910987 T2	19-05-2004
		EP 1060741 A1	20-12-2000
		ES 2207123 T3	16-05-2004
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10 July 2021 (10.07.2021)

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AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN,
KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD,
ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO,
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TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

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GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ,
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(54) Title: OCULAR DISEASE TREATMENT USING ANTI-TISSUE FACTOR ANTIBODIES

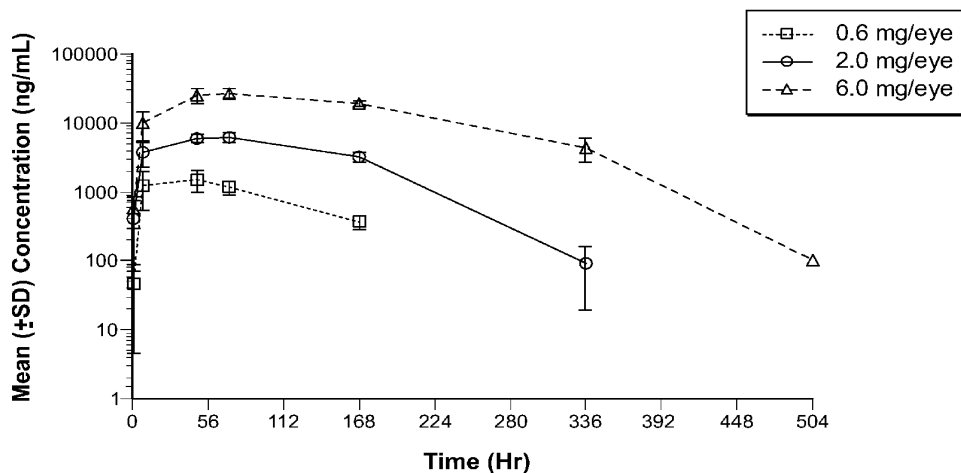


FIG. 24A

(57) Abstract: Provided herein are antibodies that specifically bind to human tissue factor (TF) and are useful for the treatment of ocular diseases. Also provided herein are methods of treating subjects having ocular diseases by administering the anti-TF antibodies.

[Continued on next page]

TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
- *of inventorship (Rule 4.17(iv))*

Published:

- *with international search report (Art. 21(3))*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
- *with sequence listing part of description (Rule 5.2(a))*

OCULAR DISEASE TREATMENT USING ANTI-TISSUE FACTOR ANTIBODIES**CROSS-REFERENCE TO RELATED APPLICATION**

[0001] This application claims priority to and the benefit of U.S. Provisional Patent Application No. 63/182,696, filed on April 30, 2021, and U.S. Provisional Patent Application No. 63/050,643, filed on July 10, 2020, the entire contents of each of which are incorporated by reference herein for all purposes.

SEQUENCE LISTING

[0001] The instant application contains a Sequence Listing which will be submitted via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on July 8, 2021, is named ITI-004WO_SL.txt and is 466,104 bytes in size.

BACKGROUND

[0002] Blood coagulation involves a complex set of processes that result in blood clotting. Tissue factor (TF) plays an important role in these coagulation processes. TF is a cell surface receptor for the serine protease factor VIIa (FVIIa). The TF/FVIIa complex catalyzes conversion of the inactive protease factor X (FX) into the active protease factor Xa (FXa). FXa and its co-factor FVa form the prothrombinase complex, which generates thrombin from prothrombin. Thrombin converts soluble fibrinogen into insoluble strands of fibrin and catalyzes many other coagulation-related processes.

[0003] Examples of diseases in which TF is overexpressed include age related macular degeneration. AMD is the most prevalent form of macular degeneration. It is a degenerative disease of the retina and is the leading cause of irreversible blindness and vision impairment in people over 65 years of age in the developed world. (Tomany *et al.*, *Ophthalmology*, 2004, which is incorporated by reference in its entirety). AMD occurs in up to 8% of individuals over the age of 60, and its prevalence increases with age. The U.S. is anticipated to have nearly 22 million cases of AMD by the year 2050, while global cases of AMD are expected to be nearly 288 million by the year 2040.

[0004] AMD is characterized by progressive loss of visual acuity in the central portion of the visual field, changes in color vision, and abnormal dark adaptation and sensitivity. The two main forms of AMD are dry AMD and wet AMD (or neovascular AMD). Dry AMD is associated with atrophic cell death of the central retina or macula, which is required for the fine vision used for activities such as reading, driving, and recognizing faces. In some instances, dry AMD can progress to wet AMD – a less common form of AMD that accounts for approximately 85% of legally blind AMD cases. Wet AMD is characterized by aberrant

vascularization under the macula (choroid neovascularization) or vascular leakage, which displaces the retina and/or results in hemorrhage and scarring.

[0005] Choroidal neovascularization (CNV) is the process in which new blood vessels grow in the choroid layer of the eye, and is associated with wet AMD.

[0006] Diabetic retinopathy (DR) is also a major cause of visual impairment in the United States and results from microvascular decompensation that begins with basement membrane thickening and ultimately results in vascular occlusion and angiogenesis. DR is observed in about 28% of diabetes patients over 40 years of age, and 4.4% suffer or are at risk of loss of vision due to DR (Zhang *et al.*, 2010, JAMA. 304: 649-656, which is incorporated by reference in its entirety). Diabetic macular edema (DME) is a symptom of DR and the most frequent cause of blindness in young and middle-aged adults (Klein *et al.*, 1984, *Ophthalmology*, 91: 1464-1474 and Moss *et al.*, 1998, *Ophthalmology*, 105: 998-1003, each of which is incorporated by reference in its entirety).

[0007] Anti-vascular endothelial growth factor (anti-VEGF) therapies (such as bevacizumab, ranibizumab, or aflibercept) are the standard therapeutic treatment for ocular diseases such as AMD and DME with neovascularization. However, the products on the market still face several challenges relating to effectiveness and safety. The majority of intravitreal injections for treatment of these ocular diseases include anti-vascular endothelial growth factor (VEGF) agents.

[0008] Aspects of the anti-TF antibodies and methods comprising use of the anti-TF antibodies of this disclosure are described in international PCT application PCT/US2019/012427, US utility application number 16/959,652, and US provisional application numbers 62/713,797; 62/713,804; 62/646,788; 62/613,545; and 62/613,564, incorporated herein by reference in their entirety for all purposes.

SUMMARY

[0009] Provided herein are antibodies that specifically bind human Tissue Factor (TF) and related methods useful for the treatment of ocular diseases.

[0010] In one aspect, provided herein is a method of treating an ocular disease in a subject in need thereof comprising administering to the subject an isolated antibody wherein the antibody binds to the extracellular domain of human Tissue Factor (TF), wherein the antibody binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa. In some embodiments, the antibody is an isolated human antibody.

[0011] In some embodiments, the antibody is administered as a dose via intraocular injection and wherein if the dose administered to the subject is greater than 6 mg of antibody/eye, the dose is administered in two or more injections. In some embodiments, the antibody is administered to the subject via a plurality of doses comprising at least a first dose and at least a second dose and wherein the time between the first and second doses is at least 28 days. In some embodiments, the antibody is administered to the subject via a plurality of doses comprising at least a first dose and at least a second dose and wherein the time between the first and second doses is at least 15 days. In some embodiments, the antibody is administered to the subject via a plurality of doses comprising at least a first dose and at least a second dose and wherein the time between the first and second doses is at least 20 days. In some embodiments, the antibody is administered to the subject via a plurality of doses comprising at least a first dose and at least a second dose and wherein the time between the first and second doses is at least 30 days. In some embodiments, the antibody is administered to the subject via a plurality of doses comprising at least a first dose and at least a second dose and wherein the time between the first and second doses is at least 40 days. In some embodiments, the antibody is administered to the subject via a plurality of doses comprising at least a first dose and at least a second dose and wherein the time between the first and second doses is at least 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41 or 42 days. In some embodiments, the antibody is administered to the subject via a plurality of doses comprising at least a first dose and at least a second dose and wherein the time between the first and second doses is at least 15-20 days, 15-25 days, 15-30 days, 15-35 days, 15-40 days, 20-25 days, 20-30 days, 20-35 days, 20-40 days, 25-30 days, 25-35 days, 25-40 days, 30-35 days, 30-40 days, 35-40 days or a range of days therein.

[0012] In some embodiments, the antibody is administered as a dose via intraocular injection, wherein if the dose administered to the subject is greater than 6 mg of antibody/eye, the dose is administered in two or more injections, and wherein the antibody is administered to the subject via a plurality of doses comprising at least a first dose and at least a second dose and wherein the time between the first and second doses is at least 28 days.

[0013] In some embodiments, the antibody is administered via intravitreal injection.

[0014] In some embodiments, the antibody (i) specifically binds to rabbit TF or (ii) is cross-reactive with rabbit TF. In some embodiments, the antibody (i) specifically binds pig TF or (ii) is cross-reactive with pig TF.

[0015] In some embodiments, the antibody does not inhibit human thrombin generation as determined by thrombin generation assay (TGA). In some embodiments, the isolated human antibody does not inhibit or inhibits human thrombin generation to a lesser extent, as determined by thrombin generation assay (TGA), compared to a comparator therapy. In some embodiments, the comparator therapy is an anti-TF protein, an anti-TF antibody, an anti-TF immunoconjugate or an anti-TF drug. In some embodiments, the comparator therapy is ICON-1. In some embodiments, the comparator therapy is an antibody comprising a V_H sequence of SEQ ID NO:821 and a V_L sequence of SEQ ID NO:822. In some embodiments, binding between the isolated antibody and a variant TF extracellular domain comprising a mutation at amino acid residue 149 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the isolated antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the isolated antibody relative to an isotype control in a live cell staining assay.

[0016] In some embodiments, the antibody comprises all three heavy chain Complementary Determining Regions (CDRs) and all three light chain CDRs from an antibody group in Table 35, wherein the all three heavy chain CDRs and the all three light chain CDRs are from the same antibody group. In some embodiments, the antibody comprises all three heavy chain Complementary Determining Regions (CDRs) and all three light chain CDRs from an antibody in any one of Tables 15-34, wherein the all three heavy chain CDRs and the all three light chain CDRs are from the same antibody.

[0017] In some embodiments, the antibody comprises all three heavy chain CDRs and all three light chain CDRs from: the antibody designated 25A, the antibody designated 25A5, the antibody designated 25A5-T, the antibody designated 25G, the antibody designated 25G1, the antibody designated 25G9, the antibody designated 43B, the antibody designated 43B1, the antibody designated 43B7, the antibody designated 43D, the antibody designated 43D7, the antibody designated 43D8, the antibody designated 43E, or the antibody designated 43Ea.

[0018] In some embodiments, the antibody comprises all three heavy chain CDRs and all three light chain CDRs from: the antibody designated 25A, the antibody designated 25A5, the antibody designated 25A5-T, the antibody designated 25G, the antibody designated 25G1, or the antibody designated 25G9. In some embodiments, the antibody comprises all three heavy chain CDRs and all three light chain CDRs from: the antibody designated 43B, the antibody designated 43B1, the antibody designated 43B7, the antibody designated 43D, the antibody designated 43D7, the antibody designated 43D8, the antibody designated 43E, or the antibody designated 43Ea. In some embodiments, the antibody comprises a V_H Domain

sequence and VL domain sequence from Table 14, wherein the VH and VL domain sequences are from the same group in Table 14.

[0019] In some embodiments, the antibody comprises a VH Domain sequence and VL domain sequence from Table 13, wherein the VH and VL domain sequences are from the same clone in Table 13. In some embodiments, the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:779; a VH-CDR2 comprising the sequence set forth in SEQ ID NO:780; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:781; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:782; a VL-CDR2 comprising the sequence set forth in SEQ ID NO:783; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:784.

[0020] In some embodiments, the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:872; a VH-CDR2 comprising the sequence set forth in SEQ ID NO:873; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:874; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:875; a VL-CDR2 comprising the sequence set forth in SEQ ID NO:876; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:877.

[0021] In some embodiments, the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:884; a VH-CDR2 comprising the sequence set forth in SEQ ID NO:885; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:886; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:887; a VL-CDR2 comprising the sequence set forth in SEQ ID NO:888; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:889.

[0022] The In some embodiments, the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:868 and a VL sequence comprising the sequence set forth in SEQ ID NO:869.

[0023] The In some embodiments, the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:189 and a VL sequence comprising the sequence set forth in SEQ ID NO:190. In some embodiments, the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:836 and a VL sequence comprising the sequence set forth in SEQ ID NO:837. In some embodiments, the antibody comprises: a heavy chain comprising the sequence set forth in SEQ ID NO:920 and a light chain comprising the sequence set forth in SEQ ID NO:921.

[0024] In some embodiments, the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:878; a VH-CDR2 comprising the sequence set forth in SEQ ID

NO:879; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:880; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:881; a VL-CDR2 comprising the sequence set forth in SEQ ID NO:882; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:883. In some embodiments, the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:267; a VH-CDR2 comprising the sequence set forth in SEQ ID NO:268; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:269; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:270; a VL-CDR2 comprising the sequence set forth in SEQ ID NO:271; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:272.

[0025] In some embodiments, the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:870 and a VL sequence comprising the sequence set forth in SEQ ID NO:871. In some embodiments, the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:303 and a VL sequence comprising the sequence set forth in SEQ ID NO:304. In some embodiments, the antibody comprises: a heavy chain comprising the sequence set forth in SEQ ID NO:922 and a light chain comprising the sequence set forth in SEQ ID NO:923.

[0026] In some embodiments, the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:797; a VH-CDR2 comprising the sequence set forth in SEQ ID NO:798; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:799; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:800; a VL-CDR2 comprising the sequence set forth in SEQ ID NO:801; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:802.

[0027] In some embodiments, the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:571; a VH-CDR2 comprising the sequence set forth in SEQ ID NO:572; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:573; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:574; a VL-CDR2 comprising the sequence set forth in SEQ ID NO:575; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:576.

[0028] In some embodiments, the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:609; a VH-CDR2 comprising the sequence set forth in SEQ ID NO:610; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:611; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:612; a VL-CDR2 comprising the sequence set forth in SEQ ID NO:613; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:614.

[0029] In some embodiments, the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:769 and a VL sequence comprising the sequence set forth in SEQ ID NO:770. In some embodiments, the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:569 and a VL sequence comprising the sequence set forth in SEQ ID NO:570. In some embodiments, the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:607 and a VL sequence comprising the sequence set forth in SEQ ID NO:608. In some embodiments, the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:645 and a VL sequence comprising the sequence set forth in SEQ ID NO:646. In some embodiments, the antibody comprises: a heavy chain comprising the sequence set forth in SEQ ID NO:924 and a light chain comprising the sequence set forth in SEQ ID NO:925. In some embodiments, the antibody comprises: a heavy chain comprising the sequence set forth in SEQ ID NO:926 and a light chain comprising the sequence set forth in SEQ ID NO:927.

[0030] In some embodiments, the antibody competes for binding to human TF with the antibody designated 25A, the antibody designated 25A5, the antibody designated 25A5-T, the antibody designated 25G, the antibody designated 25G1, the antibody designated 25G9, the antibody designated 43B, the antibody designated 43B1, the antibody designated 43B7, the antibody designated 43D, the antibody designated 43D7, the antibody designated 43D8, the antibody designated 43E, or the antibody designated 43Ea.

[0031] In some embodiments, the antibody competes for binding to human TF with the antibody designated 25A, the antibody designated 25A5, the antibody designated 25A5-T, the antibody designated 25G, the antibody designated 25G1, or the antibody designated 25G9. In some embodiments, the antibody competes for binding to human TF with the antibody designated 43B, the antibody designated 43B1, the antibody designated 43B7, the antibody designated 43D, the antibody designated 43D7, the antibody designated 43D8, the antibody designated 43E, or the antibody designated 43Ea.

[0032] In some embodiments, the antibody binds to the same human TF epitope bound by the antibody designated 25A, the antibody designated 25A5, the antibody designated 25A5-T, the antibody designated 25G, the antibody designated 25G1, the antibody designated 25G9, the antibody designated 43B, the antibody designated 43B1, the antibody designated 43B7, the antibody designated 43D, the antibody designated 43D7, the antibody designated 43D8, the antibody designated 43E, or the antibody designated 43Ea.

[0033] In some embodiments, the antibody binds to the same human TF epitope bound by the antibody designated 25A, the antibody designated 25A5, the antibody designated 25A5-T, the

antibody designated 25G, the antibody designated 25G1, or the antibody designated 25G9. In some embodiments, the antibody binds to the same human TF epitope bound by the antibody designated 43B, the antibody designated 43B1, the antibody designated 43B7, the antibody designated 43D, the antibody designated 43D7, the antibody designated 43D8, the antibody designated 43E, or the antibody designated 43Ea.

[0034] In some embodiments, the antibody does not inhibit human thrombin generation as determined by thrombin generation assay (TGA), does not reduce the thrombin peak on a thrombin generation curve (Peak IIa) compared to an isotype control, does not increase the time from the assay start to the thrombin peak on a thrombin generation curve (ttPeak) compared to an isotype control, does not decrease the endogenous thrombin potential (ETP) as determined by the area under a thrombin generation curve compared to an isotype control, allows human thrombin generation as determined by thrombin generation assay (TGA), maintains the thrombin peak on a thrombin generation curve (Peak IIa) compared to an isotype control, maintains the time from the assay start to the thrombin peak on a thrombin generation curve (ttPeak) compared to an isotype control, preserves the endogenous thrombin potential (ETP) as determined by the area under a thrombin generation curve compared to an isotype control, binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FX, does not interfere with the ability of TF:FVIIa to convert FX into FXa, and does not compete for binding to human TF with FVIIa.

[0035] The method of any one of the preceding claims, wherein the antibody (i) specifically binds to cynomolgus monkey TF or (ii) is cross-reactive with cynomolgus monkey TF.

[0036] The method of any one of the preceding claims, wherein the antibody (i) specifically binds to mouse TF or (ii) is cross-reactive with mouse TF.

[0037] In some embodiments, the three heavy chain CDRs and the three light chain CDRs are determined using exemplary, Kabat, Chothia, AbM, Contact, or IMGT numbering.

[0038] In some embodiments, the affinity of the antibody to human TF is greater compared to a comparator therapy. In some embodiments, the comparator therapy is an anti-TF protein, an anti-TF antibody, an anti-TF immunoconjugate or an anti-TF drug. In some embodiments, the comparator therapy is ICON-1.

[0039] In some embodiments, the antibody comprises an Fc region. In some embodiments, the Fc region of the antibody and comprises one or more mutations that result in enhanced effector function an increased immune response. In some embodiments, the antibody interacts with an Fcγ Receptor (FcγR) expressed by an immune cell via the Fc domain, and wherein the antibody increases an immune response in the subject upon specific binding between the

antibody and one or more TF-expressing cells, thereby treating the ocular disease. In some embodiments, the immune response is enhanced Fc effector function.

[0040] In some embodiments, the immune response increases compared to a comparator therapy. In some embodiments, the immune response is any one of: (i) increased T cell activity, (ii) increased activation of $\alpha\beta$ and/or $\gamma\delta$ T cells, (iii) increased cytotoxic T cell activity, (iv) increased NK and/or NKT cell activity, (v) alleviation of $\alpha\beta$ and/or $\gamma\delta$ T-cell suppression, (vi) increased pro-inflammatory cytokine secretion, (vii) increased IL-2 secretion; (viii) increased interferon- γ production, (ix) increased Th1 response, (x) decreased Th2 response, (xi) decreased or eliminated cell number and/or activity of at least one of regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), immature myeloid cells (iMCs), mesenchymal stromal cells, or TIE2-expressing monocytes, (xii) reduced regulatory cell activity, and/or the reduced activity of one or more of myeloid derived suppressor cells (MDSCs), iMCs, mesenchymal stromal cells, TIE2-expressing monocytes, (xiii) decreased or eliminated M2 macrophages, (xiv) reduced M2 macrophage pro-tumorigenic activity, (xv) decreased or eliminated N2 neutrophils, (xvi) reduced N2 neutrophils pro-tumorigenic activity, (xvii) reduced inhibition of T cell activation, (xviii) reduced inhibition of CTL activation, (xix) reduced inhibition of NK and/or NKT cell activation, (xx) reversed $\alpha\beta$ and/or $\gamma\delta$ T cell exhaustion, (xxi) increased $\alpha\beta$ and/or $\gamma\delta$ T cell response, (xxii) increased activity of cytotoxic cells, (xxiii) stimulated antigen-specific memory responses, (xxiv) elicitation of apoptosis or lysis of diseased or damaged cells, (xxv) stimulation of cytotoxic or cytostatic effect on diseased or damaged cells, (xxvi) induced direct killing of diseased or damaged cells, (xxvii) increased Th17 activity, (xxviii) modulated myeloid cell polarization, (xxix) modulated myeloid cell shifting toward a pro-inflammatory response, (xxx) shifted myeloid cells from M2 toward M1 phenotype, (xxxi) modulation of myeloid cells in the ocular tissues, (xxxii) increased anti-inflammatory mediator secretion, (xxxiii) enhanced myeloid and lymphoid infiltration into the diseased or damaged site, and (xxxiv) induction of complement dependent cytotoxicity and/or antibody dependent cell-mediated cytotoxicity.

[0041] In some embodiments, the immune response is any one of: (i) antibody-dependent cellular cytotoxicity (ADCC), (ii) antibody-dependent cellular phagocytosis (ADCP), and (iii) C1q binding to activate complement dependent cytotoxicity (CDC). In some embodiments, the immune response is antibody-dependent cellular cytotoxicity (ADCC). In some embodiments, the immune response is antibody-dependent cellular phagocytosis

(ADCP). In some embodiments, the immune response is C1q binding to activate complement dependent cytotoxicity (CDC).

[0042] In some embodiments, the comparator therapy is an anti-TF protein, an anti-TF antibody, an anti-TF immunoconjugate or an anti-TF drug. In some embodiments, the comparator therapy is ICON-1.

[0043] In some embodiments, the method comprises administering the antibody of any one of the preceding claims to the subject by intravitreal injection, suprachoroidal injection or subretinal injection. In some embodiments, the method comprises administering the antibody of any one of the preceding claims to the subject by intravitreal injection.

[0044] In some embodiments, the ocular disease is neovascularization, age-related macular degeneration (AMD), retinal vein occlusion, retinopathy of prematurity or diabetic retinopathy. In some embodiments, the diabetic retinopathy is proliferative diabetic retinopathy or non-proliferative diabetic retinopathy. In some embodiments, the AMD is neovascular AMD. In some embodiments, the neovascularization is macular neovascularization. In some embodiments, the AMD is wet AMD. In some embodiments, the AMD is dry AMD.

[0045] In some embodiments, upon administration to a subject, the antibody decreases the total leakiness area on the retina relative to baseline levels. In some embodiments, upon administration to a subject, the antibody decreases the total leakiness area on the retina relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug. In some embodiments, upon administration to a subject, the antibody does not alter the total leakiness area on the retina relative to baseline levels. In some embodiments, the total leakiness area is determined using fluorescein angiography or OCT-angiography.

[0046] In some embodiments, upon administration to a subject, the antibody reduces choroidal neovascularization (CNV) area relative to baseline levels. In some embodiments, upon administration to a subject, the antibody reduces choroidal neovascularization (CNV) area relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug. In some embodiments, the CNV area is reduced by at least about 10%, at least about 20%, at least about 30%, at least about 40% or at least about 50%. In some embodiments, the CNV area is determined using fluorescein angiography or OCT-angiography

[0047] In some embodiments, upon administration to a subject, the antibody does not result in ocular inflammation. In some embodiments, upon administration to a subject, the antibody results in reduced ocular inflammation relative to baseline levels. In some embodiments, upon administration to a subject, the antibody results in reduced ocular inflammation relative

to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug . In some embodiments, the ocular inflammation is as determined using direct or indirect ophthalmoscopy. In some embodiments, upon administration to a subject, the antibody results in reduced retinal thickness relative to baseline levels.

[0048] In some embodiments, upon administration to a subject, the antibody results in reduced retinal thickness relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug. In some embodiments, the retinal thickness is reduced by at least about 50 μm , at least about 100 μm , at least about 150 μm , at least about 175 μm , at least about 200 μm , at least about 225 μm or at least about 250 μm . In some embodiments, the retinal thickness is reduced by at least about 10%, at least about 20%, at least about 30%, at least about 40% or at least about 50%. In some embodiments, the reduced retinal thickness is reduced central retinal subfield thickness (CST), reduced center point thickness (CPT), or reduced central foveal thickness (CFT). In some embodiments, the retinal thickness is central retina thickness (CRT).

[0049] In some embodiments, following administration to the subject, the CRT value increases to a value within the range of 200-270 μm . In some embodiments, the retinal thickness is measured by spectral domain optical coherence tomography (SD OCT).

[0050] In some embodiments, upon administration to a subject, the antibody results in reduced mean foveal thickness relative to baseline levels. In some embodiments, upon administration to a subject, the antibody results in reduced mean foveal thickness relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug . In some embodiments, the mean foveal thickness is measured by SD OCT.

[0051] In some embodiments, upon administration to a subject, the antibody results in reduced macular edema relative to baseline levels. In some embodiments, the macular edema is determined by fluorescein angiography, or optical coherence tomography (OCT).

[0052] In some embodiments, upon administration to a subject, the antibody results in reduced leakage into the macula relative to baseline levels. In some embodiments, the leakage into the macula is determined by fluorescein angiography, or optical coherence tomography (OCT).

[0053] In some embodiments, upon administration to a subject, the antibody results in reduced or absent biomarkers of inflammation relative to baseline levels. In some embodiments, upon administration to a subject, the antibody results in reduced or absent biomarkers of inflammation relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug. In some embodiments, upon administration to a subject, the

antibody results in reduced or absent biomarkers of inflammation, as compared to a comparator therapy. In some embodiments, the comparator therapy is an anti-TF protein, an anti-TF antibody, an anti-TF immunoconjugate or an anti-TF drug. In some embodiments, the comparator therapy is ICON-1.

[0054] In some embodiments, the biomarkers of inflammation comprise one or more inflammatory cytokines. In some embodiments, the inflammatory cytokines comprise at least one of: IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF or GMCSF). In some embodiments, the biomarkers of inflammation are measured by ELISA.

[0055] In some embodiments, upon administration to a subject, the antibody results in reduced or equal anti-drug antibody (ADA) levels relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug. In some embodiments, the ADA levels are systemic ADA levels. In some embodiments, the ADA levels are within vitreous humor and/or aqueous humor. In some embodiments, the ADA levels are measured by ECL bridging assay.

[0056] In some embodiments, upon administration to a subject, the antibody does not significantly alter intraocular pressure (IOP) relative levels to baseline levels. In some embodiments, the IOP is measured by tonometry.

[0057] In some embodiments, upon administration to a subject, the antibody results in reduced mononuclear cell infiltration relative to baseline levels. In some embodiments, upon administration to a subject, the antibody results in reduced mononuclear cell infiltration relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug. In some embodiments, the mononuclear cell infiltration comprises M1 or M2 macrophages. In some embodiments, the mononuclear cell infiltration is infiltration of mononuclear cells into one or more of: retina, choroid, vitreous body, iris, ciliary body, limbus, and conjunctiva. In some embodiments, the mononuclear cell infiltration is measured by immunohistochemical (IHC) analysis.

[0058] In some embodiments, upon administration of the antibody to a subject, the exposure to the antibody in target tissues is comparable to that from a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug. In some embodiments, upon administration of the antibody to a subject, the concentration of the antibody in the vitreous humor is significantly higher than the concentration in the serum for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days following the administration.

[0059] In some embodiments, upon administration to a subject, the antibody results in less severe adverse ocular changes relative to baseline levels, wherein the adverse ocular changes

comprise the presence of one or more of: anterior chamber cells, flare and fibrin, keratic precipitates/corneal endothelial cells, cellular deposits on the anterior lens capsule, incomplete pupil dilation, cells on corneal endothelium and lens capsule, vitreous haze, vitreous cells, retinal sheathing, and chorio-retinal hemorrhages.

[0060] In some embodiments, upon administration to a subject, the antibody results in less severe adverse ocular changes relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug, wherein the adverse ocular changes comprise the presence of one or more of: anterior chamber cells, flare and fibrin, keratic precipitates/corneal endothelial cells, cellular deposits on the anterior lens capsule, incomplete pupil dilation, cells on corneal endothelium and lens capsule, vitreous haze, vitreous cells, retinal sheathing, and chorio-retinal hemorrhages.

[0061] In some embodiments, the different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug comprises: aflibercept, ranibizumab, brolucizumab, pegaptanib, bevacizumab, abicipar pegol, RG7716, KSI-301 or ICON-1.

[0062] In some embodiments, the antibody binds human TF with a K_D less than 10^{-7} M.

[0063] The method of any one of the preceding claims, wherein the antibody binds human TF with a K_D between 10^{-7} M and 10^{-11} M. In some embodiments, K_D for mouse TF as measured by ForteBio is 360 nM or less. In some embodiments, K_D for mouse TF as measured by ForteBio is between 6.12 nM and 360 nM. In some embodiments, K_D for mouse TF as measured by ForteBio is 21 nM or less. In some embodiments, K_D for mouse TF as measured by ForteBio is between about 2.4 nM and 21 nM.

[0064] In some embodiments, the subject is a mammal. In some embodiments, the subject is a human. In some embodiments, the administration is intraocular. In some embodiments, the antibody is administered by intravitreal injection.

[0065] In some embodiments, the antibody is administered once a month or once every two months. In some embodiments, the antibody is administered once every week, two weeks, three weeks or four weeks. In some embodiments, the antibody is administered biweekly. In some embodiments, the antibody is administered weekly.

[0066] In some embodiments, the antibody is administered at 0.6 mg of antibody/eye. In some embodiments, the antibody is administered at less than 0.6 mg of antibody/eye. In some embodiments, the antibody is administered at 0.6, 2, 3.6, 4, or 6 mg of antibody/eye. In some embodiments, the antibody is administered in a single injection of the antibody/eye. In some embodiments, the antibody is administered at 11.9 or 12 mg of antibody/eye. In some embodiments, the antibody is administered at less than 11.9 or 12 mg of antibody/eye. In

some embodiments, the antibody is administered in a single injection of the antibody/eye. In some embodiments, the antibody is administered at less than 0.6, 2, 3.6, 4, 6, 11.9 or 12 mg of antibody/eye.

BRIEF DESCRIPTION OF THE DRAWINGS

[0067] These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, and accompanying drawings, where:

[0068] **FIG. 1** includes a schematic showing the experimental design for swine laser CNV model studies. (*See Examples*).

[0069] **FIGs. 2A and 2B** includes plots showing the efficacy of the indicated anti-TF antibodies, ICON1, and adalimumab in a swine CNV model. **FIG. 2A** shows the mean Corrected Total Lesion Fluorescence (CTLF) at day 7 (treatment day), day 14, and day 28. **FIG. 2B** shows the percent change in CTLF relative to baseline levels (day 7).

[0070] **FIG. 3** includes a plot showing the efficacy of the indicated anti-TF antibodies, Eylea, and combination therapy in swine CNV model. WT denotes the wild-type of the antibody.

[0071] **FIG. 4** includes plots showing the percent change in CTLF from the indicated anti-TF antibodies, Eylea, and combination therapy relative to baseline levels in swine CNV model. WT denotes the wild-type of the antibody.

[0072] **FIG. 5** includes a plot showing the efficacy of the indicated anti-TF antibodies, and ICON1 in swine CNV model. DE denotes the antibody having two mutations in the human IgG1 Fc region (S239D and I332E, as determined by Kabat numbering).

[0073] **FIG. 6** includes a plot showing the percent change in CTLF from the indicated anti-TF antibodies, and ICON1 relative to baseline in swine CNV model. DE denotes the antibody having two mutations in the human IgG1 Fc region (S239D and I332E, as determined by Kabat numbering).

[0074] **FIG. 7** includes a plot showing the efficacy of the indicated anti-TF antibodies, anti-TF antigen-binding fragment, Eylea and combination therapy in swine CNV model. DE denotes the antibody having two mutations in the human IgG1 Fc region (S239D and I332E, as determined by Kabat numbering). LALAPG denotes the antibody having the combination of L₂₃₄A, L₂₃₅A, and P₃₂₉A (LALA-PG) substitutions in the human IgG1 heavy chain constant region (according to Kabat numbering).

[0075] **FIG. 8** includes a plot showing the percent change in CTLF from the indicated anti-TF antibodies, anti-TF antigen-binding fragment, Eylea and combination therapy relative to

baseline in swine CNV model. DE denotes the antibody having two mutations in the human IgG1 Fc region (S239D and I332E, as determined by Kabat numbering). LALAPG denotes the antibody having the combination of L₂₃₄A, L₂₃₅A, and P₃₂₉A (LALA-PG) substitutions in the human IgG1 heavy chain constant region (according to Kabat numbering).

[0076] FIG. 9 includes a plot showing the efficacy of different doses of Eylea and 25A5-T anti-TF antibodies in swine CNV model.

[0077] FIG. 10 includes a plot showing the percent change in CTLF from different doses of Eylea and 25A5-T anti-TF antibodies relative to baseline in swine CNV model.

[0078] FIG. 11 includes a plot showing the mean concentration profiles for 25A5-T in rabbit vitreous humor in a PK study.

[0079] FIG. 12A includes a plot showing the mean concentration profiles for 25A5-T at 600 ug dose in rabbit ocular tissues and serum in a PK study. **FIG. 12B** includes a plot showing the mean concentration profiles for 25A5-T at 3600 ug dose in rabbit ocular tissues and serum in a PK study.

[0080] FIG. 13 includes a plot showing the mean concentration profiles for ICON-1 at 600 ug dose in rabbit ocular tissues and plasma in a PK study.

[0081] FIG. 14 includes plots showing the mean concentration profiles for 25A5-T in the vitreous humor of cynomolgus monkey in a PK study.

[0082] FIG. 15A includes a plot showing the mean concentration profiles for 25A5-T in the ocular tissues and serum of cynomolgus monkey in a PK study.

[0083] FIG. 15B includes a plot showing the mean concentration profiles for 25A5-T in the ocular tissues and serum of cynomolgus monkey in a PK study.

[0084] FIG. 16 includes a plot showing the mean (SD) 25A5-T serum profiles after single IVT administration of 0.6 and 12 mg/eye.

[0085] FIG. 17 includes a schematic showing the experimental design for the pilot toxicology study conducted in cynomolgus monkeys.

[0086] FIG. 18 includes plots showing the mean concentration profiles for 25A5-T in the serum from the pilot toxicology study conducted in cynomolgus monkeys.

[0087] FIG. 19 includes plots showing the mean concentration profiles for 25A5-T in the serum from the pilot toxicology study conducted in cynomolgus monkeys.

[0088] FIG. 20 includes a plot showing the effects of 25A5-T vs. ICON-1 on the coagulation cascade, assessed using a thrombin generation assay. Peak IIa (highest thrombin concentration generated on the thrombin generation curve [nM]) is reported relative to plasma in the absence of antibody.

[0089] **FIGS. 21A and 21B** include plots showing the inhibition of cytokine secretion resulting from treatment of TF-expressing cells with 25A5-T and ICON-1. **FIG. 21A** shows the GM-CSF measurements and **FIG. 21B** shows the IL-8 measurements.

[0090] **FIGS. 22A and 22B** include plots showing the effect of 25A5-T vs. ICON-1 on antibody-dependent cell-mediated cytotoxicity (ADCC; **FIG. 22A**) and antibody-dependent cellular phagocytosis (ADCP; **FIG. 22B**).

[0091] **FIG. 23** includes a plot showing the serum concentration-time profile following IVT administration of 25A5-T.

[0092] **FIGS. 24A-24B** include plots showing summary (mean $\pm$ SD) serum 25A5-T concentrations following bilateral intravitreal injection of 0.6, 2.0 and 6.0 mg/eye 25A5-T on Day 1 in Male (**FIG. 24A**) and Female (**FIG. 24B**) cynomolgus monkeys.

[0093] **FIGS. 25A-25B** include plots showing summary (mean $\pm$ SD) serum 25A5-T concentrations following bilateral intravitreal injection of 0.6, 2.0 and 6.0 mg/eye 25A5-T on Day 1 in Male (**FIG. 25A**) and Female (**FIG. 25B**) cynomolgus monkeys.

[0094] **FIG. 26** includes sensorgrams from TF binding kinetics experiments of 25A5-T binding to the indicated TF proteins.

DETAILED DESCRIPTION

1. Definitions

[0095] Unless otherwise defined, all terms of art, notations and other scientific terminology used herein are intended to have the meanings commonly understood by those of skill in the art. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a difference over what is generally understood in the art. The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodologies by those skilled in the art, such as, for example, the widely utilized molecular cloning methodologies described in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* 4th ed. (2012) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with manufacturer-defined protocols and conditions unless otherwise noted.

[0096] As used herein, the singular forms “a,” “an,” and “the” include the plural referents unless the context clearly indicates otherwise. The terms “include,” “such as,” and the like are intended to convey inclusion without limitation, unless otherwise specifically indicated.

[0097] As used herein, the term “comprising” also specifically includes embodiments “consisting of” and “consisting essentially of” the recited elements, unless specifically indicated otherwise.

[0098] The term “about” indicates and encompasses an indicated value and a range above and below that value. In certain embodiments, the term “about” indicates the designated value $\pm 10\%$, $\pm 5\%$, or $\pm 1\%$. In certain embodiments, where applicable, the term “about” indicates the designated value(s) $\pm$ one standard deviation of that value(s).

[0099] The terms “Tissue Factor,” “TF,” “platelet tissue factor,” “factor III,” “thromboplastin,” and “CD142” are used interchangeably herein to refer to TF, or any variants (*e.g.*, splice variants and allelic variants), isoforms, and species homologs of TF that are naturally expressed by cells, or that are expressed by cells transfected with a TF gene. In some aspects, the TF protein is a TF protein naturally expressed by a primate (*e.g.*, a monkey or a human), a rodent (*e.g.*, a mouse or a rat), a dog, a camel, a cat, a cow, a goat, a horse, a pig or a sheep. In some aspects, the TF protein is human TF (hTF; SEQ ID NO:809). In some aspects, the TF protein is cynomolgus TF (cTF; SEQ ID NO:813). In some aspects, the TF protein is mouse TF (mTF; SEQ ID NO:817). In some aspects, the TF protein is pig TF (pTF; SEQ ID NO:824). TF is a cell surface receptor for the serine protease factor VIIa. It is often times constitutively expressed by certain cells surrounding blood vessels and in some disease settings.

[00100] The term “cytotoxic agent,” as used herein, refers to a substance that inhibits or prevents a cellular function and/or causes cell death or destruction. The cytotoxic agent can be an anti-angiogenic agent, a pro-apoptotic agent, an anti-mitotic agent, an anti-kinase agent, an alkylating agent, a hormone, a hormone agonist, a hormone antagonist, a chemokine, a drug, a prodrug, a toxin, an enzyme, an antimetabolite, an antibiotic, an alkaloid, or a radioactive isotope. Exemplary cytotoxic agents include calicheamycin, camptothecin, carboplatin, irinotecan, SN-38, carboplatin, camptothecin, cyclophosphamide, cytarabine, dacarbazine, docetaxel, dactinomycin, daunorubicin, doxorubicin, doxorubicin, etoposide, idarubicin, topotecan, vinca alkaloid, maytansinoid, maytansinoid analog, pyrrolobenzodiazepine, taxoid, duocarmycin, dolastatin, auristatin, and derivatives thereof.

[00101] A “linker” refers to a molecule that connects one composition to another, *e.g.*, an antibody to an agent. Linkers described herein can conjugate an antibody to a cytotoxic agent. Exemplary linkers include a labile linker, an acid labile linker, a photolabile linker, a charged linker, a disulfide-containing linker, a peptidase-sensitive linker, a β -glucuronide-linker, a

dimethyl linker, a thio-ether linker, and a hydrophilic linker. A linker can be cleavable or non-cleavable.

[00102] The term “immunoglobulin” refers to a class of structurally related proteins generally comprising two pairs of polypeptide chains: one pair of light (L) chains and one pair of heavy (H) chains. In an “intact immunoglobulin,” all four of these chains are interconnected by disulfide bonds. The structure of immunoglobulins has been well characterized. *See, e.g.,* Paul, *Fundamental Immunology* 7th ed., Ch. 5 (2013) Lippincott Williams & Wilkins, Philadelphia, PA. Briefly, each heavy chain typically comprises a heavy chain variable region (V_H) and a heavy chain constant region (C_H). The heavy chain constant region typically comprises three domains, abbreviated C_{H1}, C_{H2}, and C_{H3}. Each light chain typically comprises a light chain variable region (V_L) and a light chain constant region. The light chain constant region typically comprises one domain, abbreviated C_L.

[00103] The term “antibody” is used herein in its broadest sense and includes certain types of immunoglobulin molecules comprising one or more antigen-binding domains that specifically bind to an antigen or epitope. An antibody specifically includes intact antibodies (*e.g.*, intact immunoglobulins), antibody fragments, and multi-specific antibodies.

[00104] The term “alternative scaffold” refers to a molecule in which one or more regions may be diversified to produce one or more antigen-binding domains that specifically bind to an antigen or epitope. In some embodiments, the antigen-binding domain binds the antigen or epitope with specificity and affinity similar to that of an antibody. Exemplary alternative scaffolds include those derived from fibronectin (*e.g.*, AdnectinsTM), the β -sandwich (*e.g.*, iMab), lipocalin (*e.g.*, Anticalins[®]), EETI-II/AGRP, BPTI/LACI-D1/ITI-D2 (*e.g.*, Kunitz domains), thioredoxin peptide aptamers, protein A (*e.g.*, Affibody[®]), ankyrin repeats (*e.g.*, DARPin), gamma-B-crystallin/ubiquitin (*e.g.*, Affilins), CTLD3 (*e.g.*, Tetranectins), Fynomers, and (LDLR-A module) (*e.g.*, Avimers). Additional information on alternative scaffolds is provided in Binz *et al.*, *Nat. Biotechnol.*, 2005 23:1257-1268; Skerra, *Current Opin. in Biotech.*, 2007 18:295-304; and Silacci *et al.*, *J. Biol. Chem.*, 2014, 289:14392-14398; each of which is incorporated by reference in its entirety.

[00105] The term “antigen-binding domain” means the portion of an antibody that is capable of specifically binding to an antigen or epitope. One example of an antigen-binding domain is an antigen-binding domain formed by a V_H-V_L dimer of an antibody. Another example of an antigen-binding domain is an antigen-binding domain formed by diversification of certain loops from the tenth fibronectin type III domain of an Adnectin. Antigen-binding domains can be found in various contexts including antibodies and chimeric

antigen receptors (CARs), for example CARs derived from antibodies or antibody fragments such as scFvs.

[00106] The terms “full length antibody,” “intact antibody,” and “whole antibody” are used herein interchangeably to refer to an antibody having a structure substantially similar to a naturally occurring antibody structure and having heavy chains that comprise an Fc region. For example, when used to refer to an IgG molecule, a “full length antibody” is an antibody that comprises two heavy chains and two light chains.

[00107] The term “Fc region” means the C-terminal region of an immunoglobulin heavy chain that, in naturally occurring antibodies, interacts with Fc receptors and certain proteins of the complement system. The structures of the Fc regions of various immunoglobulins, and the glycosylation sites contained therein, are known in the art. *See* Schroeder and Cavacini, *J. Allergy Clin. Immunol.*, 2010, 125:S41-52, incorporated by reference in its entirety. The Fc region may be a naturally occurring Fc region, or an Fc region modified as described in the art or elsewhere in this disclosure.

[00108] The V_H and V_L regions may be further subdivided into regions of hypervariability (“hypervariable regions (HVRs),” also called “complementarity determining regions” (CDRs)) interspersed with regions that are more conserved. The more conserved regions are called framework regions (FRs). Each V_H and V_L generally comprises three CDRs and four FRs, arranged in the following order (from N-terminus to C-terminus): FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4. The CDRs are involved in antigen binding, and influence antigen specificity and binding affinity of the antibody. *See* Kabat *et al.*, *Sequences of Proteins of Immunological Interest 5th ed.* (1991) Public Health Service, National Institutes of Health, Bethesda, MD, incorporated by reference in its entirety.

[00109] A “Complementary Determining Region (CDR)” refers to one of three hypervariable regions (H1, H2 or H3) within the non-framework region of the immunoglobulin (Ig or antibody) V_H β -sheet framework, or one of three hypervariable regions (L1, L2 or L3) within the non-framework region of the antibody V_L β -sheet framework. CDRs are variable region sequences interspersed within the framework region sequences. CDRs are well recognized in the art and have been defined by, for example, Kabat as the regions of most hypervariability within the antibody variable (V) domains. *See* Kabat *et al.*, *J Biol Chem*, 1977, 252:6609-6616 and Kabat, *Adv Protein Chem*, 1978, 32:1-75, each of which is incorporated by reference in its entirety. CDRs have also been defined structurally by Chothia as those residues that are not part of the conserved β -sheet

framework, and thus are able to adapt different conformations. *See* Chothia and Lesk, *J Mol Biol*, 1987, 196:901-917, incorporated by reference in its entirety. Both the Kabat and Chothia nomenclatures are well known in the art. AbM, Contact and IMGT also defined CDRs. CDR positions within a canonical antibody variable domain have been determined by comparison of numerous structures. *See* Morea *et al.*, *Methods*, 2000, 20:267-279 and Al-Lazikani *et al.*, *J Mol Biol*, 1997, 273:927-48, each of which is incorporated by reference in its entirety. Because the number of residues within a hypervariable region varies in different antibodies, additional residues relative to the canonical positions are conventionally numbered with a, b, c and so forth next to the residue number in the canonical variable domain numbering scheme (Al-Lazikani *et al.*, *supra*). Such terminology is well known to those skilled in the art.

[00110] A number of hypervariable region delineations are in use and are included herein. The Kabat CDRs are based on sequence variability and are the most commonly used. *See* Kabat *et al.* (1992) *Sequences of Proteins of Immunological Interest*, DIANE Publishing: 2719, incorporated by reference in its entirety. Chothia refers instead to the location of the structural loops (Chothia and Lesk, *supra*). The AbM hypervariable regions represent a compromise between the Kabat CDRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software. The Contact hypervariable regions are based on an analysis of the available complex crystal structures. The residues from each of these hypervariable regions are noted in **Table 1**.

[00111] More recently, a universal numbering system ImMunoGeneTics (IMGT) Information System™ has been developed and widely adopted. *See* Lefranc *et al.*, *Dev Comp Immunol*, 2003, 27:55-77, incorporated by reference in its entirety. IMGT is an integrated information system specializing in immunoglobulins (IG), T cell receptors (TR) and major histocompatibility complex (MHC) of human and other vertebrates. The IMGT CDRs are referred to in terms of both the amino acid sequence and the location within the light or heavy chain. As the "location" of the CDRs within the structure of the immunoglobulin variable domain is conserved between species and present in structures called loops, by using numbering systems that align variable domain sequences according to structural features, CDR and framework residues are readily identified. Correspondence between the Kabat, Chothia and IMGT numbering is also well known in the art (Lefranc *et al.*, *supra*). An Exemplary system, shown herein, combines Kabat and Chothia CDR definitions.

Table 1

	Exemplary (Kabat + Chothia)	Kabat	Chothia	AbM	Contact	IMGT
VH CDR1	26-35	31-35	26-32	26-35	30-35	27-38
VH CDR2	50-65	50-65	52a-55	50-58	47-58	56-65
VH CDR3	95-102	95-102	96-101	95-102	93-101	105-117
VL CDR1	24-34	24-34	26-32	24-34	30-36	27-38
VL CDR2	50-56	50-56	50-52	50-56	46-55	56-65
VL CDR3	89-97	89-97	91-96	89-97	89-96	105-117

[00112] The light chain from any vertebrate species can be assigned to one of two types, called kappa (κ) and lambda (λ), based on the sequence of its constant domain.

[00113] The heavy chain from any vertebrate species can be assigned to one of five different classes (or isotypes): IgA, IgD, IgE, IgG, and IgM. These classes are also designated α , δ , ϵ , γ , and μ , respectively. The IgG and IgA classes are further divided into subclasses on the basis of differences in sequence and function. Humans express the following subclasses: IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2.

[00114] The term "constant region" or "constant domain" refers to a carboxy terminal portion of the light and heavy chain which is not directly involved in binding of the antibody to antigen but exhibits various effector function, such as interaction with the Fc receptor. The terms refer to the portion of an immunoglobulin molecule having a more conserved amino acid sequence relative to the other portion of the immunoglobulin, the variable domain, which contains the antigen-binding site. The constant domain contains the C_{H1} , C_{H2} and C_{H3} domains of the heavy chain and the C_L domain of the light chain.

[00115] The "EU numbering scheme" is generally used when referring to a residue in an antibody heavy chain constant region (*e.g.*, as reported in Kabat *et al.*, *supra*). Unless stated otherwise, the EU numbering scheme is used to refer to residues in antibody heavy chain constant regions described herein.

[00116] An "antibody fragment" comprises a portion of an intact antibody, such as the antigen-binding or variable region of an intact antibody. Antibody fragments include, for example, Fv fragments, Fab fragments, F(ab')₂ fragments, Fab' fragments, scFv (sFv) fragments, and scFv-Fc fragments.

[00117] "Fv" fragments comprise a non-covalently-linked dimer of one heavy chain variable domain and one light chain variable domain.

[00118] "Fab" fragments comprise, in addition to the heavy and light chain variable domains, the constant domain of the light chain and the first constant domain (C_{H1}) of the

heavy chain. Fab fragments may be generated, for example, by recombinant methods or by papain digestion of a full-length antibody.

[00119] “F(ab')₂” fragments contain two Fab' fragments joined, near the hinge region, by disulfide bonds. F(ab')₂ fragments may be generated, for example, by recombinant methods or by pepsin digestion of an intact antibody. The F(ab') fragments can be dissociated, for example, by treatment with β-mercaptoethanol.

[00120] “Single-chain Fv” or “sFv” or “scFv” antibody fragments comprise a V_H domain and a V_L domain in a single polypeptide chain. The V_H and V_L are generally linked by a peptide linker. *See* Plückthun A. (1994). Any suitable linker may be used. In some embodiments, the linker is a (GGGS)_n (SEQ ID NO:823). In some embodiments, n = 1, 2, 3, 4, 5, or 6. *See* Antibodies from *Escherichia coli*. In Rosenberg M. & Moore G.P. (Eds.), *The Pharmacology of Monoclonal Antibodies* vol. 113 (pp. 269-315). Springer-Verlag, New York, incorporated by reference in its entirety.

[00121] “scFv-Fc” fragments comprise an scFv attached to an Fc domain. For example, an Fc domain may be attached to the C-terminal of the scFv. The Fc domain may follow the V_H or V_L, depending on the orientation of the variable domains in the scFv (*i.e.*, V_H-V_L or V_L-V_H). Any suitable Fc domain known in the art or described herein may be used.

[00122] The term “single domain antibody” refers to a molecule in which one variable domain of an antibody specifically binds to an antigen without the presence of the other variable domain. Single domain antibodies, and fragments thereof, are described in Arabi Ghahroudi *et al.*, *FEBS Letters*, 1998, 414:521-526 and Muyldermans *et al.*, *Trends in Biochem. Sci.*, 2001, 26:230-245, each of which is incorporated by reference in its entirety. Single domain antibodies are also known as sdAbs or nanobodies.

[00123] A “multispecific antibody” is an antibody that comprises two or more different antigen-binding domains that collectively specifically bind two or more different epitopes. The two or more different epitopes may be epitopes on the same antigen (*e.g.*, a single TF molecule expressed by a cell) or on different antigens (*e.g.*, a TF molecule and a non-TF molecule). In some aspects, a multi-specific antibody binds two different epitopes (*i.e.*, a “bispecific antibody”). In some aspects, a multi-specific antibody binds three different epitopes (*i.e.*, a “trispecific antibody”). In some aspects, a multi-specific antibody binds four different epitopes (*i.e.*, a “quadspecific antibody”). In some aspects, a multi-specific antibody binds five different epitopes (*i.e.*, a “quintspecific antibody”). In some aspects, a multi-specific antibody binds 6, 7, 8, or more different epitopes. Each binding specificity may be

present in any suitable valency. Examples of multispecific antibodies are provided elsewhere in this disclosure.

[00124] A “monospecific antibody” is an antibody that comprises one or more binding sites that specifically bind to a single epitope. An example of a monospecific antibody is a naturally occurring IgG molecule which, while divalent (*i.e.*, having two antigen-binding domains), recognizes the same epitope at each of the two antigen-binding domains. The binding specificity may be present in any suitable valency.

[00125] The term “monoclonal antibody” refers to an antibody from a population of substantially homogeneous antibodies. A population of substantially homogeneous antibodies comprises antibodies that are substantially similar and that bind the same epitope(s), except for variants that may normally arise during production of the monoclonal antibody. Such variants are generally present in only minor amounts. A monoclonal antibody is typically obtained by a process that includes the selection of a single antibody from a plurality of antibodies. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, yeast clones, bacterial clones, or other recombinant DNA clones. The selected antibody can be further altered, for example, to improve affinity for the target (“affinity maturation”), to humanize the antibody, to improve its production in cell culture, and/or to reduce its immunogenicity in a subject.

[00126] The term “chimeric antibody” refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

[00127] “Humanized” forms of non-human antibodies are chimeric antibodies that contain minimal sequence derived from the non-human antibody. A humanized antibody is generally a human antibody (recipient antibody) in which residues from one or more CDRs are replaced by residues from one or more CDRs of a non-human antibody (donor antibody). The donor antibody can be any suitable non-human antibody, such as a mouse, rat, rabbit, chicken, or non-human primate antibody having a desired specificity, affinity, or biological effect. In some instances, selected framework region residues of the recipient antibody are replaced by the corresponding framework region residues from the donor antibody. Humanized antibodies may also comprise residues that are not found in either the recipient antibody or the donor antibody. Such modifications may be made to further refine antibody function. For further details, *see Jones et al., Nature*, 1986, 321:522-525; Riechmann *et al., Nature*, 1988, 332:323-329; and Presta, *Curr. Op. Struct. Biol.*, 1992, 2:593-596, each of which is incorporated by reference in its entirety.

[00128] A “human antibody” is one which possesses an amino acid sequence corresponding to that of an antibody produced by a human or a human cell, or derived from a non-human source that utilizes a human antibody repertoire or human antibody-encoding sequences (*e.g.*, obtained from human sources or designed *de novo*). Human antibodies specifically exclude humanized antibodies.

[00129] An “isolated antibody” or “isolated nucleic acid” is an antibody or nucleic acid that has been separated and/or recovered from a component of its natural environment. Components of the natural environment may include enzymes, hormones, and other proteinaceous or nonproteinaceous materials. In some embodiments, an isolated antibody is purified to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence, for example by use of a spinning cup sequenator. In some embodiments, an isolated antibody is purified to homogeneity by gel electrophoresis (*e.g.*, SDS-PAGE) under reducing or nonreducing conditions, with detection by Coomassie blue or silver stain. In some embodiments, an isolated antibody may include an antibody *in situ* within recombinant cells, since at least one component of the antibody’s natural environment is not present. In some aspects, an isolated antibody or isolated nucleic acid is prepared by at least one purification step. In some embodiments, an isolated antibody or isolated nucleic acid is purified to at least 80%, 85%, 90%, 95%, or 99% by weight. In some embodiments, an isolated antibody or isolated nucleic acid is purified to at least 80%, 85%, 90%, 95%, or 99% by volume. In some embodiments, an isolated antibody or isolated nucleic acid is provided as a solution comprising at least 85%, 90%, 95%, 98%, 99% to 100% antibody or nucleic acid by weight. In some embodiments, an isolated antibody or isolated nucleic acid is provided as a solution comprising at least 85%, 90%, 95%, 98%, 99% to 100% antibody or nucleic acid by volume.

[00130] “Affinity” refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (*e.g.*, an antibody) and its binding partner (*e.g.*, an antigen or epitope). Unless indicated otherwise, as used herein, “affinity” refers to intrinsic binding affinity, which reflects a 1:1 interaction between members of a binding pair (*e.g.*, antibody and antigen or epitope). The affinity of a molecule X for its partner Y can be represented by the dissociation equilibrium constant (K_D). The kinetic components that contribute to the dissociation equilibrium constant are described in more detail below. Affinity can be measured by common methods known in the art, including those described herein, such as surface plasmon resonance (SPR) technology (*e.g.*, BIAcore®) or biolayer interferometry (*e.g.*, FORTEBIO®).

[00131] As used, the term "VEGF" as used herein refers to human vascular endothelial growth factor (VEGF/VEGF-A). VEGF is a homodimeric glycoprotein that is known to promote angiogenesis. It includes 165-amino acid human vascular endothelial growth factor (human VEGF165) and related 121, 189 and 206 vascular endothelial growth factor isoforms (Uniprot ID No: P15692).

[00132] With regard to the binding of an antibody to a target molecule, the terms "bind," "specific binding," "specifically binds to," "specific for," "selectively binds," and "selective for" a particular antigen (*e.g.*, a polypeptide target) or an epitope on a particular antigen mean binding that is measurably different from a non-specific or non-selective interaction (*e.g.*, with a non-target molecule). Specific binding can be measured, for example, by measuring binding to a target molecule and comparing it to binding to a non-target molecule. Specific binding can also be determined by competition with a control molecule that mimics the epitope recognized on the target molecule. In that case, specific binding is indicated if the binding of the antibody to the target molecule is competitively inhibited by the control molecule. In some aspects, the affinity of a TF antibody for a non-target molecule is less than about 50% of the affinity for TF. In some aspects, the affinity of a TF antibody for a non-target molecule is less than about 40% of the affinity for TF. In some aspects, the affinity of a TF antibody for a non-target molecule is less than about 30% of the affinity for TF. In some aspects, the affinity of a TF antibody for a non-target molecule is less than about 20% of the affinity for TF. In some aspects, the affinity of a TF antibody for a non-target molecule is less than about 10% of the affinity for TF. In some aspects, the affinity of a TF antibody for a non-target molecule is less than about 1% of the affinity for TF. In some aspects, the affinity of a TF antibody for a non-target molecule is less than about 0.1% of the affinity for TF.

[00133] In some embodiments, specifically binding refers to an antibody binding with an affinity of less than 1 nM. In some embodiments, specifically binding refers to an antibody binding with an affinity of less than 10 nM. In some embodiments, specifically binding refers to an antibody binding with an affinity of less than 50 nM. In some embodiments, specifically binding refers to an antibody binding with an affinity of less than 100 nM. In some embodiments, specifically binding refers to an antibody binding with an affinity of less than 200 nM. In some embodiments, specifically binding refers to an antibody binding with an affinity of less than 300 nM. In some embodiments, specifically binding refers to an antibody binding with an affinity of less than 200 nM, 300 nM, 400 nM or 500 nM. In some embodiments, specifically binding refers to an antibody binding with an affinity of less than 0 nM, 10 nM, 20 nM, 30 nM, 40 nM, 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, or 100 nM.

[00134] The term “ k_d ” (sec^{-1}), as used herein, refers to the dissociation rate constant of a particular antibody-antigen interaction. This value is also referred to as the k_{off} value.

[00135] The term “ k_a ” ($\text{M}^{-1} \times \text{sec}^{-1}$), as used herein, refers to the association rate constant of a particular antibody-antigen interaction. This value is also referred to as the k_{on} value.

[00136] The term “ K_D ” (M), as used herein, refers to the dissociation equilibrium constant of a particular antibody-antigen interaction. $K_D = k_d/k_a$. In some embodiments, the affinity of an antibody is described in terms of the K_D for an interaction between such antibody and its antigen. For clarity, as known in the art, a smaller K_D value indicates a higher affinity interaction, while a larger K_D value indicates a lower affinity interaction.

[00137] The term “ K_A ” (M^{-1}), as used herein, refers to the association equilibrium constant of a particular antibody-antigen interaction. $K_A = k_a/k_d$.

[00138] An “affinity matured” antibody is an antibody with one or more alterations (*e.g.*, in one or more CDRs or FRs) relative to a parent antibody (*i.e.*, an antibody from which the altered antibody is derived or designed) that result in an improvement in the affinity of the antibody for its antigen, compared to the parent antibody which does not possess the alteration(s). In some embodiments, an affinity matured antibody has nanomolar or picomolar affinity for the target antigen. Affinity matured antibodies may be produced using a variety of methods known in the art. For example, Marks *et al.* (*Bio/Technology*, 1992, 10:779-783, incorporated by reference in its entirety) describes affinity maturation by V_H and V_L domain shuffling. Random mutagenesis of CDR and/or framework residues is described by, for example, Barbas *et al.*, *Proc. Nat. Acad. Sci. U.S.A.*, 1994, 91:3809-3813; Schier *et al.*, *Gene*, 1995, 169:147-155; Yelton *et al.*, *J. Immunol.*, 1995, 155:1994-2004; Jackson *et al.*, *J. Immunol.*, 1995, 154:3310-3319; and Hawkins *et al.*, *J. Mol. Biol.*, 1992, 226:889-896; each of which is incorporated by reference in its entirety.

[00139] In some embodiments, the antibodies of the present disclosure have enhanced Fc effector function. “Fc effector functions” refer to those biological activities mediated by the Fc region of an antibody, which activities may vary depending on the antibody isotype. Non-limiting examples of antibody effector functions include C1q binding to activate complement dependent cytotoxicity (CDC), Fc receptor binding to activate antibody-dependent cellular cytotoxicity (ADCC), and antibody dependent cellular phagocytosis (ADCP).

[00140] When used herein in the context of two or more antibodies, the term “competes with” or “cross-competes with” indicates that the two or more antibodies compete for binding to an antigen (*e.g.*, TF). In one exemplary assay, TF is coated on a surface and contacted with a first TF antibody, after which a second TF antibody is added. In another exemplary assay,

first a TF antibody is coated on a surface and contacted with TF, and then a second TF antibody is added. If the presence of the first TF antibody reduces binding of the second TF antibody, in either assay, then the antibodies compete with each other. The term “competes with” also includes combinations of antibodies where one antibody reduces binding of another antibody, but where no competition is observed when the antibodies are added in the reverse order. However, in some embodiments, the first and second antibodies inhibit binding of each other, regardless of the order in which they are added. In some embodiments, one antibody reduces binding of another antibody to its antigen by at least 25%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, or at least 95%. A skilled artisan can select the concentrations of the antibodies used in the competition assays based on the affinities of the antibodies for TF and the valency of the antibodies. The assays described in this definition are illustrative, and a skilled artisan can utilize any suitable assay to determine if antibodies compete with each other. Suitable assays are described, for example, in Cox *et al.*, “Immunoassay Methods,” in *Assay Guidance Manual [Internet]*, Updated December 24, 2014 (www.ncbi.nlm.nih.gov/books/NBK92434/; accessed September 29, 2015); Silman *et al.*, *Cytometry*, 2001, 44:30-37; and Finco *et al.*, *J. Pharm. Biomed. Anal.*, 2011, 54:351-358; each of which is incorporated by reference in its entirety. As provided in **Example 8**, antibodies of group 25 and antibodies of group 43 compete with each other for binding to human TF, while antibodies from groups 1, 29, 39, and 54 do not compete for binding to human TF with antibodies of groups 25 and 43.

[00141] As used herein, an antibody that binds specifically to a human antigen is considered to bind the same antigen of mouse origin when a K_D value can be measured on a ForteBio Octet with the mouse antigen. An antibody that binds specifically to a human antigen is considered to be “cross-reactive” with the same antigen of mouse origin when the K_D value for the mouse antigen is no greater than 20 times the corresponding K_D value for the respective human antigen. For example, the antibody M1593 described in U.S. Pat. Nos. 8,722,044, 8,951,525, and 8,999,333, each of which is herein incorporated by reference for all purposes, the humanized 5G9 antibody described in Ngo *et al.*, 2007, *Int J Cancer*, 120(6):1261-1267, incorporated by reference in its entirety, and chimeric ALT-836 antibody described in Hong *et al.*, 2012, *J Nucl Med*, 53(11):1748-1754, incorporated by reference in its entirety, do not bind to mouse TF. As provided in **Examples 1 and 2**, TF antibodies from groups 25 and 43 bind to mouse TF, *e.g.*, the TF antibodies 25G, 25G1, 25G9, and 43D8 are cross-reactive with mouse TF.

[00142] As used herein, an antibody that binds specifically to a human antigen is considered to be “cross-reactive” with the same antigen of cynomolgus monkey origin when the K_D value for the cynomolgus monkey antigen is no greater than 15 times the corresponding K_D value for the respective human antigen. As provided in **Example 1**, all tested antibodies from groups 1, 25, 29, 39, 43, and 54 are cross-reactive with cynomolgus monkey TF.

[00143] The term “epitope” means a portion of an antigen that is specifically bound by an antibody. Epitopes frequently include surface-accessible amino acid residues and/or sugar side chains and may have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and non-conformational epitopes are distinguished in that the binding to the former but not the latter may be lost in the presence of denaturing solvents. An epitope may comprise amino acid residues that are directly involved in the binding, and other amino acid residues, which are not directly involved in the binding. The epitope to which an antibody binds can be determined using known techniques for epitope determination such as, for example, testing for antibody binding to TF variants with different point-mutations, or to chimeric TF variants.

[00144] Percent “identity” between a polypeptide sequence and a reference sequence, is defined as the percentage of amino acid residues in the polypeptide sequence that are identical to the amino acid residues in the reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, MEGALIGN (DNASTAR), CLUSTALW, CLUSTAL OMEGA, or MUSCLE software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

[00145] A “conservative substitution” or a “conservative amino acid substitution,” refers to the substitution of an amino acid with a chemically or functionally similar amino acid. Conservative substitution tables providing similar amino acids are well known in the art. By way of example, the groups of amino acids provided in **Tables 2-4** are, in some embodiments, considered conservative substitutions for one another.

Table 2: Selected groups of amino acids that are considered conservative substitutions for one another, in certain embodiments.

<i>Acidic Residues</i>	D and E
<i>Basic Residues</i>	K, R, and H
<i>Hydrophilic Uncharged Residues</i>	S, T, N, and Q
<i>Aliphatic Uncharged Residues</i>	G, A, V, L, and I
<i>Non-polar Uncharged Residues</i>	C, M, and P
<i>Aromatic Residues</i>	F, Y, and W

Table 3: Additional selected groups of amino acids that are considered conservative substitutions for one another, in certain embodiments.

<i>Group 1</i>	A, S, and T
<i>Group 2</i>	D and E
<i>Group 3</i>	N and Q
<i>Group 4</i>	R and K
<i>Group 5</i>	I, L, and M
<i>Group 6</i>	F, Y, and W

Table 4: Further selected groups of amino acids that are considered conservative substitutions for one another, in certain embodiments.

<i>Group A</i>	A and G
<i>Group B</i>	D and E
<i>Group C</i>	N and Q
<i>Group D</i>	R, K, and H
<i>Group E</i>	I, L, M, V
<i>Group F</i>	F, Y, and W
<i>Group G</i>	S and T
<i>Group H</i>	C and M

[00146] Additional conservative substitutions may be found, for example, in Creighton, *Proteins: Structures and Molecular Properties* 2nd ed. (1993) W. H. Freeman & Co., New York, NY. An antibody generated by making one or more conservative substitutions of amino acid residues in a parent antibody is referred to as a “conservatively modified variant.”

[00147] The term “amino acid” refers to the twenty common naturally occurring amino acids. Naturally occurring amino acids include alanine (Ala; A), arginine (Arg; R), asparagine (Asn; N), aspartic acid (Asp; D), cysteine (Cys; C), glutamic acid (Glu; E), glutamine (Gln; Q), Glycine (Gly; G), histidine (His; H), isoleucine (Ile; I), leucine (Leu; L), lysine (Lys; K), methionine (Met; M), phenylalanine (Phe; F), proline (Pro; P), serine (Ser; S), threonine (Thr; T), tryptophan (Trp; W), tyrosine (Tyr; Y), and valine (Val; V).

[00148] The term “vector,” as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host

cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as “expression vectors.”

[00149] The terms “host cell,” “host cell line,” and “host cell culture” are used interchangeably and refer to cells into which an exogenous nucleic acid has been introduced, and the progeny of such cells. Host cells include “transformants” (or “transformed cells”) and “transfectants” (or “transfected cells”), which each include the primary transformed or transfected cell and progeny derived therefrom. Such progeny may not be completely identical in nucleic acid content to a parent cell, and may contain mutations.

[00150] The term “treating” (and variations thereof such as “treat” or “treatment”) refers to clinical intervention in an attempt to alter the natural course of a disease or condition in a subject in need thereof. Treatment can be performed both for prophylaxis and during the course of clinical pathology. Desirable effects of treatment include preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In connection with ocular disease such as AMD or DME, the term “treat” refers to improving the results for a subject in the early treatment of diabetic retinopathy (EDTRS) on the eye chart from the start of therapy.

[00151] As used herein, the term “therapeutically effective amount” or “effective amount” refers to an amount of an antibody or pharmaceutical composition provided herein that, when administered to a subject, is effective to treat a disease or disorder. An effective amount is sufficient to effect a desired results or benefit in a subject. An effective amount can be administered in one or more administrations, applications or dosages and is not intended to be limited to a particular formulation or administration route.

[00152] As used herein, the terms “baseline levels” and “baseline” refer to the levels for a parameter (*e.g.*, retinal thickness) immediately prior to treatment or at the time of treatment.

[00153] As used herein, the term “subject” means a mammalian subject. Exemplary subjects include humans, monkeys, dogs, cats, mice, rats, cows, horses, camels, goats, rabbits, pigs and sheep. In certain embodiments, the subject is a human. In some embodiments the subject has a disease or condition that can be treated with an antibody provided herein. In some aspects, the disease or condition involves neovascularization or vascular inflammation. In certain aspects, the disease or condition involving

neovascularization is age-related macular degeneration (AMD), diabetic retinopathy, or other ocular diseases.

[00154] As used herein, the phrase “subject in need thereof” refers to a subject that exhibits and/or is diagnosed with one or more symptoms or signs of ocular disease as described herein.

[00155] The term “package insert” is used to refer to instructions customarily included in commercial packages of therapeutic or diagnostic products (*e.g.*, kits) that contain information about the indications, usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic or diagnostic products.

[00156] A “chemotherapeutic agent” refers to a chemical compound useful in the treatment of cancer. Chemotherapeutic agents include “anti-hormonal agents” or “endocrine therapeutics” which act to regulate, reduce, block, or inhibit the effects of hormones that can promote the growth of cancer.

[00157] The term “cytostatic agent” refers to a compound or composition which arrests growth of a cell either *in vitro* or *in vivo*. In some embodiments, a cytostatic agent is an agent that reduces the percentage of cells in S phase. In some embodiments, a cytostatic agent reduces the percentage of cells in S phase by at least about 20%, at least about 40%, at least about 60%, or at least about 80%.

[00158] The term “pharmaceutical composition” refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective in treating a subject, and which contains no additional components which are unacceptably toxic to the subject in the amounts provided in the pharmaceutical composition.

[00159] The terms “modulate” and “modulation” refer to reducing or inhibiting or, alternatively, activating or increasing, a recited variable.

[00160] The terms “increase” and “activate” refer to an increase of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, or greater in a recited variable.

[00161] The terms “reduce” and “inhibit” refer to a decrease of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, or greater in a recited variable.

[00162] The term “agonize” refers to the activation of receptor signaling to induce a biological response associated with activation of the receptor. An “agonist” is an entity that binds to and agonizes a receptor.

[00163] The term “antagonize” refers to the inhibition of receptor signaling to inhibit a biological response associated with activation of the receptor. An “antagonist” is an entity that binds to and antagonizes a receptor.

2. TF Antibodies

2.1. TF Binding

[00164] Provided herein are isolated antibodies that specifically bind to TF. In some aspects, the TF is hTF (SEQ ID NO:809). In some aspects, the TF is cTF (SEQ ID NO:813). In some aspects, the TF is mTF (SEQ ID NO:817). In some aspects, the TF is rabbit TF (SEQ ID NO:832). In some aspects, the TF is pTF (SEQ ID NO:824). In some embodiments, the antibodies provided herein specifically bind to hTF (SEQ ID NO:809), cTF (SEQ ID NO:813), mTF (SEQ ID NO:817), rabbit TF (SEQ ID NO:832), and pTF (SEQ ID NO:824). In some embodiments, the antibodies provided herein specifically bind to hTF (SEQ ID NO:809), cTF (SEQ ID NO:813), mTF (SEQ ID NO:817), and pTF (SEQ ID NO:824). In some embodiments, the antibodies provided herein specifically bind to hTF (SEQ ID NO:809), cTF (SEQ ID NO:813), and mTF (SEQ ID NO:817). In some embodiments, the antibodies provided herein specifically bind to hTF (SEQ ID NO:809) and cTF (SEQ ID NO:813). In some embodiments, the antibodies provided herein do not bind mTF (SEQ ID NO:817). In some embodiments, the antibodies provided herein do not bind pTF (SEQ ID NO:824). In some embodiments, the antibodies provided herein do not bind rabbit TF (SEQ ID NO:832).

[00165] In various embodiments, the antibodies provided herein specifically bind to the extracellular domain of human TF (SEQ ID NO:810).

[00166] In some embodiments, the binding between an antibody provided herein and a variant TF extracellular domain comprising a mutation at amino acid residue 149 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody provided herein and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay. In some embodiments, the mutation at amino acid residue 149 of the sequence shown in SEQ ID NO:810 is K149N.

[00167] In some embodiments, the binding between an antibody provided herein and a variant TF extracellular domain comprising a mutation at amino acid residue 68 of the sequence shown in SEQ ID NO:810 is greater than 50% of the binding between the antibody provided herein and the extracellular domain of TF of the sequence shown in SEQ ID

NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay. In some embodiments, the mutation at amino acid residue 68 of the sequence shown in SEQ ID NO:810 is K68N.

[00168] In some embodiments, the binding between an antibody provided herein and a variant TF extracellular domain comprising mutations at amino acid residues 171 and 197 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody provided herein and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay. In some embodiments, the mutations at amino acid residues 171 and 197 of the sequence shown in SEQ ID NO:810 are N171H and T197K.

[00169] In some embodiments, the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 1-77 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 1-76 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00170] In some embodiments, the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 39-77 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 38-76 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00171] In some embodiments, the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 94-107 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 99-112 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00172] In some embodiments, the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 146-158 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 151-163 of the

sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00173] In some embodiments, the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 159-219 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-224 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00174] In some embodiments, the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 159-189 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-194 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00175] In some embodiments, the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 159-174 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-179 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00176] In some embodiments, the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 167-174 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 172-179 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00177] In some embodiments, the binding between an antibody provided herein and a rat TF extracellular domain with amino acid residues 141-194 of the sequence shown in SEQ ID

NO:838 replaced by human TF extracellular domain amino acid residues 136-189 of the sequence shown in SEQ ID NO:810 is greater than 50% of the binding between the antibody provided herein and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00178] In some embodiments, the binding between an antibody provided herein and a variant TF extracellular domain comprising a mutation at amino acid residue 149 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody provided herein and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; the binding between an antibody provided herein and a variant TF extracellular domain comprising a mutation at amino acid residue 68 of the sequence shown in SEQ ID NO:810 is greater than 50% of the binding between the antibody provided herein and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 1-77 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 1-76 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 39-77 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 38-76 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 94-107 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 99-112 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 146-158 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 151-163 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; and the binding between an antibody provided herein and a rat TF extracellular domain with amino acid residues 141-194 of the sequence shown in SEQ ID NO:838 replaced by human TF extracellular domain amino acid residues 136-189 of the sequence shown in SEQ ID NO:810

is greater than 50% of the binding between the antibody provided herein and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay. In some embodiments, the mutation at amino acid residue 149 of the sequence shown in SEQ ID NO:810 is K149N; and the mutation at amino acid residue 68 of the sequence shown in SEQ ID NO:810 is K68N.

[00179] In some embodiments, the binding between an antibody provided herein and a variant TF extracellular domain comprising a mutation at amino acid residue 149 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody provided herein and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; the binding between an antibody provided herein and a variant TF extracellular domain comprising a mutation at amino acid residue 68 of the sequence shown in SEQ ID NO:810 is greater than 50% of the binding between the antibody provided herein and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; the binding between an antibody provided herein and a variant TF extracellular domain comprising mutations at amino acid residues 171 and 197 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody provided herein and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 1-77 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 1-76 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 39-77 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 38-76 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 94-107 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 99-112 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 146-158 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 151-163 of the sequence shown in SEQ ID NO:838 is less than 50% of

the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 159-219 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-224 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 159-189 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-194 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 159-174 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-179 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; the binding between an antibody provided herein and a human TF extracellular domain with amino acid residues 167-174 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 172-179 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810; and the binding between an antibody provided herein and a rat TF extracellular domain with amino acid residues 141-194 of the sequence shown in SEQ ID NO:838 replaced by human TF extracellular domain amino acid residues 136-189 of the sequence shown in SEQ ID NO:810 is greater than 50% of the binding between the antibody provided herein and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay. In some embodiments, the mutation at amino acid residue 149 of the sequence shown in SEQ ID NO:810 is K149N; the mutation at amino acid residue 68 of the sequence shown in SEQ ID NO:810 is K68N; and the mutations at amino acid residues 171 and 197 of the sequence shown in SEQ ID NO:810 are N171H and T197K.

[00180] In some embodiments, the antibodies provided herein are inert in inhibiting human thrombin generation as determined by thrombin generation assay (TGA) compared to a reference antibody M1593, wherein the reference antibody M1593 comprises a V_H sequence of SEQ ID NO:821 and a V_L sequence of SEQ ID NO:822.

[00181] In some embodiments, the antibodies provided herein do not inhibit human thrombin generation as determined by thrombin generation assay (TGA). In certain embodiments, the antibodies provided herein allow human thrombin generation as determined by thrombin generation assay (TGA).

[00182] In some embodiments, the antibodies provided herein bind human TF at a human TF binding site that is distinct from a human TF binding site bound by human FX. In certain embodiments, the antibodies provided herein do not interfere with the ability of TF:FVIIa to convert FX into FXa.

[00183] In some embodiments, the antibodies provided herein bind human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa. In certain embodiments, the antibodies provided herein do not compete for binding to human TF with human FVIIa.

[00184] In some embodiments, the antibodies provided herein bind to the extracellular domain of human TF, bind human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa, bind human TF at a human TF binding site that is distinct from a human TF binding site bound by human FX, and allow human thrombin generation as determined by thrombin generation assay (TGA).

[00185] In some embodiments, the antibodies provided herein bind to the extracellular domain of human TF, do not inhibit human thrombin generation as determined by thrombin generation assay (TGA), do not interfere with the ability of TF:FVIIa to convert FX into FXa, and do not compete for binding to human TF with human FVIIa.

[00186] In some embodiments, the antibodies provided herein bind to the extracellular domain of human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa, do not inhibit human thrombin generation as determined by thrombin generation assay (TGA), allow human thrombin generation as determined by thrombin generation assay (TGA), bind to human TF at a human TF binding site that is distinct from a human TF binding site bound by human FX, do not interfere with the ability of TF:FVIIa to convert FX into FXa, and do not compete for binding to human TF with human FVIIa.

[00187] In some embodiments, the antibodies provided herein inhibit FVIIa-dependent TF signaling.

[00188] In some embodiments, the antibodies provided herein reduce lesion size in a swine choroidal neovascularization (CNV) model. TF is aberrantly expressed in choroidal neovascularization (CNV) found in wet age-related macular degeneration.

[00189] In some embodiments, the antibodies provided herein bind to the extracellular domain of human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa, do not inhibit human thrombin generation as determined by thrombin generation assay (TGA), allow human thrombin generation as determined by thrombin generation assay (TGA), bind to human TF at a human TF binding site that is distinct from a human TF binding site bound by human FX, do not interfere with the ability of TF:FVIIa to convert FX into FXa, do not compete for binding to human TF with human FVIIa, and bind to cynomolgus and mouse TF.

[00190] In some embodiments, the antibodies provided herein bind to the extracellular domain of human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa, do not inhibit human thrombin generation as determined by thrombin generation assay (TGA), allow human thrombin generation as determined by thrombin generation assay (TGA), bind to human TF at a human TF binding site that is distinct from a human TF binding site bound by human FX, do not interfere with the ability of TF:FVIIa to convert FX into FXa, do not compete for binding to human TF with human FVIIa, bind to cynomolgus, mouse, and pig TF, and reduce lesion size in a swine choroidal neovascularization (CNV) model.

[00191] In some embodiments, the antibodies provided herein bind to the extracellular domain of human TF, inhibit FVIIa-dependent TF signaling, and bind to cynomolgus TF.

2.2. Sequences of TF Antibodies

2.2.1. V_H Domains

[00192] In some embodiments, an antibody provided herein comprises a V_H sequence selected from SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:37. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:75. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:113. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:151. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:189. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:836. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:227. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:265. In some embodiments, an antibody provided herein comprises

a V_H sequence of SEQ ID NO:303. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:341. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:379. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:417. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:455. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:493. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:531. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:569. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:607. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:645. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:683. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:721. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:759.

[00193] In some embodiments, an antibody provided herein comprises a V_H sequence having at least about 50%, 60%, 70%, 80%, 90%, 95%, or 99% identity to an illustrative V_H sequence provided in SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759. In some embodiments, an antibody provided herein comprises a V_H sequence provided in SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759, with up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acid substitutions. In some aspects, the amino acid substitutions are conservative amino acid substitutions. In some embodiments, the antibodies described in this paragraph are referred to herein as “variants.” In some embodiments, such variants are derived from a sequence provided herein, for example, by affinity maturation, site directed mutagenesis, random mutagenesis, or any other method known in the art or described herein. In some embodiments, such variants are not derived from a sequence provided herein and may, for example, be isolated *de novo* according to the methods provided herein for obtaining antibodies.

2.2.2. V_L Domains

[00194] In some embodiments, an antibody provided herein comprises a V_L sequence selected from SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494,

532, 570, 608, 646, 684, 722, and 760. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:38. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:76. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:114. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:152. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:190. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:837. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:228. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:266. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:304. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:342. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:380. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:418. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:456. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:494. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:532. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:570. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:608. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:646. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:684. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:722. In some embodiments, an antibody provided herein comprises a V_L sequence of SEQ ID NO:760.

[00195] In some embodiments, an antibody provided herein comprises a V_L sequence having at least about 50%, 60%, 70%, 80%, 90%, 95%, or 99% identity to an illustrative V_L sequence provided in SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760. In some embodiments, an antibody provided herein comprises a V_L sequence provided in SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760, with up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acid substitutions. In some aspects, the amino acid substitutions are conservative amino acid substitutions. In some embodiments, the antibodies described in this paragraph are referred to herein as “variants.” In some embodiments, such variants are derived from a sequence

provided herein, for example, by affinity maturation, site directed mutagenesis, random mutagenesis, or any other method known in the art or described herein. In some embodiments, such variants are not derived from a sequence provided herein and may, for example, be isolated *de novo* according to the methods provided herein for obtaining antibodies.

2.2.3. V_H-V_L Combinations

[00196] In some embodiments, an antibody provided herein comprises a V_H sequence selected from SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759 and a V_L sequence selected from SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760.

[00197] In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:37 and a V_L sequence of SEQ ID NO:38. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:75 and a V_L sequence of SEQ ID NO:76. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:113 and a V_L sequence of SEQ ID NO:114. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:151 and a V_L sequence of SEQ ID NO:152. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:189 and a V_L sequence of SEQ ID NO:190. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:836 and a V_L sequence of SEQ ID NO:837. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:227 and a V_L sequence of SEQ ID NO:228. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:265 and a V_L sequence of SEQ ID NO:266. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:303 and a V_L sequence of SEQ ID NO:304. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:341 and a V_L sequence of SEQ ID NO:342. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:379 and a V_L sequence of SEQ ID NO:380. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:417 and a V_L sequence of SEQ ID NO:418. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:455 and a V_L sequence of SEQ ID NO:456. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:493 and a V_L sequence of SEQ ID NO:494. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:531 and a V_L sequence of SEQ ID NO:532. In some embodiments, an antibody

provided herein comprises a V_H sequence of SEQ ID NO:569 and a V_L sequence of SEQ ID NO:570. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:607 and a V_L sequence of SEQ ID NO:608. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:645 and a V_L sequence of SEQ ID NO:646. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:683 and a V_L sequence of SEQ ID NO:684. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:721 and a V_L sequence of SEQ ID NO:722. In some embodiments, an antibody provided herein comprises a V_H sequence of SEQ ID NO:759 and a V_L sequence of SEQ ID NO:760.

[00198] In some embodiments, an antibody provided herein comprises a V_H sequence having at least about 50%, 60%, 70%, 80%, 90%, 95%, or 99% identity to an illustrative V_H sequence provided in SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759, and a V_L sequence having at least about 50%, 60%, 70%, 80%, 90%, 95%, or 99% identity to an illustrative V_L sequence provided in SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760. In some embodiments, an antibody provided herein comprises a V_H sequence provided in SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759, with up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acid substitutions, and a V_L sequence provided in SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760, with up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acid substitutions. In some aspects, the amino acid substitutions are conservative amino acid substitutions. In some embodiments, the antibodies described in this paragraph are referred to herein as “variants.” In some embodiments, such variants are derived from a sequence provided herein, for example, by affinity maturation, site directed mutagenesis, random mutagenesis, or any other method known in the art or described herein. In some embodiments, such variants are not derived from a sequence provided herein and may, for example, be isolated *de novo* according to the methods provided herein for obtaining antibodies.

2.2.4. CDRs

[00199] In some embodiments, an antibody provided herein comprises one to three CDRs of a V_H domain selected from SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759. In some embodiments, an antibody

provided herein comprises two to three CDRs of a V_H domain selected from SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759. In some embodiments, an antibody provided herein comprises three CDRs of a V_H domain selected from SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759. In some aspects, the CDRs are Exemplary CDRs. In some aspects, the CDRs are Kabat CDRs. In some aspects, the CDRs are Chothia CDRs. In some aspects, the CDRs are AbM CDRs. In some aspects, the CDRs are Contact CDRs. In some aspects, the CDRs are IMGT CDRs.

[00200] In some embodiments, the CDRs are CDRs having at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-H1, CDR-H2, or CDR-H3 of SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759. In some embodiments, the CDR-H1 is a CDR-H1 of a V_H domain selected from SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759, with up to 1, 2, 3, 4, or 5 amino acid substitutions. In some embodiments, the CDR-H2 is a CDR-H2 of a V_H domain selected from SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions. In some embodiments, the CDR-H3 is a CDR-H3 of a V_H domain selected from SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions. In some aspects, the amino acid substitutions are conservative amino acid substitutions. In some embodiments, the antibodies described in this paragraph are referred to herein as “variants.” In some embodiments, such variants are derived from a sequence provided herein, for example, by affinity maturation, site directed mutagenesis, random mutagenesis, or any other method known in the art or described herein. In some embodiments, such variants are not derived from a sequence provided herein and may, for example, be isolated *de novo* according to the methods provided herein for obtaining antibodies.

[00201] In some embodiments, an antibody provided herein comprises one to three CDRs of a V_L domain selected from SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760. In some embodiments, an antibody provided herein comprises two to three CDRs of a V_L domain selected from SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760. In some embodiments, an antibody provided herein comprises three CDRs of a V_L domain selected from SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456,

494, 532, 570, 608, 646, 684, 722, and 760. In some aspects, the CDRs are Exemplary CDRs. In some aspects, the CDRs are Kabat CDRs. In some aspects, the CDRs are Chothia CDRs. In some aspects, the CDRs are AbM CDRs. In some aspects, the CDRs are Contact CDRs. In some aspects, the CDRs are IMGT CDRs.

[00202] In some embodiments, the CDRs are CDRs having at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-L1, CDR-L2, or CDR-L3 of SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760. In some embodiments, the CDR-L1 is a CDR-L1 of a V_L domain selected from SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760, with up to 1, 2, 3, 4, or 5 amino acid substitutions. In some embodiments, the CDR-L2 is a CDR-L2 of a V_L domain selected from SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions. In some embodiments, the CDR-L3 is a CDR-L3 of a V_L domain selected from SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions. In some aspects, the amino acid substitutions are conservative amino acid substitutions. In some embodiments, the antibodies described in this paragraph are referred to herein as “variants.” In some embodiments, such variants are derived from a sequence provided herein, for example, by affinity maturation, site directed mutagenesis, random mutagenesis, or any other method known in the art or described herein. In some embodiments, such variants are not derived from a sequence provided herein and may, for example, be isolated *de novo* according to the methods provided herein for obtaining antibodies.

[00203] In some embodiments, an antibody provided herein comprises one to three CDRs of a V_H domain selected from SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759 and one to three CDRs of a V_L domain selected from SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760. In some embodiments, an antibody provided herein comprises two to three CDRs of a V_H domain selected from SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759 and two to three CDRs of a V_L domain selected from SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760. In some embodiments, an antibody provided herein comprises three CDRs of a V_H domain selected from SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683,

721, and 759 and three CDRs of a V_L domain selected from SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760. In some aspects, the CDRs are Exemplary CDRs. In some aspects, the CDRs are Kabat CDRs. In some aspects, the CDRs are Chothia CDRs. In some aspects, the CDRs are AbM CDRs. In some aspects, the CDRs are Contact CDRs. In some aspects, the CDRs are IMGT CDRs.

[00204] In some embodiments, the CDRs are CDRs having at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-H1, CDR-H2, or CDR-H3 of SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759 and at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-L1, CDR-L2, or CDR-L3 of SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760. In some embodiments, the CDR-H1 is a CDR-H1 of a V_H domain selected from SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759, with up to 1, 2, 3, 4, or 5 amino acid substitutions; the CDR-H2 is a CDR-H2 of a V_H domain selected from SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions; the CDR-H3 is a CDR-H3 of a V_H domain selected from SEQ ID NOs: 37, 75, 113, 151, 189, 227, 265, 303, 341, 379, 417, 455, 493, 531, 569, 607, 645, 683, 721, and 759, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions; the CDR-L1 is a CDR-L1 of a V_L domain selected from SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760, with up to 1, 2, 3, 4, 5, or 6 amino acid substitutions; the CDR-L2 is a CDR-L2 of a V_L domain selected from SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760, with up to 1, 2, 3, or 4 amino acid substitutions; and the CDR-L3 is a CDR-L3 of a V_L domain selected from SEQ ID NOs: 38, 76, 114, 152, 190, 228, 266, 304, 342, 380, 418, 456, 494, 532, 570, 608, 646, 684, 722, and 760, with up to 1, 2, 3, 4, or 5 amino acid substitutions. In some aspects, the amino acid substitutions are conservative amino acid substitutions. In some embodiments, the antibodies described in this paragraph are referred to herein as “variants.” In some embodiments, such variants are derived from a sequence provided herein, for example, by affinity maturation, site directed mutagenesis, random mutagenesis, or any other method known in the art or described herein. In some embodiments, such variants are not derived from a sequence provided herein and may, for example, be isolated *de novo* according to the methods provided herein for obtaining antibodies.

[00205] In some embodiments, an antibody provided herein comprises a CDR-H3 selected from SEQ ID NOs: 3, 41, 79, 117, 155, 193, 231, 269, 307, 345, 383, 421, 459, 497, 535, 573, 611, 649, 687, and 725, as determined by the Exemplary numbering system. In some aspects, the CDR-H3 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-H3 of SEQ ID NOs: 3, 41, 79, 117, 155, 193, 231, 269, 307, 345, 383, 421, 459, 497, 535, 573, 611, 649, 687, and 725. In some embodiments, the CDR-H3 is a CDR-H3 selected from SEQ ID NOs: 3, 41, 79, 117, 155, 193, 231, 269, 307, 345, 383, 421, 459, 497, 535, 573, 611, 649, 687, and 725, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions. In some aspects, the amino acid substitutions are conservative amino acid substitutions. In some embodiments, the antibodies described in this paragraph are referred to herein as “variants.” In some embodiments, such variants are derived from a sequence provided herein, for example, by affinity maturation, site directed mutagenesis, random mutagenesis, or any other method known in the art or described herein. In some embodiments, such variants are not derived from a sequence provided herein and may, for example, be isolated *de novo* according to the methods provided herein for obtaining antibodies.

[00206] In some embodiments, an antibody provided herein comprises a CDR-H2 selected from SEQ ID NOs: 2, 40, 78, 116, 154, 192, 230, 268, 306, 344, 382, 420, 458, 496, 534, 572, 610, 648, 686, and 724, as determined by the Exemplary numbering system. In some aspects, the CDR-H2 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-H2 of SEQ ID NOs: 2, 40, 78, 116, 154, 192, 230, 268, 306, 344, 382, 420, 458, 496, 534, 572, 610, 648, 686, and 724. In some embodiments, the CDR-H2 is a CDR-H2 selected from SEQ ID NOs: 2, 40, 78, 116, 154, 192, 230, 268, 306, 344, 382, 420, 458, 496, 534, 572, 610, 648, 686, and 724, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions. In some aspects, the amino acid substitutions are conservative amino acid substitutions. In some embodiments, the antibodies described in this paragraph are referred to herein as “variants.” In some embodiments, such variants are derived from a sequence provided herein, for example, by affinity maturation, site directed mutagenesis, random mutagenesis, or any other method known in the art or described herein. In some embodiments, such variants are not derived from a sequence provided herein and may, for example, be isolated *de novo* according to the methods provided herein for obtaining antibodies.

[00207] In some embodiments, an antibody provided herein comprises a CDR-H1 selected from SEQ ID NOs: 1, 39, 77, 115, 153, 191, 229, 267, 305, 343, 381, 419, 457, 495, 533, 571, 609, 647, 685, and 723, as determined by the Exemplary numbering system. In some aspects, the CDR-H1 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a

CDR-H1 of SEQ ID NOs: 1, 39, 77, 115, 153, 191, 229, 267, 305, 343, 381, 419, 457, 495, 533, 571, 609, 647, 685, and 723. In some embodiments, the CDR-H1 is a CDR-H1 selected from SEQ ID NOs: 1, 39, 77, 115, 153, 191, 229, 267, 305, 343, 381, 419, 457, 495, 533, 571, 609, 647, 685, and 723, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions. In some aspects, the amino acid substitutions are conservative amino acid substitutions. In some embodiments, the antibodies described in this paragraph are referred to herein as “variants.” In some embodiments, such variants are derived from a sequence provided herein, for example, by affinity maturation, site directed mutagenesis, random mutagenesis, or any other method known in the art or described herein. In some embodiments, such variants are not derived from a sequence provided herein and may, for example, be isolated *de novo* according to the methods provided herein for obtaining antibodies.

[00208] In some embodiments, an antibody provided herein comprises a CDR-H3 selected from SEQ ID NOs: 3, 41, 79, 117, 155, 193, 231, 269, 307, 345, 383, 421, 459, 497, 535, 573, 611, 649, 687, and 725 and a CDR-H2 selected from SEQ ID NOs: 2, 40, 78, 116, 154, 192, 230, 268, 306, 344, 382, 420, 458, 496, 534, 572, 610, 648, 686, and 724. In some embodiments, an antibody provided herein comprises a CDR-H3 selected from SEQ ID NOs: 3, 41, 79, 117, 155, 193, 231, 269, 307, 345, 383, 421, 459, 497, 535, 573, 611, 649, 687, and 725, a CDR-H2 selected from SEQ ID NOs: 2, 40, 78, 116, 154, 192, 230, 268, 306, 344, 382, 420, 458, 496, 534, 572, 610, 648, 686, and 724, and a CDR-H1 selected from SEQ ID NOs: 1, 39, 77, 115, 153, 191, 229, 267, 305, 343, 381, 419, 457, 495, 533, 571, 609, 647, 685, and 723. In some embodiments, the CDR-H3 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-H3 of SEQ ID NOs: 3, 41, 79, 117, 155, 193, 231, 269, 307, 345, 383, 421, 459, 497, 535, 573, 611, 649, 687, and 725, the CDR-H2 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-H2 of SEQ ID NOs: 2, 40, 78, 116, 154, 192, 230, 268, 306, 344, 382, 420, 458, 496, 534, 572, 610, 648, 686, and 724, and the CDR-H1 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-H1 of SEQ ID NOs: 1, 39, 77, 115, 153, 191, 229, 267, 305, 343, 381, 419, 457, 495, 533, 571, 609, 647, 685, and 723. In some embodiments, the CDR-H3 is a CDR-H3 selected from SEQ ID NOs: 3, 41, 79, 117, 155, 193, 231, 269, 307, 345, 383, 421, 459, 497, 535, 573, 611, 649, 687, and 725, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions; the CDR-H2 is a CDR-H2 selected from SEQ ID NOs: 2, 40, 78, 116, 154, 192, 230, 268, 306, 344, 382, 420, 458, 496, 534, 572, 610, 648, 686, and 724, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions; and the CDR-H1 is a CDR-H1 selected from SEQ ID NOs: 1, 39, 77, 115, 153, 191, 229, 267, 305, 343, 381, 419, 457, 495, 533, 571, 609, 647, 685, and 723, with up to 1,

2, 3, 4, or 5 amino acid substitutions. In some aspects, the amino acid substitutions are conservative amino acid substitutions. In some embodiments, the antibody described in this paragraph are referred to herein as “variants.” In some embodiments, such variants are derived from a sequence provided herein, for example, by affinity maturation, site directed mutagenesis, random mutagenesis, or any other method known in the art or described herein. In some embodiments, such variants are not derived from a sequence provided herein and may, for example, be isolated *de novo* according to the methods provided herein for obtaining antibodies.

[00209] In some embodiments, an antibody provided herein comprises a CDR-L3 selected from SEQ ID NOs: 6, 44, 82, 120, 158, 196, 234, 272, 310, 348, 386, 424, 462, 500, 538, 576, 614, 652, 690, and 728, as determined by the Exemplary numbering system. In some aspects, the CDR-L3 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-L3 of SEQ ID NOs: 6, 44, 82, 120, 158, 196, 234, 272, 310, 348, 386, 424, 462, 500, 538, 576, 614, 652, 690, and 728. In some embodiments, the CDR-L3 is a CDR-L3 selected from SEQ ID NOs: 6, 44, 82, 120, 158, 196, 234, 272, 310, 348, 386, 424, 462, 500, 538, 576, 614, 652, 690, and 728, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions. In some aspects, the amino acid substitutions are conservative amino acid substitutions. In some embodiments, the antibodies described in this paragraph are referred to herein as “variants.” In some embodiments, such variants are derived from a sequence provided herein, for example, by affinity maturation, site directed mutagenesis, random mutagenesis, or any other method known in the art or described herein. In some embodiments, such variants are not derived from a sequence provided herein and may, for example, be isolated *de novo* according to the methods provided herein for obtaining antibodies.

[00210] In some embodiments, an antibody provided herein comprises a CDR-L2 selected from SEQ ID NOs: 5, 43, 81, 119, 157, 195, 233, 271, 309, 347, 385, 423, 461, 499, 537, 575, 613, 651, 689, and 727, as determined by the Exemplary numbering system. In some aspects, the CDR-L2 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-L2 of SEQ ID NOs: 5, 43, 81, 119, 157, 195, 233, 271, 309, 347, 385, 423, 461, 499, 537, 575, 613, 651, 689, and 727. In some embodiments, the CDR-L2 is a CDR-L2 selected from SEQ ID NOs: 5, 43, 81, 119, 157, 195, 233, 271, 309, 347, 385, 423, 461, 499, 537, 575, 613, 651, 689, and 727, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions. In some aspects, the amino acid substitutions are conservative amino acid substitutions. In some embodiments, the antibodies described in this paragraph are referred to herein as “variants.” In some embodiments, such variants are derived from a sequence provided herein, for

example, by affinity maturation, site directed mutagenesis, random mutagenesis, or any other method known in the art or described herein. In some embodiments, such variants are not derived from a sequence provided herein and may, for example, be isolated *de novo* according to the methods provided herein for obtaining antibodies.

[00211] In some embodiments, an antibody provided herein comprises a CDR-L1 selected from SEQ ID NOs: 4, 42, 80, 118, 156, 194, 232, 270, 308, 346, 384, 422, 460, 498, 536, 574, 612, 650, 688, and 726, as determined by the Exemplary numbering system. In some aspects, the CDR-L1 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-L1 of SEQ ID NOs: 4, 42, 80, 118, 156, 194, 232, 270, 308, 346, 384, 422, 460, 498, 536, 574, 612, 650, 688, and 726. In some embodiments, the CDR-L1 is a CDR-L1 selected from SEQ ID NOs: 4, 42, 80, 118, 156, 194, 232, 270, 308, 346, 384, 422, 460, 498, 536, 574, 612, 650, 688, and 726, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions. In some aspects, the amino acid substitutions are conservative amino acid substitutions. In some embodiments, the antibodies described in this paragraph are referred to herein as “variants.” In some embodiments, such variants are derived from a sequence provided herein, for example, by affinity maturation, site directed mutagenesis, random mutagenesis, or any other method known in the art or described herein. In some embodiments, such variants are not derived from a sequence provided herein and may, for example, be isolated *de novo* according to the methods provided herein for obtaining antibodies.

[00212] In some embodiments, an antibody provided herein comprises a CDR-L3 selected from SEQ ID NOs: 6, 44, 82, 120, 158, 196, 234, 272, 310, 348, 386, 424, 462, 500, 538, 576, 614, 652, 690, and 728 and a CDR-L2 selected from SEQ ID NOs: 5, 43, 81, 119, 157, 195, 233, 271, 309, 347, 385, 423, 461, 499, 537, 575, 613, 651, 689, and 727. In some embodiments, an antibody provided herein comprises a CDR-L3 selected from SEQ ID NOs: 6, 44, 82, 120, 158, 196, 234, 272, 310, 348, 386, 424, 462, 500, 538, 576, 614, 652, 690, and 728, a CDR-L2 selected from SEQ ID NOs: 5, 43, 81, 119, 157, 195, 233, 271, 309, 347, 385, 423, 461, 499, 537, 575, 613, 651, 689, and 727, and a CDR-L1 selected from SEQ ID NOs: 4, 42, 80, 118, 156, 194, 232, 270, 308, 346, 384, 422, 460, 498, 536, 574, 612, 650, 688, and 726. In some embodiments, the CDR-L3 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-L3 of SEQ ID NOs: 6, 44, 82, 120, 158, 196, 234, 272, 310, 348, 386, 424, 462, 500, 538, 576, 614, 652, 690, and 728, the CDR-L2 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-L2 of SEQ ID NOs: 5, 43, 81, 119, 157, 195, 233, 271, 309, 347, 385, 423, 461, 499, 537, 575, 613, 651, 689, and 727, and the CDR-L1 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-

L1 of SEQ ID NOs: 4, 42, 80, 118, 156, 194, 232, 270, 308, 346, 384, 422, 460, 498, 536, 574, 612, 650, 688, and 726. In some embodiments, the CDR-L3 is a CDR-L3 selected from SEQ ID NOs: 6, 44, 82, 120, 158, 196, 234, 272, 310, 348, 386, 424, 462, 500, 538, 576, 614, 652, 690, and 728, with up to 1, 2, 3, 4, or 5 amino acid substitutions; the CDR-L2 is a CDR-L2 selected from SEQ ID NOs: 5, 43, 81, 119, 157, 195, 233, 271, 309, 347, 385, 423, 461, 499, 537, 575, 613, 651, 689, and 727, with up to 1, 2, 3, or 4 amino acid substitutions; and the CDR-L1 is a CDR-L1 selected from SEQ ID NOs: 4, 42, 80, 118, 156, 194, 232, 270, 308, 346, 384, 422, 460, 498, 536, 574, 612, 650, 688, and 726, with up to 1, 2, 3, 4, 5, or 6 amino acid substitutions. In some aspects, the amino acid substitutions are conservative amino acid substitutions. In some embodiments, the antibodies described in this paragraph are referred to herein as “variants.” In some embodiments, such variants are derived from a sequence provided herein, for example, by affinity maturation, site directed mutagenesis, random mutagenesis, or any other method known in the art or described herein. In some embodiments, such variants are not derived from a sequence provided herein and may, for example, be isolated *de novo* according to the methods provided herein for obtaining antibodies.

[00213] In some embodiments, an antibody provided herein comprises a CDR-H3 selected from SEQ ID NOs: 3, 41, 79, 117, 155, 193, 231, 269, 307, 345, 383, 421, 459, 497, 535, 573, 611, 649, 687, and 725, a CDR-H2 selected from SEQ ID NOs: 2, 40, 78, 116, 154, 192, 230, 268, 306, 344, 382, 420, 458, 496, 534, 572, 610, 648, 686, and 724, a CDR-H1 selected from SEQ ID NOs: 1, 39, 77, 115, 153, 191, 229, 267, 305, 343, 381, 419, 457, 495, 533, 571, 609, 647, 685, and 723, a CDR-L3 selected from SEQ ID NOs: 6, 44, 82, 120, 158, 196, 234, 272, 310, 348, 386, 424, 462, 500, 538, 576, 614, 652, 690, and 728, a CDR-L2 selected from SEQ ID NOs: 5, 43, 81, 119, 157, 195, 233, 271, 309, 347, 385, 423, 461, 499, 537, 575, 613, 651, 689, and 727, and a CDR-L1 selected from SEQ ID NOs: 4, 42, 80, 118, 156, 194, 232, 270, 308, 346, 384, 422, 460, 498, 536, 574, 612, 650, 688, and 726. In some embodiments, the CDR-H3 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-H3 of SEQ ID NOs: 3, 41, 79, 117, 155, 193, 231, 269, 307, 345, 383, 421, 459, 497, 535, 573, 611, 649, 687, and 725, the CDR-H2 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-H2 of SEQ ID NOs: 2, 40, 78, 116, 154, 192, 230, 268, 306, 344, 382, 420, 458, 496, 534, 572, 610, 648, 686, and 724, the CDR-H1 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-H1 of SEQ ID NOs: 1, 39, 77, 115, 153, 191, 229, 267, 305, 343, 381, 419, 457, 495, 533, 571, 609, 647, 685, and 723, the CDR-L3 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-L3

of SEQ ID NOs: 6, 44, 82, 120, 158, 196, 234, 272, 310, 348, 386, 424, 462, 500, 538, 576, 614, 652, 690, and 728, the CDR-L2 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-L2 of SEQ ID NOs: 5, 43, 81, 119, 157, 195, 233, 271, 309, 347, 385, 423, 461, 499, 537, 575, 613, 651, 689, and 727, and the CDR-L1 has at least about 50%, 75%, 80%, 85%, 90%, or 95% identity with a CDR-L1 of SEQ ID NOs: 4, 42, 80, 118, 156, 194, 232, 270, 308, 346, 384, 422, 460, 498, 536, 574, 612, 650, 688, and 726. In some embodiments, the CDR-H3 is a CDR-H3 selected from SEQ ID NOs: 3, 41, 79, 117, 155, 193, 231, 269, 307, 345, 383, 421, 459, 497, 535, 573, 611, 649, 687, and 725, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions; the CDR-H2 is a CDR-H2 selected from SEQ ID NOs: 2, 40, 78, 116, 154, 192, 230, 268, 306, 344, 382, 420, 458, 496, 534, 572, 610, 648, 686, and 724, with up to 1, 2, 3, 4, 5, 6, 7, or 8 amino acid substitutions; the CDR-H1 is a CDR-H1 selected from SEQ ID NOs: 1, 39, 77, 115, 153, 191, 229, 267, 305, 343, 381, 419, 457, 495, 533, 571, 609, 647, 685, and 723, with up to 1, 2, 3, 4, or 5 amino acid substitutions; the CDR-L3 is a CDR-L3 selected from SEQ ID NOs: 6, 44, 82, 120, 158, 196, 234, 272, 310, 348, 386, 424, 462, 500, 538, 576, 614, 652, 690, and 728, with up to 1, 2, 3, 4, or 5 amino acid substitutions; the CDR-L2 is a CDR-L2 selected from SEQ ID NOs: 5, 43, 81, 119, 157, 195, 233, 271, 309, 347, 385, 423, 461, 499, 537, 575, 613, 651, 689, and 727, with up to 1, 2, 3, or 4 amino acid substitutions; and the CDR-L1 is a CDR-L1 selected from SEQ ID NOs: 4, 42, 80, 118, 156, 194, 232, 270, 308, 346, 384, 422, 460, 498, 536, 574, 612, 650, 688, and 726, with up to 1, 2, 3, 4, 5, or 6 amino acid substitutions. In some aspects, the amino acid substitutions are conservative amino acid substitutions. In some embodiments, the antibodies described in this paragraph are referred to herein as “variants.” In some embodiments, such variants are derived from a sequence provided herein, for example, by affinity maturation, site directed mutagenesis, random mutagenesis, or any other method known in the art or described herein. In some embodiments, such variants are not derived from a sequence provided herein and may, for example, be isolated *de novo* according to the methods provided herein for obtaining antibodies.

[00214] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:1, a CDR-H2 of SEQ ID NO:2, a CDR-H3 of SEQ ID NO:3, a CDR-L1 of SEQ ID NO:4, a CDR-L2 of SEQ ID NO:5, and a CDR-L1 of SEQ ID NO:6, as determined by the Exemplary numbering system.

[00215] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:39, a CDR-H2 of SEQ ID NO:40, a CDR-H3 of SEQ ID NO:41, a CDR-L1 of SEQ

ID NO:42, a CDR-L2 of SEQ ID NO:43, and a CDR-L1 of SEQ ID NO:44, as determined by the Exemplary numbering system.

[00216] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:77, a CDR-H2 of SEQ ID NO:78, a CDR-H3 of SEQ ID NO:79, a CDR-L1 of SEQ ID NO:80, a CDR-L2 of SEQ ID NO:81, and a CDR-L1 of SEQ ID NO:82, as determined by the Exemplary numbering system.

[00217] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:115, a CDR-H2 of SEQ ID NO:116, a CDR-H3 of SEQ ID NO:117, a CDR-L1 of SEQ ID NO:118, a CDR-L2 of SEQ ID NO:119, and a CDR-L1 of SEQ ID NO:120, as determined by the Exemplary numbering system.

[00218] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:153, a CDR-H2 of SEQ ID NO:154, a CDR-H3 of SEQ ID NO:155, a CDR-L1 of SEQ ID NO:156, a CDR-L2 of SEQ ID NO:157, and a CDR-L1 of SEQ ID NO:158, as determined by the Exemplary numbering system.

[00219] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:884, a CDR-H2 of SEQ ID NO:885, a CDR-H3 of SEQ ID NO:886, a CDR-L1 of SEQ ID NO:887, a CDR-L2 of SEQ ID NO:888, and a CDR-L1 of SEQ ID NO:889, as determined by the Exemplary numbering system.

[00220] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:191, a CDR-H2 of SEQ ID NO:192, a CDR-H3 of SEQ ID NO:193, a CDR-L1 of SEQ ID NO:194, a CDR-L2 of SEQ ID NO:195, and a CDR-L1 of SEQ ID NO:196, as determined by the Exemplary numbering system.

[00221] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:229, a CDR-H2 of SEQ ID NO:230, a CDR-H3 of SEQ ID NO:231, a CDR-L1 of SEQ ID NO:232, a CDR-L2 of SEQ ID NO:233, and a CDR-L1 of SEQ ID NO:234, as determined by the Exemplary numbering system.

[00222] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:267, a CDR-H2 of SEQ ID NO:268, a CDR-H3 of SEQ ID NO:269, a CDR-L1 of SEQ ID NO:270, a CDR-L2 of SEQ ID NO:271, and a CDR-L1 of SEQ ID NO:272, as determined by the Exemplary numbering system.

[00223] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:305, a CDR-H2 of SEQ ID NO:306, a CDR-H3 of SEQ ID NO:307, a CDR-L1 of SEQ ID NO:308, a CDR-L2 of SEQ ID NO:309, and a CDR-L1 of SEQ ID NO:310, as determined by the Exemplary numbering system.

[00224] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:343, a CDR-H2 of SEQ ID NO:344, a CDR-H3 of SEQ ID NO:345, a CDR-L1 of SEQ ID NO:346, a CDR-L2 of SEQ ID NO:347, and a CDR-L1 of SEQ ID NO:348, as determined by the Exemplary numbering system.

[00225] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:381, a CDR-H2 of SEQ ID NO:382, a CDR-H3 of SEQ ID NO:383, a CDR-L1 of SEQ ID NO:384, a CDR-L2 of SEQ ID NO:385, and a CDR-L1 of SEQ ID NO:386, as determined by the Exemplary numbering system.

[00226] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:419, a CDR-H2 of SEQ ID NO:420, a CDR-H3 of SEQ ID NO:421, a CDR-L1 of SEQ ID NO:422, a CDR-L2 of SEQ ID NO:423, and a CDR-L1 of SEQ ID NO:424, as determined by the Exemplary numbering system.

[00227] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:457, a CDR-H2 of SEQ ID NO:458, a CDR-H3 of SEQ ID NO:459, a CDR-L1 of SEQ ID NO:460, a CDR-L2 of SEQ ID NO:461, and a CDR-L1 of SEQ ID NO:462, as determined by the Exemplary numbering system.

[00228] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:495, a CDR-H2 of SEQ ID NO:496, a CDR-H3 of SEQ ID NO:497, a CDR-L1 of SEQ ID NO:498, a CDR-L2 of SEQ ID NO:499, and a CDR-L1 of SEQ ID NO:500, as determined by the Exemplary numbering system.

[00229] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:533, a CDR-H2 of SEQ ID NO:534, a CDR-H3 of SEQ ID NO:535, a CDR-L1 of SEQ ID NO:536, a CDR-L2 of SEQ ID NO:537, and a CDR-L1 of SEQ ID NO:538, as determined by the Exemplary numbering system.

[00230] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:571, a CDR-H2 of SEQ ID NO:572, a CDR-H3 of SEQ ID NO:573, a CDR-L1 of SEQ ID NO:574, a CDR-L2 of SEQ ID NO:575, and a CDR-L1 of SEQ ID NO:576, as determined by the Exemplary numbering system.

[00231] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:609, a CDR-H2 of SEQ ID NO:610, a CDR-H3 of SEQ ID NO:611, a CDR-L1 of SEQ ID NO:612, a CDR-L2 of SEQ ID NO:613, and a CDR-L1 of SEQ ID NO:614, as determined by the Exemplary numbering system.

[00232] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:647, a CDR-H2 of SEQ ID NO:648, a CDR-H3 of SEQ ID NO:649, a CDR-L1 of

SEQ ID NO:650, a CDR-L2 of SEQ ID NO:651, and a CDR-L1 of SEQ ID NO:652, as determined by the Exemplary numbering system.

[00233] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:685, a CDR-H2 of SEQ ID NO:686, a CDR-H3 of SEQ ID NO:687, a CDR-L1 of SEQ ID NO:688, a CDR-L2 of SEQ ID NO:689, and a CDR-L1 of SEQ ID NO:690, as determined by the Exemplary numbering system.

[00234] In some embodiments, an antibody provided herein comprises a CDR-H1 of SEQ ID NO:723, a CDR-H2 of SEQ ID NO:724, a CDR-H3 of SEQ ID NO:725, a CDR-L1 of SEQ ID NO:726, a CDR-L2 of SEQ ID NO:727, and a CDR-L1 of SEQ ID NO:728, as determined by the Exemplary numbering system.

2.2.5. Consensus Sequences

[00235] In some embodiments, provided herein is a first family of antibodies, wherein an antibody of such family comprises the following six CDR sequences: (a) a CDR-H1 having the sequence G-F-T-F-S-X₁-Y-A-M-X₂, wherein X₁ is D or S and X₂ is A or G (SEQ ID NO:773); (b) a CDR-H2 having the sequence X₃-I-S-G-S-G-G-L-T-Y-Y-A-D-S-V-K-G, wherein X₃ is A or T (SEQ ID NO:774); (c) a CDR-H3 having the sequence APYGYMDV (SEQ ID NO:775); (d) a CDR-L1 having the sequence RASQSISSWLA (SEQ ID NO:776); (e) a CDR-L2 having the sequence KASSLES (SEQ ID NO:777); and (f) a CDR-L3 having the sequence QQYKSYIT (SEQ ID NO:778). In some embodiments, an antibody of such family comprises a V_H sequence of SEQ ID NO:761 and a V_L sequence of SEQ ID NO:762. In some embodiments, provided herein is an antibody within such first family.

[00236] In some embodiments, provided herein is a second family of antibodies, wherein an antibody of such family comprises the following six CDR sequences: (a) a CDR-H1 having the sequence G-Y-T-F-X₁-X₂-Y-G-I-S, wherein X₁ is D or R and X₂ is S or V (SEQ ID NO:779); (b) a CDR-H2 having the sequence W-X₃-A-P-Y-X₄-G-N-T-N-Y-A-Q-K-L-Q-G, wherein X₃ is I or V and X₄ is S or N (SEQ ID NO:780); (c) a CDR-H3 having the sequence D-A-G-T-Y-S-P-X₅-G-Y-G-M-D-V, wherein X₅ is F or Y (SEQ ID NO:781); (d) a CDR-L1 having the sequence X₆-A-S-X₇-S-I-X₈-X₉-W-L-A, wherein X₆ is R or Q, X₇ is Q, E, or H, X₈ is S, D, or N, and X₉ is S or N (SEQ ID NO:782); (e) a CDR-L2 having the sequence X₁₀-A-X₁₁-X₁₂-L-E-X₁₃, wherein X₁₀ is K or S, X₁₁ is S or Y, X₁₂ is S, Y, or N, and X₁₃ is S or Y (SEQ ID NO:783); and (f) a CDR-L3 having the sequence Q-X₁₄-F-Q-X₁₅-L-P-P-F-T, wherein X₁₄ is Q, L, or R, and X₁₅ is S or K (SEQ ID NO:784). In some embodiments, an antibody of such family comprises a V_H sequence of SEQ ID NO:763 and a V_L sequence

of SEQ ID NO:764. In some embodiments, provided herein is an antibody within such second family.

[00237] In some embodiments, provided herein is a third family of antibodies, wherein an antibody of such family comprises the following six CDR sequences: (a) a CDR-H1 having the sequence G-F-T-F-X₁-S-X₂-G-M-H, wherein X₁ is H or R and X₂ is R or Y (SEQ ID NO:785); (b) a CDR-H2 having the sequence VITYDGINKYYADSVEG (SEQ ID NO:786); (c) a CDR-H3 having the sequence DGVYYGVYDY (SEQ ID NO:787); (d) a CDR-L1 having the sequence KSSQSVLFSSNNKNYLA (SEQ ID NO:788); (e) a CDR-L2 having the sequence WASTRES (SEQ ID NO:789); and (f) a CDR-L3 having the sequence QQFHSYPLT (SEQ ID NO:790). In some embodiments, an antibody of such family comprises a V_H sequence of SEQ ID NO:765 and a V_L sequence of SEQ ID NO:766. In some embodiments, provided herein is an antibody within such third family.

[00238] In some embodiments, provided herein is a fourth family of antibodies, wherein an antibody of such family comprises the following six CDR sequences: (a) a CDR-H1 having the sequence GGTFSSNAIG (SEQ ID NO:791); (b) a CDR-H2 having the sequence SIPIIGFANYAQKFQG (SEQ ID NO:792); (c) a CDR-H3 having the sequence DSGYYYGASSFGMDV (SEQ ID NO:793); (d) a CDR-L1 having the sequence RASQSVSSNLA (SEQ ID NO:794); (e) a CDR-L2 having the sequence GASTRAT (SEQ ID NO:795); and (f) a CDR-L3 having the sequence EQYNNLPLT (SEQ ID NO:796). In some embodiments, an antibody of such family comprises a V_H sequence of SEQ ID NO:767 and a V_L sequence of SEQ ID NO:768. In some embodiments, provided herein is an antibody within such fourth family.

[00239] In some embodiments, provided herein is a fifth family of antibodies, wherein an antibody of such family comprises the following six CDR sequences: (a) a CDR-H1 having the sequence G-G-S-X₁-S-S-G-X₂-Y-W-S, wherein X₁ is I or L and X₂ is Q or Y (SEQ ID NO:797); (b) a CDR-H2 having the sequence E-I-X₃-X₄-S-G-S-T-R-Y-N-P-S-L-K-S, wherein X₃ is Y or G and X₄ is Y or A (SEQ ID NO:798); (c) a CDR-H3 having the sequence D-X₅-P-Y-Y-Y-X₆-G-G-Y-Y-Y-Y-M-D-V, wherein X₅ is T or A and X₆ is E, G, or D (SEQ ID NO:799); (d) a CDR-L1 having the sequence R-A-S-X₇-S-V-X₈-S-S-X₉-L-A, wherein X₇ is Q, E, or D, X₈ is S or D, and X₉ is Y or F (SEQ ID NO:800); (e) a CDR-L2 having the sequence G-A-X₁₀-X₁₁-R-X₁₂-X₁₃, wherein X₁₀ is S, D, F, or Y, X₁₁ is S or T, X₁₂ is A or Q, and X₁₃ is T or N (SEQ ID NO:801); and (f) a CDR-L3 having the sequence Q-Q-X₁₄-G-V-V-P-Y-T, wherein X₁₄ is V, A, or D (SEQ ID NO:802). In some embodiments, an antibody of

such family comprises a V_H sequence of SEQ ID NO:769 and a V_L sequence of SEQ ID NO:770. In some embodiments, provided herein is an antibody within such fifth family.

[00240] In some embodiments, provided herein is a sixth family of antibodies, wherein an antibody of such family comprises the following six CDR sequences: (a) a CDR-H1 having the sequence GYTFANYMH (SEQ ID NO:803); (b) a CDR-H2 having the sequence IINPSGGITVYAQKFQG (SEQ ID NO:804); (c) a CDR-H3 having the sequence GGSKVAALAFDI (SEQ ID NO:805); (d) a CDR-L1 having the sequence QASQDISNSLN (SEQ ID NO:806); (e) a CDR-L2 having the sequence DASNLET (SEQ ID NO:807); and (f) a CDR-L3 having the sequence QQYNFHPLT (SEQ ID NO:808). In some embodiments, an antibody of such family comprises a V_H sequence of SEQ ID NO:771 and a V_L sequence of SEQ ID NO:772. In some embodiments, provided herein is an antibody within such sixth family.

[00241] In some embodiments, provided herein is a seventh family of antibodies, wherein an antibody of such family comprises the following six CDR sequences: (a) a CDR-H1 having the sequence G-Y-T-F-D-X₁-Y-G-I-S, wherein X₁ is V or A (SEQ ID NO:872); (b) a CDR-H2 having the sequence W-I-A-P-Y-X₂-G-N-T-N-Y-A-Q-K-L-Q-G, wherein X₂ is N or S (SEQ ID NO:873); (c) a CDR-H3 having the sequence D-A-G-T-Y-S-P-F-G-Y-G-M-D-V (SEQ ID NO:874); (d) a CDR-L1 having the sequence X₃-A-S-X₄-S-I-X₅-X₆-W-L-A, wherein X₃ is R or Q, X₄ is Q or E, X₅ is S or N, and X₆ is S or N (SEQ ID NO:875); (e) a CDR-L2 having the sequence K-A-X₇-X₈-L-E-X₉, wherein X₇ is S or Y, X₈ is S or N, and X₉ is S or Y (SEQ ID NO:876); and (f) a CDR-L3 having the sequence Q-X₁₀-F-Q-X₁₁-L-P-P-F-T, wherein X₁₀ is Q or L, and X₁₁ is S or K (SEQ ID NO:877). In some embodiments, an antibody of such family comprises a V_H sequence of SEQ ID NO:868 and a V_L sequence of SEQ ID NO:869. In some embodiments, provided herein is an antibody within such seventh family.

[00242] In some embodiments, provided herein is an eighth family of antibodies, wherein an antibody of such family comprises the following six CDR sequences: (a) a CDR-H1 having the sequence G-Y-T-F-R-S-Y-G-I-S (SEQ ID NO:878); (b) a CDR-H2 having the sequence W-V-A-P-Y-X₁-G-N-T-N-Y-A-Q-K-L-Q-G, wherein X₁ is S or N (SEQ ID NO:879); (c) a CDR-H3 having the sequence D-A-G-T-Y-S-P-Y-G-Y-G-M-D-V (SEQ ID NO:880); (d) a CDR-L1 having the sequence X₂-A-S-X₃-S-I-X₄-S-W-L-A, wherein X₂ is R or Q, X₃ is Q or H, X₄ is S or D (SEQ ID NO:881); (e) a CDR-L2 having the sequence X₅-A-S-X₆-L-E-S, wherein X₅ is K or S, X₆ is S or Y (SEQ ID NO:882); and (f) a CDR-L3 having the sequence Q-X₇-F-Q-S-L-P-P-F-T, wherein X₇ is Q, L, or R (SEQ ID NO:883). In some

embodiments, an antibody of such family comprises a VH sequence of SEQ ID NO:870 and a VL sequence of SEQ ID NO:871. In some embodiments, provided herein is an antibody within such eighth family.

2.2.6. Functional Properties of Antibody Variants

[00243] As described above, and elsewhere in this disclosure, provided herein are antibody variants defined based on percent identity to an illustrative antibody sequence provided herein, or substitution of amino acid residues in comparison to an illustrative antibody sequence provided herein.

[00244] In some embodiments, a variant of an antibody provided herein has specificity for hTF. In some embodiments, a variant of an antibody provided herein has specificity for cTF. In some embodiments, a variant of an antibody provided herein has specificity for mTF. In some embodiments, a variant of an antibody provided herein has specificity for hTF and cTF. In some embodiments, a variant of an antibody provided herein has specificity for hTF and mTF. In some embodiments, a variant of an antibody provided herein has specificity for cTF and mTF. In some embodiments, a variant of an antibody provided herein has specificity for hTF, cTF and mTF.

[00245] In some embodiments, a variant of an antibody that is derived from an illustrative antibody sequence provided herein retains affinity, as measured by K_D , for hTF that is within about 1.5-fold, about 2-fold, about 3-fold, about 4-fold, about 5-fold, about 6-fold, about 7-fold, about 8-fold, about 9-fold or about 10-fold the affinity of such illustrative antibody. In some embodiments, a variant of an antibody that is derived from an illustrative antibody sequence provided herein retains affinity, as measured by K_D , for cTF that is within about 1.5-fold, about 2-fold, about 3-fold, about 4-fold, about 5-fold, about 6-fold, about 7-fold, about 8-fold, about 9-fold or about 10-fold the affinity of such illustrative antibody. In some embodiments, a variant of an antibody that is derived from an illustrative antibody sequence provided herein retains affinity, as measured by K_D , for mTF that is within about 1.5-fold, about 2-fold, about 3-fold, about 4-fold, about 5-fold, about 6-fold, about 7-fold, about 8-fold, about 9-fold or about 10-fold the affinity of such illustrative antibody. In some embodiments, a variant of an antibody that is derived from an illustrative antibody sequence provided herein retains affinity, as measured by K_D , for both hTF and cTF that is within about 1.5-fold, about 2-fold, about 3-fold, about 4-fold, about 5-fold, about 6-fold, about 7-fold, about 8-fold, about 9-fold or about 10-fold the affinity of such illustrative antibody. In some embodiments, a variant of an antibody that is derived from an illustrative antibody

sequence provided herein retains affinity, as measured by K_D , for both hTF and mTF that is within about 1.5-fold, about 2-fold, about 3-fold, about 4-fold, about 5-fold, about 6-fold, about 7-fold, about 8-fold, about 9-fold or about 10-fold the affinity of such illustrative antibody. In some embodiments, a variant of an antibody that is derived from an illustrative antibody sequence provided herein retains affinity, as measured by K_D , for both cTF and mTF that is within about 1.5-fold, about 2-fold, about 3-fold, about 4-fold, about 5-fold, about 6-fold, about 7-fold, about 8-fold, about 9-fold or about 10-fold the affinity of such illustrative antibody. In some embodiments, a variant of an antibody that is derived from an illustrative antibody sequence provided herein retains affinity, as measured by K_D , for all three of hTF, cTF and mTF that is within about 1.5-fold, about 2-fold, about 3-fold, about 4-fold, about 5-fold, about 6-fold, about 7-fold, about 8-fold, about 9-fold or about 10-fold the affinity of such illustrative antibody.

[00246] In some embodiments, a variant of an antibody provided herein retains the ability to inhibit TF signaling, as measured by one or more assays or biological effects described herein. In some embodiments, a variant of an antibody provided herein retains the normal function of TF in the blood coagulation processes.

[00247] In some embodiments, a variant of an antibody provided herein competes for binding to TF with an antibody selected from 1F, 1G, 25A, 25A3, 25A5, 25A5-T, 25G, 25G1, 25G9, 29D, 29E, 39A, 43B, 43B1, 43B7, 43D, 43D7, 43D8, 43E, 43Ea, and 54E, each as provided in **Table 13** of this disclosure. In some embodiments, a variant of an antibody provided herein competes for binding to TF with an antibody selected from 25A, 25A3, 25A5, 25A5-T, 25G, 25G1, and 25G9. In some embodiments, a variant of an antibody provided herein competes for binding to TF with an antibody selected from 43B, 43B1, 43B7, 43D, 43D7, 43D8, 43E, and 43Ea. In some embodiments, a variant of an antibody provided herein competes for binding to TF with an antibody selected from 25A, 25A3, 25A5, 25A5-T, 25G, 25G1, 25G9, 43B, 43B1, 43B7, 43D, 43D7, 43D8, 43E, and 43Ea. In some embodiments, a variant of an antibody provided herein competes for binding to TF with an antibody selected from 1F, 1G, 29D, 29E, 39A, or 54E.

[00248] In some embodiments, a variant of an antibody provided herein allows human thrombin generation as determined by thrombin generation assay (TGA). In some embodiments, a variant of an antibody provided herein does not inhibit human thrombin generation as determined by thrombin generation assay (TGA).

[00249] In some embodiments, a variant of an antibody provided herein binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FX.

In some embodiments, a variant of an antibody provided herein does not interfere with the ability of TF:FVIIa to convert FX into FXa.

[00250] In some embodiments, a variant of an antibody provided herein binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa. In some embodiments, a variant of an antibody provided herein does not compete for binding to human TF with human FVIIa.

[00251] In some embodiments, a variant of an antibody provided herein inhibits FVIIa-dependent TF signaling.

[00252] In some embodiments, a variant of an antibody provided herein binds mouse TF (SEQ ID NO:817). In some embodiments, a variant of an antibody provided herein binds mouse TF with an affinity lower (as indicated by higher K_D) than the affinity of the antibody for hTF. In some embodiments, a variant of an antibody provided herein does not bind mTF.

[00253] In some embodiments, a variant of an antibody provided herein binds pig TF (SEQ ID NO:824). In some embodiments, a variant of an antibody provided herein binds pig TF with an affinity lower (as indicated by higher K_D) than the affinity of the antibody for hTF. In some embodiments, a variant of an antibody provided herein does not bind pTF.

[00254] In some embodiments, a variant of an antibody provided herein binds the same epitope of TF as such antibody.

2.2.7. Other Functional Properties of Antibodies

[00255] In some embodiments, an antibody provided herein has one or more of the characteristics listed in the following (a)-(dd): (a) binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa; (b) does not inhibit human thrombin generation as determined by thrombin generation assay (TGA); (c) does not reduce the thrombin peak on a thrombin generation curve (Peak IIa) compared to an isotype control; (d) does not increase the time from the assay start to the thrombin peak on a thrombin generation curve (ttPeak) compared to an isotype control; (e) does not decrease the endogenous thrombin potential (ETP) as determined by the area under a thrombin generation curve compared to an isotype control; (f) allows human thrombin generation as determined by thrombin generation assay (TGA); (g) maintains the thrombin peak on a thrombin generation curve (Peak IIa) compared to an isotype control; (h) maintains the time from the assay start to the thrombin peak on a thrombin generation curve (ttPeak) compared to an isotype control; (i) preserves the endogenous thrombin potential (ETP) as determined by the area under a thrombin generation curve compared to an isotype control; (j) binds human TF

at a human TF binding site that is distinct from a human TF binding site bound by human FX; (k) does not interfere with the ability of TF:FVIIa to convert FX into FXa; (l) does not compete for binding to human TF with human FVIIa; (m) inhibits FVIIa-dependent TF signaling; (n) binds to cynomolgus TF; (o) binds to mouse TF; (p) binds to rabbit TF; (q) binds to pig TF; (r) reduces lesion size in a swine choroidal neovascularization (CNV) model; (s) the binding between the antibody and a variant TF extracellular domain comprising a mutation at amino acid residue 149 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (t) the binding between the antibody and a variant TF extracellular domain comprising a mutation at amino acid residue 68 of the sequence shown in SEQ ID NO:810 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (u) the binding between the antibody and a variant TF extracellular domain comprising mutations at amino acid residues 171 and 197 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (v) the binding between the antibody and a human TF extracellular domain with amino acid residues 1-77 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 1-76 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (w) the binding between the antibody and a human TF extracellular domain with amino acid residues 39-77 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 38-76 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (x) the binding between the antibody and a human TF extracellular domain with amino acid residues 94-107 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 99-112 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding

between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (y) the binding between the antibody and a human TF extracellular domain with amino acid residues 146-158 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 151-163 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (z) the binding between the antibody and a human TF extracellular domain with amino acid residues 159-219 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-224 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (aa) the binding between the antibody and a human TF extracellular domain with amino acid residues 159-189 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-194 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (bb) the binding between the antibody and a human TF extracellular domain with amino acid residues 159-174 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-179 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (cc) the binding between the antibody and a human TF extracellular domain with amino acid residues 167-174 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 172-179 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; and (dd) the binding between the antibody and a rat TF extracellular domain with amino acid residues 141-194 of the sequence shown in SEQ ID NO:838 replaced by human TF extracellular domain amino acid residues 136-189 of

the sequence shown in SEQ ID NO:810 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay. In some embodiments, an antibody provided herein has two or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has three or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has four or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has five or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has six or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has seven or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has eight or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has nine or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has ten or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has eleven or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twelve or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has thirteen or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has fourteen or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has fifteen or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has sixteen or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has seventeen or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has eighteen or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has nineteen or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-one or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-two or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-three of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an

antibody provided herein has twenty-four of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-five of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-six of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-seven of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-eight of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-nine of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has all thirty of the characteristics listed in the foregoing (a)-(dd).

[00256] In some embodiments, an antibody provided herein has one or more of the characteristics listed in the following (a)-(dd): (a) binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa; (b) does not inhibit human thrombin generation as determined by thrombin generation assay (TGA); (c) does not reduce the thrombin peak on a thrombin generation curve (Peak IIa) compared to an isotype control; (d) does not increase the time from the assay start to the thrombin peak on a thrombin generation curve (ttPeak) compared to an isotype control; (e) does not decrease the endogenous thrombin potential (ETP) as determined by the area under a thrombin generation curve compared to an isotype control; (f) allows human thrombin generation as determined by thrombin generation assay (TGA); (g) maintains the thrombin peak on a thrombin generation curve (Peak IIa) compared to an isotype control; (h) maintains the time from the assay start to the thrombin peak on a thrombin generation curve (ttPeak) compared to an isotype control; (i) preserves the endogenous thrombin potential (ETP) as determined by the area under a thrombin generation curve compared to an isotype control; (j) binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FX; (k) does not interfere with the ability of TF:FVIIa to convert FX into FXa; (l) does not compete for binding to human TF with human FVIIa; (m) inhibits FVIIa-dependent TF signaling; (n) binds to cynomolgus TF; (o) binds to mouse TF; (p) binds to rabbit TF; (q) binds to pig TF; (r) reduces lesion size in a swine choroidal neovascularization (CNV) model; (s) the binding between the antibody and a variant TF extracellular domain comprising a mutation K149N of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (t) the binding between the antibody and a

variant TF extracellular domain comprising a mutation K68N of the sequence shown in SEQ ID NO:810 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (u) the binding between the antibody and a variant TF extracellular domain comprising mutations N171H and T197K of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (v) the binding between the antibody and a human TF extracellular domain with amino acid residues 1-77 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 1-76 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (w) the binding between the antibody and a human TF extracellular domain with amino acid residues 39-77 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 38-76 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (x) the binding between the antibody and a human TF extracellular domain with amino acid residues 94-107 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 99-112 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (y) the binding between the antibody and a human TF extracellular domain with amino acid residues 146-158 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 151-163 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (z) the binding between the antibody and a human TF extracellular domain with amino acid residues 159-219 of the sequence shown in SEQ ID NO:810 replaced by rat

TF extracellular domain amino acid residues 164-224 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (aa) the binding between the antibody and a human TF extracellular domain with amino acid residues 159-189 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-194 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (bb) the binding between the antibody and a human TF extracellular domain with amino acid residues 159-174 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-179 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (cc) the binding between the antibody and a human TF extracellular domain with amino acid residues 167-174 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 172-179 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; and (dd) the binding between the antibody and a rat TF extracellular domain with amino acid residues 141-194 of the sequence shown in SEQ ID NO:838 replaced by human TF extracellular domain amino acid residues 136-189 of the sequence shown in SEQ ID NO:810 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay. In some embodiments, an antibody provided herein has two or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has three or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has four or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has five or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has six or more of the characteristics listed in the foregoing (a)-(dd). In some

embodiments, an antibody provided herein has seven or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has eight or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has nine or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has ten or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has eleven or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twelve or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has thirteen or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has fourteen or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has fifteen or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has sixteen or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has seventeen or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has eighteen or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has nineteen or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-one or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-two or more of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-three of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-four of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-five of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-six of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-seven of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-eight of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has twenty-nine of the characteristics listed in the foregoing (a)-(dd). In some embodiments, an antibody provided herein has all thirty of the characteristics listed in the foregoing (a)-(dd).

[00257] In some embodiments, an antibody provided herein exhibits a combination of characteristics comprising two or more of characteristics listed in the following (a)-(dd): (a) binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa; (b) does not inhibit human thrombin generation as determined by thrombin generation assay (TGA); (c) does not reduce the thrombin peak on a thrombin generation curve (Peak IIa) compared to an isotype control; (d) does not increase the time from the assay start to the thrombin peak on a thrombin generation curve (ttPeak) compared to an isotype control; (e) does not decrease the endogenous thrombin potential (ETP) as determined by the area under a thrombin generation curve compared to an isotype control; (f) allows human thrombin generation as determined by thrombin generation assay (TGA); (g) maintains the thrombin peak on a thrombin generation curve (Peak IIa) compared to an isotype control; (h) maintains the time from the assay start to the thrombin peak on a thrombin generation curve (ttPeak) compared to an isotype control; (i) preserves the endogenous thrombin potential (ETP) as determined by the area under a thrombin generation curve compared to an isotype control; (j) binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FX; (k) does not interfere with the ability of TF:FVIIa to convert FX into FXa; (l) does not compete for binding to human TF with human FVIIa; (m) inhibits FVIIa-dependent TF signaling; (n) binds to cynomolgus TF; (o) binds to mouse TF; (p) binds to rabbit TF; (q) binds to pig TF; (r) reduces lesion size in a swine choroidal neovascularization (CNV) model; (s) the binding between the antibody and a variant TF extracellular domain comprising a mutation at amino acid residue 149 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (t) the binding between the antibody and a variant TF extracellular domain comprising a mutation at amino acid residue 68 of the sequence shown in SEQ ID NO:810 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (u) the binding between the antibody and a variant TF extracellular domain comprising mutations at amino acid residues 171 and 197 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (v) the binding between

the antibody and a human TF extracellular domain with amino acid residues 1-77 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 1-76 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (w) the binding between the antibody and a human TF extracellular domain with amino acid residues 39-77 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 38-76 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (x) the binding between the antibody and a human TF extracellular domain with amino acid residues 94-107 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 99-112 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (y) the binding between the antibody and a human TF extracellular domain with amino acid residues 146-158 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 151-163 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (z) the binding between the antibody and a human TF extracellular domain with amino acid residues 159-219 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-224 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (aa) the binding between the antibody and a human TF extracellular domain with amino acid residues 159-189 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-194 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live

cell staining assay; (bb) the binding between the antibody and a human TF extracellular domain with amino acid residues 159-174 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-179 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (cc) the binding between the antibody and a human TF extracellular domain with amino acid residues 167-174 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 172-179 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; and (dd) the binding between the antibody and a rat TF extracellular domain with amino acid residues 141-194 of the sequence shown in SEQ ID NO:838 replaced by human TF extracellular domain amino acid residues 136-189 of the sequence shown in SEQ ID NO:810 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00258] In some embodiments, an antibody provided herein exhibits a combination of characteristics comprising two or more of characteristics listed in the following (a)-(dd): (a) binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa; (b) does not inhibit human thrombin generation as determined by thrombin generation assay (TGA); (c) does not reduce the thrombin peak on a thrombin generation curve (Peak IIa) compared to an isotype control; (d) does not increase the time from the assay start to the thrombin peak on a thrombin generation curve (ttPeak) compared to an isotype control; (e) does not decrease the endogenous thrombin potential (ETP) as determined by the area under a thrombin generation curve compared to an isotype control; (f) allows human thrombin generation as determined by thrombin generation assay (TGA); (g) maintains the thrombin peak on a thrombin generation curve (Peak IIa) compared to an isotype control; (h) maintains the time from the assay start to the thrombin peak on a thrombin generation curve (ttPeak) compared to an isotype control; (i) preserves the endogenous thrombin potential (ETP) as determined by the area under a thrombin generation curve compared to an isotype control; (j) binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FX; (k) does not interfere with the

ability of TF:FVIIa to convert FX into FXa; (l) does not compete for binding to human TF with human FVIIa; (m) inhibits FVIIa-dependent TF signaling; (n) binds to cynomolgus TF; (o) binds to mouse TF; (p) binds to rabbit TF; (q) binds to pig TF; (r) reduces lesion size in a swine choroidal neovascularization (CNV) model; (s) the binding between the antibody and a variant TF extracellular domain comprising a mutation K149N of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (t) the binding between the antibody and a variant TF extracellular domain comprising a mutation K68N of the sequence shown in SEQ ID NO:810 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (u) the binding between the antibody and a variant TF extracellular domain comprising mutations N171H and T197K of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (v) the binding between the antibody and a human TF extracellular domain with amino acid residues 1-77 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 1-76 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (w) the binding between the antibody and a human TF extracellular domain with amino acid residues 39-77 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 38-76 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (x) the binding between the antibody and a human TF extracellular domain with amino acid residues 94-107 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 99-112 of the sequence shown in SEQ ID NO:838 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody

relative to an isotype control in a live cell staining assay; (y) the binding between the antibody and a human TF extracellular domain with amino acid residues 146-158 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 151-163 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (z) the binding between the antibody and a human TF extracellular domain with amino acid residues 159-219 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-224 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (aa) the binding between the antibody and a human TF extracellular domain with amino acid residues 159-189 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-194 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (bb) the binding between the antibody and a human TF extracellular domain with amino acid residues 159-174 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 164-179 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; (cc) the binding between the antibody and a human TF extracellular domain with amino acid residues 167-174 of the sequence shown in SEQ ID NO:810 replaced by rat TF extracellular domain amino acid residues 172-179 of the sequence shown in SEQ ID NO:838 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; and (dd) the binding between the antibody and a rat TF extracellular domain with amino acid residues 141-194 of the sequence shown in SEQ ID NO:838 replaced by human TF extracellular domain amino acid residues 136-189 of the sequence shown in SEQ ID NO:810 is greater than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in

SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00259] In some embodiments, an antibody provided herein exhibits a combination of the characteristics listed in the following: binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa; does not inhibit human thrombin generation as determined by thrombin generation assay (TGA); and the binding between the antibody and a variant TF extracellular domain comprising mutations at amino acid residues 171 and 197 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00260] In some embodiments, an antibody provided herein exhibits a combination of the characteristics listed in the following: binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa; does not inhibit human thrombin generation as determined by thrombin generation assay (TGA); and the binding between the antibody and a variant TF extracellular domain comprising mutations N171H and T197K of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00261] In some embodiments, an antibody provided herein exhibits a combination of the characteristics listed in the following: binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa; allows human thrombin generation as determined by thrombin generation assay (TGA); and the binding between the antibody and a variant TF extracellular domain comprising mutations at amino acid residues 171 and 197 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00262] In some embodiments, an antibody provided herein exhibits a combination of the characteristics listed in the following: binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa; allows human thrombin generation as determined by thrombin generation assay (TGA); and the binding between the antibody and a variant TF extracellular domain comprising mutations N171H and T197K of

the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00263] In some embodiments, an antibody provided herein exhibits a combination of the characteristics listed in the following: binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa; does not inhibit human thrombin generation as determined by thrombin generation assay (TGA); the binding between the antibody and a variant TF extracellular domain comprising a mutation at amino acid residue 149 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; and the binding between the antibody and a variant TF extracellular domain comprising mutations at amino acid residues 171 and 197 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00264] In some embodiments, an antibody provided herein exhibits a combination of the characteristics listed in the following: binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa; does not inhibit human thrombin generation as determined by thrombin generation assay (TGA); the binding between the antibody and a variant TF extracellular domain comprising a mutation K149N of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; and the binding between the antibody and a variant TF extracellular domain comprising mutations N171H and T197K of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00265] In some embodiments, an antibody provided herein exhibits a combination of the characteristics listed in the following: binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa; allows human thrombin

generation as determined by thrombin generation assay (TGA); the binding between the antibody and a variant TF extracellular domain comprising a mutation at amino acid residue 149 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; and the binding between the antibody and a variant TF extracellular domain comprising mutations at amino acid residues 171 and 197 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00266] In some embodiments, an antibody provided herein exhibits a combination of the characteristics listed in the following: binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa; allows human thrombin generation as determined by thrombin generation assay (TGA); the binding between the antibody and a variant TF extracellular domain comprising a mutation K149N of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; and the binding between the antibody and a variant TF extracellular domain comprising mutations N171H and T197K of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00267] In some embodiments, an antibody provided herein exhibits a combination of the characteristics listed in the following: binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa; does not inhibit human thrombin generation as determined by thrombin generation assay (TGA); binds to cynomolgus TF; the binding between the antibody and a variant TF extracellular domain comprising a mutation at amino acid residue 149 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; and the binding between the antibody and a variant TF extracellular domain comprising mutations at

amino acid residues 171 and 197 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00268] In some embodiments, an antibody provided herein exhibits a combination of the characteristics listed in the following: binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa; does not inhibit human thrombin generation as determined by thrombin generation assay (TGA); binds to cynomolgus TF; the binding between the antibody and a variant TF extracellular domain comprising a mutation K149N of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; and the binding between the antibody and a variant TF extracellular domain comprising mutations N171H and T197K of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00269] In some embodiments, an antibody provided herein exhibits a combination of the characteristics listed in the following: binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa; allows human thrombin generation as determined by thrombin generation assay (TGA); binds to cynomolgus TF; the binding between the antibody and a variant TF extracellular domain comprising a mutation at amino acid residue 149 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; and the binding between the antibody and a variant TF extracellular domain comprising mutations at amino acid residues 171 and 197 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

[00270] In some embodiments, an antibody provided herein exhibits a combination of the characteristics listed in the following: binds human TF at a human TF binding site that is

distinct from a human TF binding site bound by human FVIIa; allows human thrombin generation as determined by thrombin generation assay (TGA); binds to cynomolgus TF; the binding between the antibody and a variant TF extracellular domain comprising a mutation K149N of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay; and the binding between the antibody and a variant TF extracellular domain comprising mutations N171H and T197K of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the antibody relative to an isotype control in a live cell staining assay.

2.3. Affinity and Other Properties of TF Antibodies

2.3.1. Affinity of TF Antibodies

[00271] In some embodiments, the affinity of an antibody provided herein for TF as indicated by K_D , is less than about 10^{-5} M, less than about 10^{-6} M, less than about 10^{-7} M, less than about 10^{-8} M, less than about 10^{-9} M, less than about 10^{-10} M, less than about 10^{-11} M, or less than about 10^{-12} M. In some embodiments, the affinity of the antibody is between about 10^{-7} M and 10^{-12} M. In some embodiments, the affinity of the antibody is between about 10^{-7} M and 10^{-11} M. In some embodiments, the affinity of the antibody is between about 10^{-7} M and 10^{-10} M. In some embodiments, the affinity of the antibody is between about 10^{-7} M and 10^{-9} M. In some embodiments, the affinity of the antibody is between about 10^{-7} M and 10^{-8} M. In some embodiments, the affinity of the antibody is between about 10^{-8} M and 10^{-12} M. In some embodiments, the affinity of the antibody is between about 10^{-8} M and 10^{-11} M. In some embodiments, the affinity of the antibody is between about 10^{-9} M and 10^{-11} M. In some embodiments, the affinity of the antibody is between about 10^{-10} M and 10^{-11} M. In some embodiments, the affinity of the antibody is less than about 0.5 nM, 0.6 nM, 0.7 nM, 0.8 nM, 0.9 nM or 1.0 nM.

[00272] In some embodiments, the K_D value of an antibody provided herein for cTF is no more than $15\times$ of the K_D value of the antibody for hTF. In some embodiments, the K_D value of an antibody provided herein for cTF is no more than $10\times$ of the K_D value of the antibody for hTF. In some embodiments, the K_D value of an antibody provided herein for cTF is no more than $8\times$ of the K_D value of the antibody for hTF. In some embodiments, the K_D value of

an antibody provided herein for cTF is no more than $5\times$ of the K_D value of the antibody for hTF. In some embodiments, the K_D value of an antibody provided herein for cTF is no more than $3\times$ of the K_D value of the antibody for hTF. In some embodiments, the K_D value of an antibody provided herein for cTF is no more than $2\times$ of the K_D value of the antibody for hTF.

[00273] In some embodiments, the K_D value of an antibody provided herein for mTF is no more than $20\times$ of the K_D value of the antibody for hTF. In some embodiments, the K_D value of an antibody provided herein for mTF is no more than $15\times$ of the K_D value of the antibody for hTF. In some embodiments, the K_D value of an antibody provided herein for mTF is no more than $10\times$ of the K_D value of the antibody for hTF. In some embodiments, the K_D value of an antibody provided herein for mTF is no more than $5\times$ of the K_D value of the antibody for hTF. In some embodiments, the K_D value of an antibody provided herein for mTF is no more than $2\times$ of the K_D value of the antibody for hTF.

[00274] In some embodiments, the affinity of an antibody provided herein for hTF as indicated by K_D measured by Biacore, as set forth in **Table 5** is selected from about 0.31 nM, about 6.20 nM, about 0.36 nM, about 0.08 nM, about 23.0 nM, about 0.94 nM, about 13.3 nM, about 0.47 nM, about 0.09 nM, about 1.75 nM, about 0.07 nM, about 0.14 nM, about 2.09 nM, about 0.06 nM, about 0.15 nM, about 1.46 nM, about 1.60 nM, and about 0.42 nM. In some embodiments, such affinity as indicated by K_D ranges from about 23.0 nM to about 0.06 nM. In some embodiments, such is about 23.0 nM or less.

[00275] In some embodiments, the affinity of an antibody provided herein for hTF as indicated by K_D measured by ForteBio, as set forth in **Table 5** is selected from about 1.28 nM, about 2.20 nM, about 8.45 nM, about 1.67 nM, about 0.64 nM, about 21.9 nM, about 3.97 nM, about 35.8 nM, about 3.30 nM, about 2.32 nM, about 0.83 nM, about 2.40 nM, about 0.96 nM, about 0.86 nM, about 3.84 nM, about 1.02 nM, about 1.61 nM, about 2.52 nM, about 2.28 nM, and about 1.59 nM. In some embodiments, such affinity as indicated by K_D ranges from about 35.8 nM to about 0.64 nM. In some embodiments, such K_D is about 35.8 nM or less.

[00276] In some embodiments, the affinity of an antibody provided herein for cTF as indicated by K_D measured by Biacore, as set forth in **Table 5** is selected from about 0.26 nM, about 5.42 nM, about 0.21 nM, about 0.04 nM, about 18.0 nM, about 0.78 nM, about 16.4 nM, about 5.06 nM, about 0.08 nM, about 5.64 nM, about 0.12 nM, about 0.24 nM, about 5.66 nM, about 0.39 nM, about 5.69 nM, about 6.42 nM, and about 1.83 nM. In some embodiments, such affinity as indicated by K_D ranges from about 18.0 nM to about 0.04 nM. In some embodiments, such K_D is about 18.0 nM or less.

[00277] In some embodiments, the affinity of an antibody provided herein for cTF as indicated by K_D measured by ForteBio, as set forth in **Table 5** is selected from about 1.43 nM, about 2.70 nM, about 7.65 nM, about 1.36 nM, about 0.76 nM, about 17.5 nM, about 4.99 nM, about 42.9 nM, about 12.0 nM, about 15.0 nM, about 0.57 nM, about 3.40 nM, about 1.05 nM, about 0.94 nM, about 4.12 nM, about 1.11 nM, about 1.96 nM, about 4.07 nM, about 2.71 nM, and about 4.16 nM. In some embodiments, such affinity as indicated by K_D ranges from about 42.9 nM to about 0.57 nM. In some embodiments, such K_D is about 42.9 nM or less.

[00278] In some embodiments, the affinity of an antibody provided herein for mTF as indicated by K_D measured by Biacore, as set forth in **Table 5** is selected from about 5.4 nM, about 2.9 nM, about 21 nM, and about 2.4 nM. In some embodiments, such affinity as indicated by K_D ranges from about 21 nM to about 2.4 nM. In some embodiments, such K_D is about 21 nM or less.

[00279] In some embodiments, the affinity of an antibody provided herein for mTF as indicated by K_D measured by ForteBio, as set forth in **Table 5** is selected from about 263 nM, about 131 nM, about 188 nM, about 114 nM, about 34.2 nM, about 9.16 nM, about 161 nM, about 72.1 nM, about 360 nM, about 281 nM, about 41.4 nM, about 6.12 nM, about 121 nM, and about 140 nM. In some embodiments, such affinity as indicated by K_D ranges from about 360 nM to about 6.12 nM. In some embodiments, such K_D is about 360 nM or less.

[00280] In some embodiments, the affinity of an antibody provided herein for hTF as indicated by EC_{50} measured with human TF-positive HCT-116 cells, (as set forth in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety) is selected from about 50 pM, about 58 pM, about 169 pM, about 77 pM, about 88 pM, about 134 pM, about 85 pM, about 237 pM, about 152 pM, about 39 pM, about 559 pM, about 280 pM, about 255 pM, about 147 pM, about 94 pM, about 117 pM, about 687 pM, about 532 pM, and about 239 pM. In some embodiments, such affinity ranges from about 687 pM to about 39 pM. In some embodiments, such EC_{50} is about 687 pM or less.

[00281] In some embodiments, the affinity of an antibody provided herein for mTF as indicated by EC_{50} measured with mouse TF-positive CHO cells, (as set forth in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety) is selected from about 455 nM, about 87 nM, about 11 nM, about 3.9 nM, about 3.0 nM, about 3.4 nM, about 255 nM, about 2.9 nM,

about 3.6 nM, and about 4.0 nM. In some embodiments, such affinity ranges from about 455 nM to about 2.9 nM. In some embodiments, such EC_{50} is about 455 pM or less.

[00282] In some embodiments, the K_D value of an antibody provided herein for pTF is no more than $20\times$ of the K_D value of the antibody for hTF. In some embodiments, the K_D value of an antibody provided herein for pTF is no more than $15\times$ of the K_D value of the antibody for hTF. In some embodiments, the K_D value of an antibody provided herein for pTF is no more than $10\times$ of the K_D value of the antibody for hTF. In some embodiments, the K_D value of an antibody provided herein for pTF is no more than $5\times$ of the K_D value of the antibody for hTF. In some embodiments, the K_D value of an antibody provided herein for pTF is no more than $2\times$ of the K_D value of the antibody for hTF.

[00283] In some embodiments, the affinity of an antibody provided herein for pTF as indicated by K_D measured by Biacore, as set forth in Table 40 is 3.31 nM or 12.9 nM.

2.3.2. Thrombin Generation in the Presence of TF Antibodies

[00284] In some embodiments, the TF antibodies provided herein do not inhibit human thrombin generation as determined by thrombin generation assay (TGA). In certain embodiments, the TF antibodies provided herein allow human thrombin generation as determined by thrombin generation assay (TGA).

[00285] In some embodiments, the percent peak thrombin generation (% Peak IIa) is at least 40% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 50% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 60% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 70% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 80% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 90% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 95% in the presence of no less than 100 nM TF antibody compared to the control

conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 99% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA).

[00286] In some embodiments, the % Peak IIa is at least 40% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 50% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 60% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 70% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 80% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 90% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 95% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 99% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA).

[00287] In some embodiments, the % Peak IIa is at least 60% in the presence of no less than 10 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 70% in the presence of no less than 10 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 80% in the presence of no less than 10 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 90% in the presence of no less than 10 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 95% in the presence of no less than 10 nM TF antibody compared to the control

conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % Peak IIa is at least 99% in the presence of no less than 10 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA).

[00288] In some embodiments, the % Peak IIa in the presence of 100 nM TF antibody, as set forth in **Table 6** and **Table 37** is selected from about 99%, about 100%, about 103%, about 64%, about 52%, about 87%, about 96%, about 98%, and about 53% compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA) without antibody pre-incubation. In some embodiments, such % Peak IIa ranges from about 52% to about 103%. In some embodiments, such % Peak IIa is about 52% or more.

[00289] In some embodiments, the % Peak IIa in the presence of 50 nM TF antibody, as set forth in **Table 6** and **Table 37** is selected from about 99%, about 100%, about 103%, about 67%, about 58%, about 89%, about 96%, about 98%, about 68%, about 62%, and about 88% compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA) without antibody pre-incubation. In some embodiments, such % Peak IIa ranges from about 58% to about 103%. In some embodiments, such % Peak IIa is about 58% or more.

[00290] In some embodiments, the % Peak IIa in the presence of 10 nM TF antibody, as set forth in **Table 6** and **Table 37** is selected from about 100%, about 99%, about 103%, about 87%, about 83%, about 95%, about 98%, about 86%, and about 96% compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA) without antibody pre-incubation. In some embodiments, such % Peak IIa ranges from about 83% to about 103%. In some embodiments, such % Peak IIa is about 83% or more.

[00291] In some embodiments, the % Peak IIa in the presence of 100 nM TF antibody, as set forth in **Table 7** and **Table 38** is selected from about 108%, about 105%, about 111%, about 58%, about 47%, about 91%, about 103%, about 109%, about 107%, and about 45% compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA) with 10 min antibody pre-incubation. In some embodiments, such % Peak IIa ranges from about 45% to about 111%. In some embodiments, such % Peak IIa is about 45% or more.

[00292] In some embodiments, the % Peak IIa in the presence of 50 nM TF antibody, as set forth in **Table 7** and **Table 38** is selected from about 107%, about 104%, about 114%, about 62%, about 49%, about 87%, about 105%, about 109%, about 55%, and about 92% compared to the control conditions without the antibody, as determined by thrombin

generation assay (TGA) with 10 min antibody pre-incubation. In some embodiments, such % Peak IIa ranges from about 49% to about 114%. In some embodiments, such % Peak IIa is about 49% or more.

[00293] In some embodiments, the % Peak IIa in the presence of 10 nM TF antibody, as set forth in **Table 7** and **Table 38** is selected from about 105%, about 114%, about 76%, about 68%, about 94%, about 108%, about 104%, about 74%, and about 93% compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA) with 10 min antibody pre-incubation. In some embodiments, such % Peak IIa ranges from about 68% to about 114%. In some embodiments, such % Peak IIa is about 68% or more.

[00294] In some embodiments, the percent endogenous thrombin potential (% ETP) is at least 80% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % ETP is at least 90% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % ETP is at least 95% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % ETP is at least 99% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA).

[00295] In some embodiments, the % ETP is at least 80% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % ETP is at least 90% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % ETP is at least 95% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % ETP is at least 99% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA).

[00296] In some embodiments, the % ETP is at least 80% in the presence of no less than 10 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % ETP is at least 90% in the presence of no less than 10 nM TF antibody compared to the control conditions without the

antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % ETP is at least 95% in the presence of no less than 10 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA). In some embodiments, the % ETP is at least 99% in the presence of no less than 10 nM TF antibody compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA).

[00297] In some embodiments, the % ETP in the presence of 100 nM TF antibody, as set forth in **Table 6** and **Table 37** is selected from about 108%, about 103%, about 109%, about 100%, about 96%, about 102%, about 105%, and about 92% compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA) without antibody pre-incubation. In some embodiments, such % ETP ranges from about 92% to about 109%. In some embodiments, such % ETP is about 92% or more.

[00298] In some embodiments, the % ETP in the presence of 50 nM TF antibody, as set forth in **Table 6** and **Table 37** is selected from about 108%, about 103%, about 111%, about 101%, about 97%, about 104%, about 106%, about 93%, about 96%, and about 105% compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA) without antibody pre-incubation. In some embodiments, such % ETP ranges from about 93% to about 111%. In some embodiments, such % ETP is about 93% or more.

[00299] In some embodiments, the % ETP in the presence of 10 nM TF antibody, as set forth in **Table 6** and **Table 37** is selected from about 106%, about 109%, about 105%, about 104%, about 107%, about 99%, about 101%, and about 102% compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA) without antibody pre-incubation. In some embodiments, such % ETP ranges from about 99% to about 109%. In some embodiments, such % ETP is about 99% or more.

[00300] In some embodiments, the % ETP in the presence of 100 nM TF antibody, as set forth in **Table 7** and **Table 38** is selected from about 110%, about 104%, about 106%, about 98%, about 95%, about 108%, about 107%, about 96%, about 92%, and about 103% compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA) with 10 min antibody pre-incubation. In some embodiments, such % ETP ranges from about 92% to about 110%. In some embodiments, such % ETP is about 92% or more.

[00301] In some embodiments, the % ETP in the presence of 50 nM TF antibody, as set forth in **Table 7** and **Table 38** is selected from about 110%, about 106%, about 108%, about

103%, about 96%, about 109%, about 102%, about 104%, about 94%, and about 98% compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA) with 10 min antibody pre-incubation. In some embodiments, such % ETP ranges from about 94% to about 110%. In some embodiments, such % ETP is about 94% or more.

[00302] In some embodiments, the % ETP in the presence of 10 nM TF antibody, as set forth in **Table 7** and **Table 38** is selected from about 107%, about 106%, about 110%, about 103%, about 100%, about 105%, about 102%, and about 101% compared to the control conditions without the antibody, as determined by thrombin generation assay (TGA) with 10 min antibody pre-incubation. In some embodiments, such % ETP ranges from about 100% to about 110%. In some embodiments, such % ETP is about 100% or more.

2.3.3. FXa Conversion in the Presence of TF Antibodies

[00303] In some embodiments, the antibodies provided herein bind human TF at a human TF binding site that is distinct from a human TF binding site bound by human FX. In certain embodiments, the antibodies provided herein do not interfere with the ability of TF:FVIIa to convert FX into FXa.

[00304] In some embodiments, the percentage of FXa conversion (% FXa) is at least 75% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FXa is at least 80% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FXa is at least 85% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FXa is at least 90% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FXa is at least 95% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody.

[00305] In some embodiments, the % FXa is at least 75% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FXa is at least 80% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FXa is at least 85% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FXa is at least 90% in the presence of no less than 50 nM TF antibody compared to the control conditions without the

antibody. In some embodiments, the % FXa is at least 95% in the presence of no less than 50 nM TF antibody compared to the control conditions without the antibody.

[00306] In some embodiments, the % FXa is at least 75% in the presence of no less than 25 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FXa is at least 80% in the presence of no less than 25 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FXa is at least 85% in the presence of no less than 25 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FXa is at least 90% in the presence of no less than 25 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FXa is at least 95% in the presence of no less than 25 nM TF antibody compared to the control conditions without the antibody.

[00307] In some embodiments, the % FXa is at least 75% in the presence of no less than 12.5 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FXa is at least 80% in the presence of no less than 12.5 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FXa is at least 85% in the presence of no less than 12.5 nM TF antibody compared to the control conditions without the antibody. In some embodiments, % FXa is at least 90% in the presence of no less than 12.5 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FXa is at least 95% in the presence of no less than 12.5 nM TF antibody compared to the control conditions without the antibody.

[00308] In some embodiments, the % FXa in the presence of 100 nM TF antibody, as set forth in **Table 8** is selected from about 89%, about 96%, about 116%, about 108%, about 117%, about 105%, about 112%, about 106%, about 103%, about 111%, about 98%, and about 101% compared to the control conditions without the antibody. In some embodiments, such % FXa ranges from about 89% to about 117%. In some embodiments, such % FXa is about 89% or more.

[00309] In some embodiments, the % FXa in the presence of 50 nM TF antibody, as set forth in **Table 8** is selected from about 94%, about 93%, about 78%, about 102%, about 99%, about 104%, about 105%, about 108%, about 107%, about 97%, and about 106% compared to the control conditions without the antibody. In some embodiments, such % FXa ranges from about 78% to about 108%. In some embodiments, such % FXa is about 78% or more.

[00310] In some embodiments, the % FXa in the presence of 25 nM TF antibody, as set forth in **Table 8** is selected from about 81%, about 89%, about 85%, about 109%, about 96%, about 97%, about 108%, about 104%, about 103%, about 112%, and about 89% compared to

the control conditions without the antibody. In some embodiments, such % FXa ranges from about 81% to about 112%. In some embodiments, such % FXa is about 81% or more.

[00311] In some embodiments, the % FXa in the presence of 12.5 nM TF antibody, as set forth in **Table 8** is selected from about 87%, about 89%, about 82%, about 99%, about 101%, about 98%, about 113%, about 106%, about 115%, about 110%, about 120%, about 85%, and about 108% compared to the control conditions without the antibody. In some embodiments, such % FXa ranges from about 82% to about 120%. In some embodiments, such % FXa is about 82% or more.

2.3.4. FVIIa Binding in the Presence of TF Antibodies

[00312] In some embodiments, the antibodies provided herein bind human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa. In certain embodiments, the antibodies provided herein do not compete for binding to human TF with human FVIIa.

[00313] In some embodiments, the percentage of FVIIa binding (% FVIIa) is at least 75% in the presence of no less than 250 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 80% in the presence of no less than 250 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 85% in the presence of no less than 250 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 90% in the presence of no less than 250 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 95% in the presence of no less than 250 nM TF antibody compared to the control conditions without the antibody.

[00314] In some embodiments, the % FVIIa is at least 75% in the presence of no less than 83 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 80% in the presence of no less than 83 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 85% in the presence of no less than 83 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 90% in the presence of no less than 83 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 95% in the presence of no less than 83 nM TF antibody compared to the control conditions without the antibody.

[00315] In some embodiments, the % FVIIa is at least 75% in the presence of no less than 28 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 80% in the presence of no less than 28 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 85% in the presence of no less than 28 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 90% in the presence of no less than 28 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 95% in the presence of no less than 28 nM TF antibody compared to the control conditions without the antibody.

[00316] In some embodiments, the % FVIIa is at least 75% in the presence of no less than 9.25 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 80% in the presence of no less than 9.25 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 85% in the presence of no less than 9.25 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 90% in the presence of no less than 9.25 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the % FVIIa is at least 95% in the presence of no less than 9.25 nM TF antibody compared to the control conditions without the antibody.

[00317] In some embodiments, the % FVIIa in the presence of 250 nM TF antibody, as set forth in **Table 9** is selected from about 98%, about 87%, about 80%, about 92%, about 95%, about 89%, about 91%, about 97%, about 94%, about 101%, and about 96% compared to the control conditions without the antibody. In some embodiments, such % FVIIa ranges from about 80% to about 101%. In some embodiments, such % FVIIa is about 80% or more.

[00318] In some embodiments, the % FVIIa in the presence of 83 nM TF antibody, as set forth in **Table 9** is selected from about 97%, about 88%, about 77%, about 93%, about 94%, about 91%, about 98%, about 100%, and about 92% compared to the control conditions without the antibody. In some embodiments, such % FVIIa ranges from about 77% to about 100%. In some embodiments, such % FVIIa is about 77% or more.

[00319] In some embodiments, the % FVIIa in the presence of 28 nM TF antibody, as set forth in **Table 9** is selected from about 101%, about 87%, about 79%, about 96%, about 93%, about 95%, about 98%, about 100%, about 102%, about 99%, about 92%, and about 91% compared to the control conditions without the antibody. In some embodiments, such % FVIIa ranges from about 79% to about 102%. In some embodiments, such % FVIIa is about 79% or more.

[00320] In some embodiments, the % FVIIa in the presence of 9.25 nM TF antibody, as set forth in **Table 9** is selected from about 100%, about 90%, about 76%, about 97%, about 93%, about 99%, about 98%, about 102%, about 101%, and about 95% compared to the control conditions without the antibody. In some embodiments, such % FVIIa ranges from about 76% to about 102%. In some embodiments, such % FVIIa is about 76% or more.

2.3.5. FVIIa-dependent TF Signaling in the Presence of TF Antibodies

[00321] In some embodiments, the antibodies provided herein inhibit FVIIa-dependent TF signaling. In some embodiments, the inhibition of FVIIa-dependent TF signaling is measured by the reduction of IL8. In some embodiments, the inhibition of FVIIa-dependent TF signaling is measured by the reduction of GM-CSF.

[00322] In some embodiments, the Interleukin 8 concentration (IL8 conc) is reduced by at least 70% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the IL8 conc is reduced by at least 80% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the IL8 conc is reduced by at least 90% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody.

[00323] In some embodiments, the IL8 conc is reduced by at least 70% in the presence of no less than 40 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the IL8 conc is reduced by at least 80% in the presence of no less than 40 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the IL8 conc is reduced by at least 90% in the presence of no less than 40 nM TF antibody compared to the control conditions without the antibody.

[00324] In some embodiments, the IL8 conc is reduced by at least 60% in the presence of no less than 16 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the IL8 conc is reduced by at least 70% in the presence of no less than 16 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the IL8 conc is reduced by at least 80% in the presence of no less than 16 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the IL8 conc is reduced by at least 90% in the presence of no less than 16 nM TF antibody compared to the control conditions without the antibody.

[00325] In some embodiments, the IL8 conc is reduced by at least 50% in the presence of no less than 6.4 nM TF antibody compared to the control conditions without the antibody. In

some embodiments, the IL8 conc is reduced by at least 60% in the presence of no less than 6.4 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the IL8 conc is reduced by at least 70% in the presence of no less than 6.4 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the IL8 conc is reduced by at least 80% in the presence of no less than 6.4 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the IL8 conc is reduced by at least 90% in the presence of no less than 6.4 nM TF antibody compared to the control conditions without the antibody.

[00326] In some embodiments, the Granulocyte-Macrophage Colony-Stimulating Factor concentration (GM-CSF conc) is reduced by at least 70% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the GM-CSF conc is reduced by at least 80% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the GM-CSF conc is reduced by at least 90% in the presence of no less than 100 nM TF antibody compared to the control conditions without the antibody.

[00327] In some embodiments, the GM-CSF conc is reduced by at least 70% in the presence of no less than 40 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the GM-CSF conc is reduced by at least 80% in the presence of no less than 40 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the GM-CSF conc is reduced by at least 90% in the presence of no less than 40 nM TF antibody compared to the control conditions without the antibody.

[00328] In some embodiments, the GM-CSF conc is reduced by at least 60% in the presence of no less than 16 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the GM-CSF conc is reduced by at least 70% in the presence of no less than 16 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the GM-CSF conc is reduced by at least 80% in the presence of no less than 16 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the GM-CSF conc is reduced by at least 90% in the presence of no less than 16 nM TF antibody compared to the control conditions without the antibody.

[00329] In some embodiments, the GM-CSF conc is reduced by at least 50% in the presence of no less than 6.4 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the GM-CSF conc is reduced by at least 60% in the

presence of no less than 6.4 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the GM-CSF conc is reduced by at least 70% in the presence of no less than 6.4 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the GM-CSF conc is reduced by at least 80% in the presence of no less than 6.4 nM TF antibody compared to the control conditions without the antibody. In some embodiments, the GM-CSF conc is reduced by at least 90% in the presence of no less than 6.4 nM TF antibody compared to the control conditions without the antibody.

[00330] In some embodiments, the percentage of Interleukin 8 (% IL8) in the presence of 100 nM TF antibody, as set forth in **Table 10** is selected from about 2%, about 9%, about 8%, about 6%, about 13%, about 1%, about 3%, about 4%, and about 5% compared to the control conditions without the antibody. In some embodiments, such % IL8 ranges from about 1% to about 13%. In some embodiments, such % IL8 is about 13% or less.

[00331] In some embodiments, the % IL8 in the presence of 40 nM TF antibody, as set forth in **Table 10** is selected from about 2%, about 8%, about 7%, about 10%, about 14%, about 4%, about 5%, and about 6% compared to the control conditions without the antibody. In some embodiments, such % IL8 ranges from about 2% to about 14%. In some embodiments, such % IL8 is about 14% or less.

[00332] In some embodiments, the % IL8 in the presence of 16 nM TF antibody, as set forth in **Table 10** is selected from about 2%, about 3%, about 10%, about 8%, about 7%, about 16%, about 9%, about 15%, about 5%, and about 6% compared to the control conditions without the antibody. In some embodiments, such % IL8 ranges from about 2% to about 16%. In some embodiments, such % IL8 is about 16% or less.

[00333] In some embodiments, the % IL8 in the presence of 6.4 nM TF antibody, as set forth in **Table 10** is selected from about 3%, about 4%, about 11%, about 9%, about 14%, about 22%, about 12%, about 6%, about 5%, about 15%, about 21%, and about 8% compared to the control conditions without the antibody. In some embodiments, such % IL8 ranges from about 3% to about 22%. In some embodiments, such % IL8 is about 22% or less.

[00334] In some embodiments, the percentage of Granulocyte-Macrophage Colony-Stimulating Factor (% GM-CSF) in the presence of 100 nM TF antibody, as set forth in **Table 11** is selected from about 6%, about 7%, about 22%, about 20%, about 12%, about 19%, about 17%, about 25%, about 5%, about 14%, about 11%, and about 10% compared to the control conditions without the antibody. In some embodiments, such % GM-CSF ranges from about 5% to about 25%. In some embodiments, such % GM-CSF is about 25% or less.

[00335] In some embodiments, the % GM-CSF in the presence of 40 nM TF antibody, as set forth in **Table 11** is selected from about 6%, about 7%, about 19%, about 15%, about 18%, about 16%, about 26%, about 5%, about 13%, about 11%, and about 10% compared to the control conditions without the antibody. In some embodiments, such % GM-CSF ranges from about 5% to about 26%. In some embodiments, such % GM-CSF is about 26% or less.

[00336] In some embodiments, the % GM-CSF in the presence of 16 nM TF antibody, as set forth in **Table 11** is selected from about 6%, about 7%, about 22%, about 19%, about 14%, about 32%, about 17%, about 26%, about 5%, about 12%, about 13%, about 9%, about 11%, and about 15% compared to the control conditions without the antibody. In some embodiments, such % GM-CSF ranges from about 5% to about 32%. In some embodiments, such % GM-CSF is about 32% or less.

[00337] In some embodiments, the % GM-CSF in the presence of 6.4 nM TF antibody, as set forth in **Table 11** is selected from about 8%, about 9%, about 24%, about 20%, about 18%, about 39%, about 34%, about 15%, about 21%, about 16%, about 17%, and about 10% compared to the control conditions without the antibody. In some embodiments, such % GM-CSF ranges from about 8% to about 39%. In some embodiments, such % GM-CSF is about 39% or less.

2.3.6. Lesion Size Reduction in Swine Choroidal Neovascularization (CNV) Model

[00338] In some embodiments, the antibodies provided herein reduce lesion size in a swine choroidal neovascularization (CNV) model. In some embodiments, the reduction in lesion size is measured by Fluorescein Angiography (FA).

[00339] In some embodiments, the lesion size in a swine CNV model is reduced by at least 5% 7 days after administration of the anti-TF antibody. In some embodiments, the lesion size in a swine CNV model is reduced by at least 10% 7 days after administration of the anti-TF antibody. In some embodiments, the lesion size in a swine CNV model is reduced by at least 20% 7 days after administration of the anti-TF antibody. In some embodiments, the lesion size in a swine CNV model is reduced by at least 40% 7 days after administration of the anti-TF antibody. In some embodiments, the lesion size in a swine CNV model is reduced by at least 60% 7 days after administration of the anti-TF antibody.

[00340] In some embodiments, the lesion size in a swine CNV model is reduced by at least 10% 21 days after administration of the anti-TF antibody. In some embodiments, the lesion size in a swine CNV model is reduced by at least 20% 21 days after administration of the anti-TF antibody. In some embodiments, the lesion size in a swine CNV model is reduced by

at least 40% 21 days after administration of the anti-TF antibody. In some embodiments, the lesion size in a swine CNV model is reduced by at least 60% 21 days after administration of the anti-TF antibody. In some embodiments, the lesion size in a swine CNV model is reduced by at least 80% 21 days after administration of the anti-TF antibody.

2.4. Germlines

[00341] The antibodies provided herein may comprise any suitable V_H and V_L germline sequences.

[00342] In some embodiments, the V_H region of an antibody provided herein is from the VH3 germline. In some embodiments, the V_H region of an antibody provided herein is from the VH1 germline. In some embodiments, the V_H region of an antibody provided herein is from the VH4 germline.

[00343] In some embodiments, the V_H region of an antibody provided herein is from the VH3-23 germline. In some embodiments, the V_H region of an antibody provided herein is from the VH1-18 germline. In some embodiments, the V_H region of an antibody provided herein is from the VH3-30 germline. In some embodiments, the V_H region of an antibody provided herein is from the VH1-69 germline. In some embodiments, the V_H region of an antibody provided herein is from the VH4-31 germline. In some embodiments, the V_H region of an antibody provided herein is from the VH4-34 germline. In some embodiments, the V_H region of an antibody provided herein is from the VH1-46 germline.

[00344] In some embodiments, the V_L region of an antibody provided herein is from the VK1 germline. In some embodiments, the V_L region of an antibody provided herein is from the VK4 germline. In some embodiments, the V_L region of an antibody provided herein is from the VK3 germline.

[00345] In some embodiments, the V_L region of an antibody provided herein is from the VK1-05 germline. In some embodiments, the V_L region of an antibody provided herein is from the VK4-01 germline. In some embodiments, the V_L region of an antibody provided herein is from the VK3-15 germline. In some embodiments, the V_L region of an antibody provided herein is from the VK3-20 germline. In some embodiments, the V_L region of an antibody provided herein is from the VK1-33 germline.

2.5. Monospecific and Multispecific TF Antibodies

[00346] In some embodiments, the antibodies provided herein are monospecific antibodies.

[00347] In some embodiments, the antibodies provided herein are multispecific antibodies.

[00348] In some embodiments, a multispecific antibody provided herein binds more than one antigen. In some embodiments, a multispecific antibody binds two antigens. In some embodiments, a multispecific antibody binds three antigens. In some embodiments, a multispecific antibody binds four antigens. In some embodiments, a multispecific antibody binds five antigens.

[00349] In some embodiments, a multispecific antibody provided herein binds more than one epitope on a TF antigen. In some embodiments, a multispecific antibody binds two epitopes on a TF antigen. In some embodiments, a multispecific antibody binds three epitopes on a TF antigen.

[00350] Many multispecific antibody constructs are known in the art, and the antibodies provided herein may be provided in the form of any suitable multispecific suitable construct.

[00351] In some embodiments, the multispecific antibody comprises an immunoglobulin comprising at least two different heavy chain variable regions each paired with a common light chain variable region (*i.e.*, a “common light chain antibody”). The common light chain variable region forms a distinct antigen-binding domain with each of the two different heavy chain variable regions. *See Merchant et al., Nature Biotechnol.*, 1998, 16:677-681, incorporated by reference in its entirety.

[00352] In some embodiments, the multispecific antibody comprises an immunoglobulin comprising an antibody or fragment thereof attached to one or more of the N- or C-termini of the heavy or light chains of such immunoglobulin. *See Coloma and Morrison, Nature Biotechnol.*, 1997, 15:159-163, incorporated by reference in its entirety. In some aspects, such antibody comprises a tetravalent bispecific antibody.

[00353] In some embodiments, the multispecific antibody comprises a hybrid immunoglobulin comprising at least two different heavy chain variable regions and at least two different light chain variable regions. *See Milstein and Cuello, Nature*, 1983, 305:537-540; and Staerz and Bevan, *Proc. Natl. Acad. Sci. USA*, 1986, 83:1453-1457; each of which is incorporated by reference in its entirety.

[00354] In some embodiments, the multispecific antibody comprises immunoglobulin chains with alterations to reduce the formation of side products that do not have multispecificity. In some aspects, the antibodies comprise one or more “knobs-into-holes” modifications as described in U.S. Pat. No. 5,731,168, incorporated by reference in its entirety.

[00355] In some embodiments, the multispecific antibody comprises immunoglobulin chains with one or more electrostatic modifications to promote the assembly of Fc heteromultimers. *See* WO 2009/089004, incorporated by reference in its entirety.

[00356] In some embodiments, the multispecific antibody comprises a bispecific single chain molecule. *See* Traunecker *et al.*, *EMBO J.*, 1991, 10:3655-3659; and Gruber *et al.*, *J. Immunol.*, 1994, 152:5368-5374; each of which is incorporated by reference in its entirety.

[00357] In some embodiments, the multispecific antibody comprises a heavy chain variable domain and a light chain variable domain connected by a polypeptide linker, where the length of the linker is selected to promote assembly of multispecific antibodies with the desired multispecificity. For example, monospecific scFvs generally form when a heavy chain variable domain and light chain variable domain are connected by a polypeptide linker of more than 12 amino acid residues. *See* U.S. Pat. Nos. 4,946,778 and 5,132,405, each of which is incorporated by reference in its entirety. In some embodiments, reduction of the polypeptide linker length to less than 12 amino acid residues prevents pairing of heavy and light chain variable domains on the same polypeptide chain, thereby allowing pairing of heavy and light chain variable domains from one chain with the complementary domains on another chain. The resulting antibodies therefore have multispecificity, with the specificity of each binding site contributed by more than one polypeptide chain. Polypeptide chains comprising heavy and light chain variable domains that are joined by linkers between 3 and 12 amino acid residues form predominantly dimers (termed diabodies). With linkers between 0 and 2 amino acid residues, trimers (termed triabodies) and tetramers (termed tetrabodies) are favored. However, the exact type of oligomerization appears to depend on the amino acid residue composition and the order of the variable domain in each polypeptide chain (*e.g.*, V_H-linker-V_L vs. V_L-linker-V_H), in addition to the linker length. A skilled person can select the appropriate linker length based on the desired multispecificity.

[00358] In some embodiments, the multispecific antibody comprises a diabody. *See* Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 1993, 90:6444-6448, incorporated by reference in its entirety. In some embodiments, the multispecific antibody comprises a triabody. *See* Todorovska *et al.*, *J. Immunol. Methods*, 2001, 248:47-66, incorporated by reference in its entirety. In some embodiments, the multispecific antibody comprises a tetrabody. *See id.*, incorporated by reference in its entirety.

[00359] In some embodiments, the multispecific antibody comprises a trispecific F(ab')₃ derivative. *See* Tutt *et al.* *J. Immunol.*, 1991, 147:60-69, incorporated by reference in its entirety.

[00360] In some embodiments, the multispecific antibody comprises a cross-linked antibody. *See* U.S. Patent No. 4,676,980; Brennan *et al.*, *Science*, 1985, 229:81-83; Staerz, *et al. Nature*, 1985, 314:628-631; and EP 0453082; each of which is incorporated by reference in its entirety.

[00361] In some embodiments, the multispecific antibody comprises antigen-binding domains assembled by leucine zippers. *See* Kostelny *et al.*, *J. Immunol.*, 1992, 148:1547-1553, incorporated by reference in its entirety.

[00362] In some embodiments, the multispecific antibody comprises complementary protein domains. In some aspects, the complementary protein domains comprise an anchoring domain (AD) and a dimerization and docking domain (DDD). In some embodiments, the AD and DDD bind to each other and thereby enable assembly of multispecific antibody structures via the “dock and lock” (DNL) approach. Antibodies of many specificities may be assembled, including bispecific antibodies, trispecific antibodies, tetraspecific antibodies, quintspecific antibodies, and hexaspecific antibodies. Multispecific antibodies comprising complementary protein domains are described, for example, in U.S. Pat. Nos. 7,521,056; 7,550,143; 7,534,866; and 7,527,787; each of which is incorporated by reference in its entirety.

[00363] In some embodiments, the multispecific antibody comprises a dual action Fab (DAF) antibody as described in U.S. Pat. Pub. No. 2008/0069820, incorporated by reference in its entirety.

[00364] In some embodiments, the multispecific antibody comprises an antibody formed by reduction of two parental molecules followed by mixing of the two parental molecules and reoxidation to assembly a hybrid structure. *See* Carlring *et al.*, *PLoS One*, 2011, 6:e22533, incorporated by reference in its entirety.

[00365] In some embodiments, the multispecific antibody comprises a DVD-IgTM. A DVD-IgTM is a dual variable domain immunoglobulin that can bind to two or more antigens. DVD-IgsTM are described in U.S. Pat. No. 7,612,181, incorporated by reference in its entirety.

[00366] In some embodiments, the multispecific antibody comprises a DARTTM. DARTsTM are described in Moore *et al.*, *Blood*, 2011, 117:454-451, incorporated by reference in its entirety.

[00367] In some embodiments, the multispecific antibody comprises a DuoBody[®]. DuoBodies[®] are described in Labrijn *et al.*, *Proc. Natl. Acad. Sci. USA*, 2013, 110:5145-5150; Gramer *et al.*, *mAbs*, 2013, 5:962-972; and Labrijn *et al.*, *Nature Protocols*, 2014, 9:2450-2463; each of which is incorporated by reference in its entirety.

[00368] In some embodiments, the multispecific antibody comprises an antibody fragment attached to another antibody or fragment. The attachment can be covalent or non-covalent. When the attachment is covalent, it may be in the form of a fusion protein or via a chemical linker. Illustrative examples of multispecific antibodies comprising antibody fragments attached to other antibodies include tetravalent bispecific antibodies, where an scFv is fused to the C-terminus of the C_{H3} from an IgG. *See Coloma and Morrison, Nature Biotechnol.*, 1997, 15:159-163. Other examples include antibodies in which a Fab molecule is attached to the constant region of an immunoglobulin. *See Miler et al., J. Immunol.*, 2003, 170:4854-4861, incorporated by reference in its entirety. Any suitable fragment may be used, including any of the fragments described herein or known in the art.

[00369] In some embodiments, the multispecific antibody comprises a CovX-Body. CovX-Bodies are described, for example, in Doppalapudi *et al.*, *Proc. Natl. Acad. Sci. USA*, 2010, 107:22611-22616, incorporated by reference in its entirety.

[00370] In some embodiments, the multispecific antibody comprises an Fcab antibody, where one or more antigen-binding domains are introduced into an Fc region. Fcab antibodies are described in Wozniak-Knopp *et al.*, *Protein Eng. Des. Sel.*, 2010, 23:289-297, incorporated by reference in its entirety.

[00371] In some embodiments, the multispecific antibody comprises a TandAb[®] antibody. TandAb[®] antibodies are described in Kipriyanov *et al.*, *J. Mol. Biol.*, 1999, 293:41-56 and Zhukovsky *et al.*, *Blood*, 2013, 122:5116, each of which is incorporated by reference in its entirety.

[00372] In some embodiments, the multispecific antibody comprises a tandem Fab. Tandem Fabs are described in WO 2015/103072, incorporated by reference in its entirety.

[00373] In some embodiments, the multispecific antibody comprises a Zybody[™]. Zybody[™]s are described in LaFleur *et al.*, *mAbs*, 2013, 5:208-218, incorporated by reference in its entirety.

[00374] In some embodiments, administration of the multispecific antibody results in cell-mediated cytotoxicity.

2.6. Glycosylation Variants

[00375] In certain embodiments, an antibody provided herein may be altered to increase, decrease or eliminate the extent to which it is glycosylated. Glycosylation of polypeptides is typically either “N-linked” or “O-linked.”

[00376] “N-linked” glycosylation refers to the attachment of a carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site.

[00377] “O-linked” glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

[00378] Addition or deletion of N-linked glycosylation sites to or from an antibody provided herein may be accomplished by altering the amino acid sequence such that one or more of the above-described tripeptide sequences is created or removed. Addition or deletion of O-linked glycosylation sites may be accomplished by addition, deletion, or substitution of one or more serine or threonine residues in or to (as the case may be) the sequence of an antibody.

[00379] In some embodiments, an antibody provided herein comprises a glycosylation motif that is different from a naturally occurring antibody. Any suitable naturally occurring glycosylation motif can be modified in the antibodies provided herein. The structural and glycosylation properties of immunoglobulins, for example, are known in the art and summarized, for example, in Schroeder and Cavacini, *J. Allergy Clin. Immunol.*, 2010, 125:S41-52, incorporated by reference in its entirety.

[00380] In some embodiments, an antibody provided herein comprises an IgG1 Fc region with modification to the oligosaccharide attached to asparagine 297 (Asn 297). Naturally occurring IgG1 antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn 297 of the C_{H2} domain of the Fc region. *See Wright et al., TIBTECH*, 1997, 15:26-32, incorporated by reference in its entirety. The oligosaccharide attached to Asn 297 may include various carbohydrates such as mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the “stem” of the biantennary oligosaccharide structure.

[00381] In some embodiments, the oligosaccharide attached to Asn 297 is modified to create antibodies having altered ADCC. In some embodiments, the oligosaccharide is altered to improve ADCC. In some embodiments, the oligosaccharide is altered to reduce ADCC.

[00382] In some aspects, an antibody provided herein comprises an IgG1 domain with reduced fucose content at position Asn 297 compared to a naturally occurring IgG1 domain. Such Fc domains are known to have improved ADCC. *See* Shields *et al.*, *J. Biol. Chem.*, 2002, 277:26733-26740, incorporated by reference in its entirety. In some aspects, such antibodies do not comprise any fucose at position Asn 297. The amount of fucose may be determined using any suitable method, for example as described in WO 2008/077546, incorporated by reference in its entirety.

[00383] In some embodiments, an antibody provided herein comprises a bisected oligosaccharide, such as a biantennary oligosaccharide attached to the Fc region of the antibody that is bisected by GlcNAc. Such antibody variants may have reduced fucosylation and/or improved ADCC function. Examples of such antibody variants are described, for example, in WO 2003/011878; U.S. Pat. No. 6,602,684; and U.S. Pat. Pub. No. 2005/0123546; each of which is incorporated by reference in its entirety.

[00384] Other illustrative glycosylation variants which may be incorporated into the antibodies provided herein are described, for example, in U.S. Pat. Pub. Nos. 2003/0157108, 2004/0093621, 2003/0157108, 2003/0115614, 2002/0164328, 2004/0093621, 2004/0132140, 2004/0110704, 2004/0110282, 2004/0109865; International Pat. Pub. Nos. 2000/61739, 2001/29246, 2003/085119, 2003/084570, 2005/035586, 2005/035778; 2005/053742, 2002/031140; Okazaki *et al.*, *J. Mol. Biol.*, 2004, 336:1239-1249; and Yamane-Ohnuki *et al.*, *Biotech. Bioeng.*, 2004, 87: 614-622; each of which is incorporated by reference in its entirety.

[00385] In some embodiments, an antibody provided herein comprises an Fc region with at least one galactose residue in the oligosaccharide attached to the Fc region. Such antibody variants may have improved CDC function. Examples of such antibody variants are described, for example, in WO 1997/30087; WO 1998/58964; and WO 1999/22764; each of which is incorporated by reference in its entirety.

[00386] Examples of cell lines capable of producing defucosylated antibodies include Lec13 CHO cells, which are deficient in protein fucosylation (*see* Ripka *et al.*, *Arch. Biochem. Biophys.*, 1986, 249:533-545; U.S. Pat. Pub. No. 2003/0157108; WO 2004/056312; each of which is incorporated by reference in its entirety), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene or FUT8 knockout CHO cells (*see* Yamane-Ohnuki *et al.*, *Biotech. Bioeng.*, 2004, 87: 614-622; Kanda *et al.*, *Biotechnol. Bioeng.*, 2006, 94:680-688; and WO 2003/085107; each of which is incorporated by reference in its entirety).

[00387] In some embodiments, an antibody provided herein is an aglycosylated antibody. An aglycosylated antibody can be produced using any method known in the art or described herein. In some aspects, an aglycosylated antibody is produced by modifying the antibody to remove all glycosylation sites. In some aspects, the glycosylation sites are removed only from the Fc region of the antibody. In some aspects, an aglycosylated antibody is produced by expressing the antibody in an organism that is not capable of glycosylation, such as *E. coli*, or by expressing the antibody in a cell-free reaction mixture.

[00388] In some embodiments, an antibody provided herein has a constant region with reduced effector function compared to a native IgG1 antibody. In some embodiments, the affinity of a constant region of an Fc region of an antibody provided herein for Fc receptor is less than the affinity of a native IgG1 constant region for such Fc receptor.

2.7. Fc Region Amino Acid Sequence Variants

[00389] In certain embodiments, an antibody provided herein comprises an Fc region with one or more amino acid substitutions, insertions, or deletions in comparison to a naturally occurring Fc region. In some aspects, such substitutions, insertions, or deletions yield antibodies with altered stability, glycosylation, or other characteristics. In some aspects, such substitutions, insertions, or deletions yield aglycosylated antibodies.

[00390] In some aspects, the Fc region of an antibody provided herein is modified to yield an antibody with altered affinity for an Fc receptor, or an antibody that is more immunologically inert. In some embodiments, the antibody variants provided herein possess some, but not all, effector functions. Such antibodies may be useful, for example, when the half-life of the antibody is important *in vivo*, but when certain effector functions (*e.g.*, complement activation and ADCC) are unnecessary or deleterious.

[00391] In some embodiments, the Fc region of an antibody provided herein is a human IgG4 Fc region comprising one or more of the hinge stabilizing mutations S228P and L235E. *See Aalberse et al., Immunology*, 2002, 105:9-19, incorporated by reference in its entirety. In some embodiments, the IgG4 Fc region comprises one or more of the following mutations: E233P, F234V, and L235A. *See Armour et al., Mol. Immunol.*, 2003, 40:585-593, incorporated by reference in its entirety. In some embodiments, the IgG4 Fc region comprises a deletion at position G236.

[00392] In some embodiments, the Fc region of an antibody provided herein is a human IgG1 Fc region comprising one or more mutations to reduce Fc receptor binding. In some aspects, the one or more mutations are in residues selected from S228 (*e.g.*, S228A), L234

(*e.g.*, L234A), L235 (*e.g.*, L235A), D265 (*e.g.*, D265A), and N297 (*e.g.*, N297A). In some aspects, the antibody comprises a PVA236 mutation. PVA236 means that the amino acid sequence ELLG (SEQ ID NO: 928), from amino acid position 233 to 236 of IgG1 or EFLG (SEQ ID NO: 929) of IgG4, is replaced by PVA. *See* U.S. Pat. No. 9,150,641, incorporated by reference in its entirety.

[00393] In some embodiments, the Fc region of an antibody provided herein is modified as described in Armour *et al.*, *Eur. J. Immunol.*, 1999, 29:2613-2624; WO 1999/058572; and/or U.K. Pat. App. No. 98099518; each of which is incorporated by reference in its entirety.

[00394] In some embodiments, the Fc region of an antibody provided herein is a human IgG2 Fc region comprising one or more of mutations A330S and P331S.

[00395] In some embodiments, the Fc region of an antibody provided herein has an amino acid substitution at one or more positions selected from 238, 265, 269, 270, 297, 327 and 329. *See* U.S. Pat. No. 6,737,056, incorporated by reference in its entirety. Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called “DANA” Fc mutant with substitution of residues 265 and 297 with alanine. *See* U.S. Pat. No. 7,332,581, incorporated by reference in its entirety. In some embodiments, the antibody comprises an alanine at amino acid position 265. In some embodiments, the antibody comprises an alanine at amino acid position 297.

[00396] In certain embodiments, an antibody provided herein comprises an Fc region with one or more amino acid substitutions which improve ADCC, such as a substitution at one or more of positions 298, 333, and 334 of the Fc region. In some embodiments, an antibody provided herein comprises an Fc region with one or more amino acid substitutions at positions 239, 332, and 330, as described in Lazar *et al.*, *Proc. Natl. Acad. Sci. USA*, 2006, 103:4005-4010, incorporated by reference in its entirety. For example, in some certain embodiments, an antibody provided herein has one or more of S239D, A330L, and I332E substitutions in the Fc region, as determined by Kabat numbering. In some aspects, the FC region is a human IgG1 Fc region.

[00397] In certain embodiments, an antibody provided herein comprises the combination of L234A, L235A, and P329A (LALA-PG) substitutions in the human IgG1 heavy chain constant region (as determined by Kabat numbering) that have been shown to effectively silence the effector function of human IgG1 antibodies. (*See*, for example, Schlothauer *et al.* *Protein Engineering, Design and Selection* 29.10 (2016): 457-466, which is incorporated by reference in its entirety).

[00398] In some embodiments, an antibody provided herein comprises one or more alterations that improves or diminishes C1q binding and/or CDC. *See* U.S. Pat. No. 6,194,551; WO 99/51642; and Idusogie *et al.*, *J. Immunol.*, 2000, 164:4178-4184; each of which is incorporated by reference in its entirety.

[00399] In some embodiments, an antibody provided herein comprises one or more alterations to increase half-life. Antibodies with increased half-lives and improved binding to the neonatal Fc receptor (FcRn) are described, for example, in Hinton *et al.*, *J. Immunol.*, 2006, 176:346-356; and U.S. Pat. Pub. No. 2005/0014934; each of which is incorporated by reference in its entirety. Such Fc variants include those with substitutions at one or more of Fc region residues: 238, 250, 256, 265, 272, 286, 303, 305, 307, 311, 312, 314, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424, 428, and 434 of an IgG.

[00400] In some embodiments, an antibody provided herein comprises one or more Fc region variants as described in U.S. Pat. Nos. 7,371,826, 5,648,260, and 5,624,821; Duncan and Winter, *Nature*, 1988, 322:738-740; and WO 94/29351; each of which is incorporated by reference in its entirety.

2.8. Pyroglutamate

[00401] As is known in the art, both glutamate (E) and glutamine (Q) at the N-termini of recombinant proteins can cyclize spontaneously to form pyroglutamate (pE) *in vitro* and *in vivo*. *See* Liu *et al.*, *J. Biol. Chem.*, 2011, 286:11211-11217, incorporated by reference in its entirety.

[00402] In some embodiments, provided herein are antibodies comprising a polypeptide sequence having a pE residue at the N-terminal position. In some embodiments, provided herein are antibodies comprising a polypeptide sequence in which the N-terminal residue has been converted from Q to pE. In some embodiments, provided herein are antibodies comprising a polypeptide sequence in which the N-terminal residue has been converted from E to pE.

2.9. Cysteine Engineered Antibody Variants

[00403] In certain embodiments, provided herein are cysteine engineered antibodies, also known as “thioMAbs,” in which one or more residues of the antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at solvent accessible sites of the antibody. By substituting such residues with cysteine, reactive thiol groups are introduced at solvent accessible sites of the antibody and may be used to conjugate

the antibody to other moieties, such as drug moieties or linker-drug moieties, for example, to create an immunoconjugate.

[00404] In certain embodiments, any one or more of the following residues may be substituted with cysteine: V205 of the light chain; A118 of the heavy chain Fc region; and S400 of the heavy chain Fc region. Cysteine engineered antibodies may be generated as described, for example, in U.S. Pat. No. 7,521,541, which is incorporated by reference in its entirety.

3. Comparator Therapies for Treatment of Ocular Diseases

[00405] The antibodies of the present disclosure are useful for the treatment of ocular diseases. In certain embodiments, the antibodies described herein have superior properties (*e.g.*, retinal thickness reduction, macular edema reduction, inflammatory cytokine reduction, etc.) relative to comparator therapies. In some embodiments, a comparator therapy is a reference antibody as described herein. These comparator therapies can be, for example, vascular endothelial growth factor (VEGF) inhibitors, VEGF receptor inhibitors, TF inhibitors, platelet derived growth factor (PDGF) inhibitors or PDGF receptor inhibitors, etc. In certain embodiments, the comparator therapies are anti-TF antibodies, anti-TF drugs, anti-VEGF antibodies, or anti-VEGF drugs. In some embodiments, the comparator therapies are anti-TF proteins or anti-TF immunoconjugates. In some embodiments, the comparator therapies are anti-VEGF proteins or anti-VF immunoconjugates. Herein, when comparisons are made to “anti-TF antibodies, anti-TF drugs, anti-VEGF antibodies, or anti-VEGF drugs” other comparator therapies may be contemplated as well.

[00406] Non-limiting examples of different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug that may be compared to the anti-TF antibodies of the present disclosure include: aflibercept (Eylea), ranibizumab, brolucizumab, pegaptanib, bevacizumab, abicipar pegol, Faricimab (RG7716), KSI-301, hI-con1 (also referred to as “ICON1” or “ICON-1”) and M1593.

[00407] Aflibercept (trade name: Eylea) is a recombinant protein composed of the binding domains of two human vascular endothelial growth factor (VEGF) receptors fused with the Fc region of human immunoglobulin gamma 1 (IgG1). It is a dimeric glycoprotein and is in a class of medications called vascular endothelial growth factor-A (VEGF-A) and placental growth factor (PlGF) antagonists. It functions by stopping abnormal blood vessel growth and leakage in the eye(s). Aflibercept, as an ophthalmic agent, is used in the treatment of macular

edema following Central Retinal Vein Occlusion (CRVO) and neovascular Age-Related Macular Degeneration (AMD). An exemplary sequence for aflibercept is provided below:
 SDTGRPFVEMYSEIPEIIHMTGRELVIPCRVTSPNITVTLKKFPLDTLIPDGKRIIWDSR
 KGFIISNATYKEIGLLTCEATVNGHLYKTNLYLTHRQTNTIIDVVLSPSHGIELSVGEKL
 VLNCTARTELNVGIDFNWEYPSSKHQHKLVNRDLKTQSGSEMKKFLSTLTIDGVTR
 SDQGLYTCAASSGLMTKKNSTFVRVHEKDKTHTCPPCPAPELLGGPSVFLFPPKPKD
 TLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV
 LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ
 VSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQ
 GNVFSCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 930).

[00408] Ranibizumab (Ranibizumab, trade name: Lucentis®) is a monoclonal antibody fragment derived from the same parental murine antibody as bevacizumab (Avastin) that is approved for the treatment of ocular diseases such as wet AMD. However, it has been affinity matured to provide stronger binding to VEGF-A. Because VEGF-A blockade is associated with some systemic toxicity, ranibizumab lacks the Fc portion that reduces serum half-life and thus systemic toxicity. It is indicated for the treatment of macular edema after retinal vein occlusion, age-related macular degeneration (wet), and diabetic macular edema. An exemplary sequences for light chain and heavy chain of Ranibizumab is provided below:

Ranibizumab Light Chain: DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPG
 KAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFLTISLQPEDFATYYCQQYSTVPWTFGQ
 GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNFFYPREAKVQWKVDNALQSG
 NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
 (SEQ ID NO: 931).

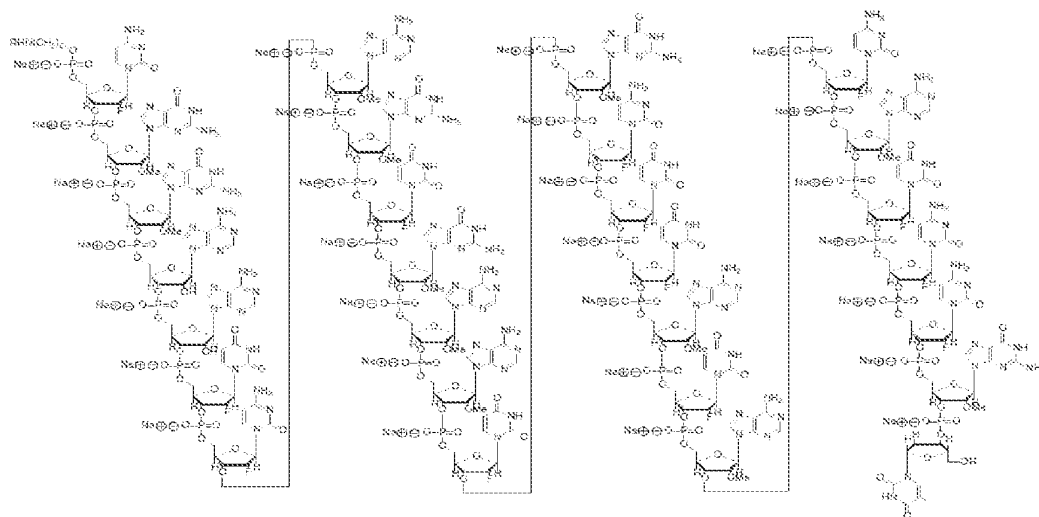
Ranibizumab Heavy Chain: EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVR
 QAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVY
 YCAKYPYYGTSHWYFDVWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGC
 LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNV
 NHKPSNTKVDKKVEPKSCDKTHL (SEQ ID NO: 932).

[00409] Brolucizumab (trade name: Beovu®) is a humanized single chain monoclonal antibody indicated to treat neovascular age related macular degeneration. It is in a class of medications called vascular endothelial growth factor A (VEGF-A) antagonists and has a high affinity to all VEGF-A isoforms. It inhibits the activation of VEGF receptors through the prevention of ligand-receptor interaction resulting in the suppression of endothelial cell

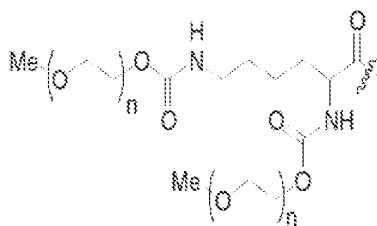
proliferation and vascular permeability. An exemplary sequence for brolucizumab is provided below:

MEIVMTQSPSTLSASVGDRVIITCQASEIIHSWLAWYQQKPGKAPKLLIYLASTLASG
VPSRFSGSGSGAEFTLTISLQPDDEFATYYCQNVYLASTNGANFGQGTKLTVLGGGG
GSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWV
RQAPGKGLEWVGFIDPDDDPYYATWAKGRFTISRDNSKNTLYLQMNSLRAEDTAV
YYCAGGDHNSGWGLDIWGQGTLVTVSS (SEQ ID NO: 933).

[00410] Pegaptanib (also referred to as macugen) is a polynucleotide aptamer. Pegaptanib aids neovascular age-related macular degeneration by binding to VEGF which in order reduces angiogenesis and vessel permeability. It is approved as an aqueous sodium solution for intravitreal injection. Pegaptanib sodium is a covalent conjugate of an oligonucleotide of twenty-eight nucleotides in length that terminates in a pentylamino linker, to which two 20-kilodalton monomethoxy polyethylene glycol (PEG) units are covalently attached via the two amino groups on a lysine residue. An exemplary structural formula for pegaptanib sodium is provided below:



, where R is



[00411] Bevacizumab (trade name: Avastin) was the first antiangiogenic agent introduced to the US market. It is a humanized monoclonal IgG antibody, and inhibits angiogenesis by binding and neutralizing VEGF-A. Bevacizumab is generally indicated for use in combination with different chemotherapy regimens which are specific to the type, severity, and stage of cancer. Its use in ophthalmology application is off-label, and in such cases is used for the treatment of CNV (in AMD and other diseases), diabetic macular edema (DME), and macular edema due to retinal vein occlusions. An exemplary sequence for the light chain and heavy chain of bevacizumab is provided below:

Bevacizumab light chain: DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPG KAPKVLIIYFTSSLHSGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQYSTVPWTFGQ GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVCLLNFPYPREAKVQWKVDNALQSG NSQESVTEQDSKDYSLSTLTLSKADYEEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 934).

Bevacizumab heavy chain: EVQLVESGGGLVQPGGSLRLSCAASGYFTFTNYGMNWVRQ APGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYY CAKYPHYHYGSSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN HKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPGK (SEQ ID NO: 935).

[00412] Abicipar pegol is a designed ankyrin repeat proteins (DARPin) directed to bind all VEGF-A isoforms, similar to ranibizumab. It has a higher affinity and a longer intraocular half-life than ranibizumab (>13 days vs. 7.2 days), making it a potential anti-VEGF therapy with longer duration and need for less frequent injections. (See Moisseiev, E. & Loewenstein, A. *Eye* 2020, 34:605–606, incorporated by reference in its entirety). It is an investigational drug indicated for treatment of macular edema and macular degeneration. An exemplary sequence for abicipar pegol is provided below:

GSDLDDKKLLEAARAGQDDEVRILMANGADVNDSTGWTPHLAAPWGHPEIVEV LLKNGADVNAADFQGWTPHLAAAVGHLEIVEVLLKYGADVNAQDKFGKTAFDISI DNGNEDLAEILQKAAGGGSGGGSC (SEQ ID NO: 936).

[00413] Faricimab (also identified as RG7716) is a bispecific antibody designed specifically for the treatment of retinal eye diseases that simultaneously binds to and

inactivates Angiopoietin-2 (Ang-2) and vascular endothelial growth factor A (VEGF-A). It is an investigational drug indicated for treatment of wet age-related macular degeneration (AMD). An exemplary sequence for faricimab is provided below:

A chain:

QVQLVQSGAEVKKPGASVKVSCKASGYTFTGYYMHWVRQAPGQGLEWMGWINPN
SGGTNYAQKFQGRVTMTRDTSISTAYMELSRLSDDTAVYYCARSPNPYYYDSSGY
YYPGAFDIWGQGTMTVSSASVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK
VQWKVDNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGL
SSPVTKSFNRGEC DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMASRTPEVTCVVVD
VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLAQDWLNGKEY
KCKVSNKALGAPIEK TISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPSDI
AVEWESNGQPENNYKTTTPVLDS DGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALH
NAYTQKSLSLSPGK (SEQ ID NO: 937)

B chain:

EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVRQAPGKGLEWVGWINTY
TGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPYYYGTSHWYF
DVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG
ALTSKVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
CDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMASRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLAQDWLNGKEYKCKVSNKALGA
PIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPE
NNYKTTTPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNAYTQKSLSL
PGK (SEQ ID NO: 938)

C chain:

DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIIYFTSSLHSGVP
SRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTVAAPSVFIF
PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSL
LSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 931)

D chain:

SYVLTQPPSVSVAPGQTARITCGGNNIGSKSVHWYQQKPGQAPVLVYDDSDRPSGI
PERFSGSNSGNTATLTISRVEAGDEADYYCQVWDSSSDHWVFGGGTKLTVLSSASTK
GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGL
YSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC (SEQ ID NO: 939)

[00414] KSI-301 (Kodac Sciences, Palo Alto, CA) is a full-length anti-VEGF antibody stably linked to a biopolymer to create an antibody-biopolymer conjugate (ABC). It uses a large molecular structure to bind to and inhibit vascular endothelial growth factor. The biopolymer's high molecular weight is intended to help retain the drug in the vitreous and enhance durability. It is an investigational drug indicated for treatment of wet AMD, diabetic eye disease, DME, and uveitis.

[00415] ICON1 (also referred to as "hI-con1" or "ICON-1") is an anti-TF fusion protein. It combines a targeting domain with a modified Factor VII, which binds to TF, with an effector domain that can engage Fc bearing cells. The drug is in development for the treatment of patients with wet age-related macular degeneration (AMD), which is characterized by TF overexpression. The sequence for ICON-1 is provided below (underlining indicates the mutant FVII sequence and the bold indicates the Fc hinge region).

ICON-1:

ANAFLEELRPGSLERECKEEQCSFEEAREIFKDAERTKLFWISYSDGDQCASSPCQNG
GSCKDQLQSYICFCLPAFEGRNCETHKDDQLICVNENGGCEQYCSDHTGTRSCRCH
EGYSLLADGVSCTPTVEYPCGKIPILEKRNASKPQGRIVGGKVC PKGEC PWQVLLLV
NGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLI AVLGEHDLSEHDGDEQSRRVAQVI
IPSTYVPGTTNHDIALRLHQPVVLTDHVVPLCLPERTFSERTLAFVRFSLVSGWGQL
LDRGATALELMVLNVPRLMTQDCLQQSRKVGDS PNITEYMF CAGYSDGSKDSCAG
DSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKL MRSEPRP
GVLLRAPFP**GSAEPKSCDKTHTC****PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC**
 VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN
 GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGF
 YPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSV M
 HEALHNHYTQKSLSLSPGK (SEQ ID NO: 940)

[00416] ICON-1 is described in US Application No. 15/746,545, which is incorporated by reference in its entirety for all purposes.

[00417] M1593 is an anti-TF monoclonal antibody. (See US Application No.: 14/230,128, which is incorporated by reference in its entirety). The sequences for its light and heavy chains are provided below:

M1593 light chain:

DIVMTQTPLSLPVTGPGEPAISCKSSQSLSSGNQKNYLTWYLQKPGQSPQLLIYWA
 STRESGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCQNDYTYPLTFGQGKLEIKR
 TVAAPS VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVT

EQDSKDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 941)

M1593 heavy chain:

EVQLVQSGAEVKKPGESLRISCKGSGYTFAPYWIEWVRQMPGKGLEWMGDILPGT
GFTTYSFSFQGHVTISADKSISTAYLQWSSLKASDTAMYYCARSGYYGNSGFAYWG
QGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK
THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE
KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN
YKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSVSMHEALHNHYTQKSLSLSPG
K (SEQ ID NO: 942)

[00418] In certain embodiments, the antibodies of present disclosure can be administered at higher doses than comparator therapies (*e.g.*, those disclosed herein) and result in a better safety profile than the comparator therapies administered at the same dose or a lower dose. For example, in some embodiments, upon administration to a subject, the antibody results in less severe adverse ocular changes relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug, wherein the adverse ocular changes comprise the presence of one or more of: inflammation in the anterior and/or posterior chambers of the eye, anterior chamber cells, flare and fibrin, keratic precipitates/corneal endothelial cells, cellular deposits on the anterior lens capsule, incomplete pupil dilation, cells on corneal endothelium and lens capsule, vitreous haze, vitreous cells, retinal sheathing, and chorio-retinal hemorrhages.

[00419] In some embodiments, upon administration to a subject, the antibody results in reduced mononuclear cell infiltration than comparator drugs administered at the same dose or a lower dose than the antibody. This mononuclear inflammation may be in the vitreous humor, aqueous humor, retinal tissues, or other tissues within the eye(s). Macrophages play a role in inflammation and in angiogenesis. (*see Jager MJ, J Ophthalmic Vis Res.* 2014;9(1):1-2, which is incorporated by reference in its entirety). In some embodiments, the predominant type of macrophage is M2 macrophages. In such cases, in some embodiments, upon administration to a subject, the antibody results in reduced infiltration of M2 macrophages than comparator drugs.

4. Ophthalmologic Examinations and Measurements

[00420] Ophthalmologic examinations can be conducted on subjects before and after administration of an antibody provided herein. Various tests/examinations that can be included in an ophthalmologic examination are exemplified in the Example section. For example, an ophthalmologic examination may include best-corrected visual acuity testing with Early Treatment Diabetic Retinopathy Study (ETDRS) charts, tonometry (to measure intraocular pressure), slit-lamp biomicroscopy, and fundus examination. Additionally, it may include measurement of ocular tissue thickness values (e.g., macular thickness, central retinal thickness, foveal thickness, choroidal thickness, central retinal subfield thickness (CST), center point thickness (CPT), or central foveal thickness (CFT)). Typically the central area (the macula) of the retina is of interest in ocular diseases (e.g., AMD and DME). An average of this central 1000 μm retina is assessed, the central retina thickness (CRT), and fovea is at the centerpoint of this area. For example, to measure ocular tissue thickness, following pupil dilation, a subject undergoes spectral domain optical coherence tomography (SD OCT) imaging by the Spectralis (Heidelberg Engineering Germany, Version 1.6.4.0), which uses an internal fixation source and centers on the patient's fovea. Retinal thickness in the 9 ETDRS subfields can be analyzed by the RT map analysis protocol. Choroidal thickness can be measured with EDI scans in the center of each ETDRS subfield. Methods for calculating retinal thickness, choroidal thickness, central foveal subfield thickness, central point thickness, *etc.* are known in the art and are provided, for example, in Grover, Sandeep, et al., *Investigative ophthalmology & visual science* 51.5 (2010): 2644-2647; Géhl, Zsuzsanna, et al., *BMC ophthalmology* 14.1 (2014): 103; and Jacobsen, Agnes Galbo, et al., *Investigative Ophthalmology & Visual Science* 56.6 (2015): 3875-3882, each of which are incorporated by reference in their entirety. Examples of normal ocular tissue thickness values (in healthy eyes) in humans are provided in Chan, Annie, et al., *Archives of ophthalmology* 124.2 (2006): 193-198 and Mitkova-Hristova, V. T., & Konareva-Kostyanova, M. I. (2011), *Folia medica*, 53(4), 28-33, each of which are incorporated by reference in their entirety. For example, normal human CRT averages are approximately 200-270 μM .

[00421] In certain embodiments, an ocular disease is known to increase ocular tissue thickness. For example, in the presence of exudative disease (e.g., AMD, DME), the CRT values can be above 300-500 μM . In such cases, upon administration of an antibody provided herein, the ocular tissue thickness (e.g. CRT) is reduced relative to a comparator therapy and/or baseline levels. In some cases, upon administration of an antibody provided herein, the ocular tissue thickness (e.g. CRT) is restored to normal (non-diseased) thickness values.

[00422] In certain embodiments, an ocular disease is known to reduce ocular tissue thickness. For example, in the presence of atrophic disease of the macula the thickness can decrease below 200 μM . In such cases, upon administration of an antibody provided herein, the ocular tissue thickness (e.g. CRT) is increased relative to a comparator therapy and/or baseline levels. In some cases, upon administration of an antibody provided herein, the ocular tissue thickness (e.g. CRT) is restored to normal (non-diseased) thickness values.

[00423] In certain embodiments, an antibody provided herein is administered by IVT injection and the IOP is measured immediately prior to administration (to establish baseline levels) and following administration (e.g., approximately 30 minutes after treatment). The IOP measurement prior to treatment can be bilateral (measurement in both eyes), regardless of whether both eyes will receive the IVT injection. The IOP measurement(s) following treatment can be in the test eye only (*i.e.* the eye receiving IVT injection). In certain embodiments, the IOP in the eye of a subject is measured about 20 minutes, about 30 minutes, about 40 minutes, about 50 minutes or about 1 hour prior to administration.

[00424] In humans, normal intraocular pressure ranges from 12-22 mmHg (Glaucoma Research Foundation, “High Eye Pressure and Glaucoma”, October 2017). Generally, increases in IOP are expected immediately following IVT injections and are usually resolved within minutes after the injection (e.g., about 20, 22, 24, 26, 28, 30, 32, 34, 36, 38 or 40 minutes after the IVT injection). Typically, this temporary increase in IOP after an IVT injection (e.g., of 50-100 μl) is resolved and the majority of patients leave the office with the IOP returning to pre-injection values. Decrease in intraocular pressure after an IVT are not common and may be indicative of leaking or inflammation.

[00425] In certain embodiments, upon administration to a subject, the antibody provided herein does not significantly alter IOP. As used, “significantly alter” refers to a change in IOP levels other than that expected within the first 30 minutes following IVT injection. In cases where IOP is not “significantly altered” the IOP levels are restored to pre-injection levels in less than, for example, 10 minutes, 12 minutes, 14 minutes, 16 minutes, 18 minutes, 20 minutes, 22 minutes, 24 minutes, 26 minutes, 28 minutes, 30 minutes, 32 minutes, 34 minutes, 36 minutes, 38 minutes or 40 minutes.

5. Inflammatory Cytokines and Chemokines

[00426] In certain embodiments, upon administration to a subject, the antibody provided herein result in reduced or absent biomarkers of inflammation relative to baseline levels or a comparator therapy (e.g., a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or

anti-VEGF drug). Examples of these biomarkers of inflammation include inflammatory cytokines and chemokines (also referred to as proinflammatory cytokines and chemokines), which are proteins secreted from immune cells to promote inflammation either systemically or locally. Non-limiting examples of inflammatory cytokines and chemokines that are reduced upon administration of the antibodies provided herein, relative to baseline levels or a comparator therapy, include: IL-1 beta, IL-6, IL-8, IL-9, IL-10, IL-12, MCP-1 (CCL2), MCP-3 (CCL7), VEGF, TNF, and MMP9. These are examples of cytokines that are elevated in cases of subjects suffering from ocular diseases such as AMD. In certain embodiments, upon administration to a subject, the antibody provided herein result in reduced or absent inflammatory cytokine relative to baseline levels or a comparator therapy, wherein the inflammatory cytokine is associated with the diseased state for an ocular disease, or wherein the inflammatory cytokine is known to be elevated in a subject having an ocular disease.

[00427] The biomarkers of inflammation (*e.g.*, inflammatory cytokines, chemokines, and other inflammatory mediators) can be measured using immunohistochemistry, the assays for which are known in the art and are exemplified elsewhere in this disclosure. Biomarkers of inflammation can also be measure in samples using ELISA or various Olink panels (*e.g.* custom Olink panels). Alternatively, the expression levels for biomarkers of inflammation (*e.g.*, inflammatory cytokines) in ocular tissues or samples can be measured using RT-PCR.

6. Methods for Making TF Antibodies

6.1. TF Antigen Preparation

[00428] The TF antigen used for isolation of the antibodies provided herein may be intact TF or a fragment of TF. The TF antigen may be, for example, in the form of an isolated protein or a protein expressed on the surface of a cell.

[00429] In some embodiments, the TF antigen is a non-naturally occurring variant of TF, such as a TF protein having an amino acid sequence or post-translational modification that does not occur in nature.

[00430] In some embodiments, the TF antigen is truncated by removal of, for example, intracellular or membrane-spanning sequences, or signal sequences. In some embodiments, the TF antigen is fused at its C-terminus to a human IgG1 Fc domain or a polyhistidine tag.

6.2. Methods of Making Monoclonal Antibodies

[00431] Monoclonal antibodies may be obtained, for example, using the hybridoma method first described by Kohler *et al.*, *Nature*, 1975, 256:495-497 (incorporated by

reference in its entirety), and/or by recombinant DNA methods (*see e.g.*, U.S. Patent No. 4,816,567, incorporated by reference in its entirety). Monoclonal antibodies may also be obtained, for example, using phage-display libraries (*see e.g.*, U.S. Patent No. 8,258,082, which is incorporated by reference in its entirety) or, alternatively, using yeast-based libraries (*see e.g.*, U.S. Patent No. 8,691,730, which is incorporated by reference in its entirety).

[00432] In the hybridoma method, a mouse or other appropriate host animal is immunized to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes are then fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. *See Goding J.W., Monoclonal Antibodies: Principles and Practice* 3rd ed. (1986) Academic Press, San Diego, CA, incorporated by reference in its entirety.

[00433] The hybridoma cells are seeded and grown in a suitable culture medium that contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

[00434] Useful myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive media conditions, such as the presence or absence of HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOP-21 and MC-11 mouse tumors (available from the Salk Institute Cell Distribution Center, San Diego, CA), and SP-2 or X63-Ag8-653 cells (available from the American Type Culture Collection, Rockville, MD). Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies. *See e.g.*, Kozbor, *J. Immunol.*, 1984, 133:3001, incorporated by reference in its entirety.

[00435] After the identification of hybridoma cells that produce antibodies of the desired specificity, affinity, and/or biological activity, selected clones may be subcloned by limiting dilution procedures and grown by standard methods. *See Goding, supra*. Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

[00436] DNA encoding the monoclonal antibodies may be readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of

binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). Thus, the hybridoma cells can serve as a useful source of DNA encoding antibodies with the desired properties. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as bacteria (*e.g.*, *E. coli*), yeast (*e.g.*, *Saccharomyces* or *Pichia sp.*), COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce antibody, to produce the monoclonal antibodies.

6.3. Methods of Making Chimeric Antibodies

[00437] Illustrative methods of making chimeric antibodies are described, for example, in U.S. Pat. No. 4,816,567; and Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*, 1984, 81:6851-6855; each of which is incorporated by reference in its entirety. In some embodiments, a chimeric antibody is made by using recombinant techniques to combine a non-human variable region (*e.g.*, a variable region derived from a mouse, rat, hamster, rabbit, or non-human primate, such as a monkey) with a human constant region.

6.4. Methods of Making Humanized Antibodies

[00438] Humanized antibodies may be generated by replacing most, or all, of the structural portions of a non-human monoclonal antibody with corresponding human antibody sequences. Consequently, a hybrid molecule is generated in which only the antigen-specific variable, or CDR, is composed of non-human sequence. Methods to obtain humanized antibodies include those described in, for example, Winter and Milstein, *Nature*, 1991, 349:293-299; Rader *et al.*, *Proc. Nat. Acad. Sci. U.S.A.*, 1998, 95:8910-8915; Steinberger *et al.*, *J. Biol. Chem.*, 2000, 275:36073-36078; Queen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1989, 86:10029-10033; and U.S. Patent Nos. 5,585,089, 5,693,761, 5,693,762, and 6,180,370; each of which is incorporated by reference in its entirety.

6.5. Methods of Making Human Antibodies

[00439] Human antibodies can be generated by a variety of techniques known in the art, for example by using transgenic animals (*e.g.*, humanized mice). *See, e.g.*, Jakobovits *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1993, 90:2551; Jakobovits *et al.*, *Nature*, 1993, 362:255-258; Bruggermann *et al.*, *Year in Immuno.*, 1993, 7:33; and U.S. Patent Nos. 5,591,669, 5,589,369 and 5,545,807; each of which is incorporated by reference in its entirety. Human antibodies can also be derived from phage-display libraries (*see e.g.*, Hoogenboom *et al.*, *J. Mol. Biol.*, 1991, 227:381-388; Marks *et al.*, *J. Mol. Biol.*, 1991, 222:581-597; and U.S. Pat. Nos. 5,565,332 and 5,573,905; each of which is incorporated by reference in its entirety). Human antibodies may also be generated by *in vitro* activated B cells (*see e.g.*, U.S. Patent. Nos.

5,567,610 and 5,229,275, each of which is incorporated by reference in its entirety). Human antibodies may also be derived from yeast-based libraries (*see e.g.*, U.S. Patent No. 8,691,730, incorporated by reference in its entirety).

6.6. Methods of Making Antibody Fragments

[00440] The antibody fragments provided herein may be made by any suitable method, including the illustrative methods described herein or those known in the art. Suitable methods include recombinant techniques and proteolytic digestion of whole antibodies. Illustrative methods of making antibody fragments are described, for example, in Hudson *et al.*, *Nat. Med.*, 2003, 9:129-134, incorporated by reference in its entirety. Methods of making scFv antibodies are described, for example, in Plückthun, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994); WO 93/16185; and U.S. Pat. Nos. 5,571,894 and 5,587,458; each of which is incorporated by reference in its entirety.

6.7. Methods of Making Alternative Scaffolds

[00441] The alternative scaffolds provided herein may be made by any suitable method, including the illustrative methods described herein or those known in the art. For example, methods of preparing AdnectinsTM are described in Emanuel *et al.*, *mAbs*, 2011, 3:38-48, incorporated by reference in its entirety. Methods of preparing iMabs are described in U.S. Pat. Pub. No. 2003/0215914, incorporated by reference in its entirety. Methods of preparing Anticalins[®] are described in Vogt and Skerra, *Chem. Biochem.*, 2004, 5:191-199, incorporated by reference in its entirety. Methods of preparing Kunitz domains are described in Wagner *et al.*, *Biochem. & Biophys. Res. Comm.*, 1992, 186:118-1145, incorporated by reference in its entirety. Methods of preparing thioredoxin peptide aptamers are provided in Geyer and Brent, *Meth. Enzymol.*, 2000, 328:171-208, incorporated by reference in its entirety. Methods of preparing Affibodies are provided in Fernandez, *Curr. Opinion in Biotech.*, 2004, 15:364-373, incorporated by reference in its entirety. Methods of preparing DARPins are provided in Zahnd *et al.*, *J. Mol. Biol.*, 2007, 369:1015-1028, incorporated by reference in its entirety. Methods of preparing Affilins are provided in Ebersbach *et al.*, *J. Mol. Biol.*, 2007, 372:172-185, incorporated by reference in its entirety. Methods of preparing Tetranectins are provided in Graversen *et al.*, *J. Biol. Chem.*, 2000, 275:37390-37396, incorporated by reference in its entirety. Methods of preparing Avimers are provided in Silverman *et al.*, *Nature Biotech.*, 2005, 23:1556-1561, incorporated by reference in its

entirety. Methods of preparing Fynomers are provided in Silacci *et al.*, *J. Biol. Chem.*, 2014, 289:14392-14398, incorporated by reference in its entirety.

[00442] Further information on alternative scaffolds is provided in Binz *et al.*, *Nat. Biotechnol.*, 2005 23:1257-1268; and Skerra, *Current Opin. in Biotech.*, 2007 18:295-304, each of which is incorporated by reference in its entirety.

6.8. Methods of Making Multispecific Antibodies

[00443] The multispecific antibodies provided herein may be made by any suitable method, including the illustrative methods described herein or those known in the art. Methods of making common light chain antibodies are described in Merchant *et al.*, *Nature Biotechnol.*, 1998, 16:677-681, incorporated by reference in its entirety. Methods of making tetravalent bispecific antibodies are described in Coloma and Morrison, *Nature Biotechnol.*, 1997, 15:159-163, incorporated by reference in its entirety. Methods of making hybrid immunoglobulins are described in Milstein and Cuello, *Nature*, 1983, 305:537-540; and Staerz and Bevan, *Proc. Natl. Acad. Sci. USA*, 1986, 83:1453-1457; each of which is incorporated by reference in its entirety. Methods of making immunoglobulins with knobs-into-holes modification are described in U.S. Pat. No. 5,731,168, incorporated by reference in its entirety. Methods of making immunoglobulins with electrostatic modifications are provided in WO 2009/089004, incorporated by reference in its entirety. Methods of making bispecific single chain antibodies are described in Traunecker *et al.*, *EMBO J.*, 1991, 10:3655-3659; and Gruber *et al.*, *J. Immunol.*, 1994, 152:5368-5374; each of which is incorporated by reference in its entirety. Methods of making single-chain antibodies, whose linker length may be varied, are described in U.S. Pat. Nos. 4,946,778 and 5,132,405, each of which is incorporated by reference in its entirety. Methods of making diabodies are described in Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 1993, 90:6444-6448, incorporated by reference in its entirety. Methods of making triabodies and tetrabodies are described in Todorovska *et al.*, *J. Immunol. Methods*, 2001, 248:47-66, incorporated by reference in its entirety. Methods of making trispecific F(ab')₃ derivatives are described in Tutt *et al.*, *J. Immunol.*, 1991, 147:60-69, incorporated by reference in its entirety. Methods of making cross-linked antibodies are described in U.S. Patent No. 4,676,980; Brennan *et al.*, *Science*, 1985, 229:81-83; Staerz, *et al. Nature*, 1985, 314:628-631; and EP 0453082; each of which is incorporated by reference in its entirety. Methods of making antigen-binding domains assembled by leucine zippers are described in Kostelny *et al.*, *J. Immunol.*, 1992, 148:1547-1553, incorporated by reference in its entirety. Methods of making antibodies via the DNL

approach are described in U.S. Pat. Nos. 7,521,056; 7,550,143; 7,534,866; and 7,527,787; each of which is incorporated by reference in its entirety. Methods of making hybrids of antibody and non-antibody molecules are described in WO 93/08829, incorporated by reference in its entirety, for examples of such antibodies. Methods of making DAF antibodies are described in U.S. Pat. Pub. No. 2008/0069820, incorporated by reference in its entirety. Methods of making antibodies via reduction and oxidation are described in Carlring *et al.*, *PLoS One*, 2011, 6:e22533, incorporated by reference in its entirety. Methods of making DVD-IgsTM are described in U.S. Pat. No. 7,612,181, incorporated by reference in its entirety. Methods of making DARTsTM are described in Moore *et al.*, *Blood*, 2011, 117:454-451, incorporated by reference in its entirety. Methods of making DuoBodies[®] are described in Labrijn *et al.*, *Proc. Natl. Acad. Sci. USA*, 2013, 110:5145-5150; Gramer *et al.*, *mAbs*, 2013, 5:962-972; and Labrijn *et al.*, *Nature Protocols*, 2014, 9:2450-2463; each of which is incorporated by reference in its entirety. Methods of making antibodies comprising scFvs fused to the C-terminus of the CH₃ from an IgG are described in Coloma and Morrison, *Nature Biotechnol.*, 1997, 15:159-163, incorporated by reference in its entirety. Methods of making antibodies in which a Fab molecule is attached to the constant region of an immunoglobulin are described in Miler *et al.*, *J. Immunol.*, 2003, 170:4854-4861, incorporated by reference in its entirety. Methods of making CovX-Bodies are described in Doppalapudi *et al.*, *Proc. Natl. Acad. Sci. USA*, 2010, 107:22611-22616, incorporated by reference in its entirety. Methods of making Fcab antibodies are described in Wozniak-Knopp *et al.*, *Protein Eng. Des. Sel.*, 2010, 23:289-297, incorporated by reference in its entirety. Methods of making TandAb[®] antibodies are described in Kipriyanov *et al.*, *J. Mol. Biol.*, 1999, 293:41-56 and Zhukovsky *et al.*, *Blood*, 2013, 122:5116, each of which is incorporated by reference in its entirety. Methods of making tandem Fabs are described in WO 2015/103072, incorporated by reference in its entirety. Methods of making ZybodiesTM are described in LaFleur *et al.*, *mAbs*, 2013, 5:208-218, incorporated by reference in its entirety.

6.9. Methods of Making Variants

[00444] In some embodiments, an antibody provided herein is an affinity matured variant of a parent antibody, which may be generated, for example, using phage display-based affinity maturation techniques. Briefly, one or more CDR residues may be mutated and the variant antibodies, or portions thereof, displayed on phage and screened for affinity. Such alterations may be made in CDR “hotspots,” or residues encoded by codons that undergo

mutation at high frequency during the somatic maturation process (*see* Chowdhury, *Methods Mol. Biol.*, 2008, 207:179-196, incorporated by reference in its entirety), and/or residues that contact the antigen.

[00445] Any suitable method can be used to introduce variability into a polynucleotide sequence(s) encoding an antibody, including error-prone PCR, chain shuffling, and oligonucleotide-directed mutagenesis such as trinucleotide-directed mutagenesis (TRIM). In some aspects, several CDR residues (*e.g.*, 4-6 residues at a time) are randomized. CDR residues involved in antigen binding may be specifically identified, for example, using alanine scanning mutagenesis or modeling. CDR-H3 and CDR-L3 in particular are often targeted for mutation.

[00446] The introduction of diversity into the variable regions and/or CDRs can be used to produce a secondary library. The secondary library is then screened to identify antibody variants with improved affinity. Affinity maturation by constructing and reselecting from secondary libraries has been described, for example, in Hoogenboom *et al.*, *Methods in Molecular Biology*, 2001, 178:1-37, incorporated by reference in its entirety.

6.10. Vectors, Host Cells, and Recombinant Methods

[00447] Also provided are isolated nucleic acids encoding TF antibodies, vectors comprising the nucleic acids, and host cells comprising the vectors and nucleic acids, as well as recombinant techniques for the production of the antibodies.

[00448] For recombinant production of an antibody, the nucleic acid(s) encoding it may be isolated and inserted into a replicable vector for further cloning (*i.e.*, amplification of the DNA) or expression. In some aspects, the nucleic acid may be produced by homologous recombination, for example as described in U.S. Patent No. 5,204,244, incorporated by reference in its entirety.

[00449] Many different vectors are known in the art. The vector components generally include one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, for example as described in U.S. Patent No. 5,534,615, incorporated by reference in its entirety.

[00450] Illustrative examples of suitable host cells are provided below. These host cells are not meant to be limiting, and any suitable host cell may be used to produce the antibodies provided herein.

[00451] Suitable host cells include any prokaryotic (*e.g.*, bacterial), lower eukaryotic (*e.g.*, yeast), or higher eukaryotic (*e.g.*, mammalian) cells. Suitable prokaryotes include eubacteria,

such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia* (*E. coli*), *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella* (*S. typhimurium*), *Serratia* (*S. marcescans*), *Shigella*, *Bacilli* (*B. subtilis* and *B. licheniformis*), *Pseudomonas* (*P. aeruginosa*), and *Streptomyces*. One useful *E. coli* cloning host is *E. coli* 294, although other strains such as *E. coli* B, *E. coli* X1776, and *E. coli* W3110 are also suitable.

[00452] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are also suitable cloning or expression hosts for TF antibody-encoding vectors.

Saccharomyces cerevisiae, or common baker's yeast, is a commonly used lower eukaryotic host microorganism. However, a number of other genera, species, and strains are available and useful, such as *Schizosaccharomyces pombe*, *Kluyveromyces* (*K. lactis*, *K. fragilis*, *K. bulgaricus*, *K. wickerhamii*, *K. waltii*, *K. drosophilum*, *K. thermotolerans*, and *K. marxianus*), *Yarrowia*, *Pichia pastoris*, *Candida* (*C. albicans*), *Trichoderma reesia*, *Neurospora crassa*, *Schwanniomyces* (*S. occidentalis*), and filamentous fungi such as, for example *Penicillium*, *Tolypocladium*, and *Aspergillus* (*A. nidulans* and *A. niger*).

[00453] Useful mammalian host cells include COS-7 cells, HEK293 cells, baby hamster kidney (BHK) cells, Chinese hamster ovary (CHO), mouse sertoli cells, African green monkey kidney cells (VERO-76), and the like.

[00454] The host cells used to produce the TF antibody of this invention may be cultured in a variety of media. Commercially available media such as, for example, Ham's F10, Minimal Essential Medium (MEM), RPMI-1640, and Dulbecco's Modified Eagle's Medium (DMEM) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al.*, *Meth. Enz.*, 1979, 58:44; Barnes *et al.*, *Anal. Biochem.*, 1980, 102:255; and U.S. Patent Nos. 4,767,704, 4,657,866, 4,927,762, 4,560,655, and 5,122,469; or WO 90/03430 and WO 87/00195 may be used. Each of the foregoing references is incorporated by reference in its entirety.

[00455] Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics, trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art.

[00456] The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

[00457] When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. For example, Carter *et al.* (*Bio/Technology*, 1992, 10:163-167, incorporated by reference in its entirety) describes a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation.

[00458] In some embodiments, the antibody is produced in a cell-free system. In some aspects, the cell-free system is an *in vitro* transcription and translation system as described in Yin *et al.*, *mAbs*, 2012, 4:217-225, incorporated by reference in its entirety. In some aspects, the cell-free system utilizes a cell-free extract from a eukaryotic cell or from a prokaryotic cell. In some aspects, the prokaryotic cell is *E. coli*. Cell-free expression of the antibody may be useful, for example, where the antibody accumulates in a cell as an insoluble aggregate, or where yields from periplasmic expression are low.

[00459] Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon[®] or Millipore[®] Pellicon[®] ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

[00460] The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being a particularly useful purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that comprise human $\gamma 1$, $\gamma 2$, or $\gamma 4$ heavy chains (Lindmark *et al.*, *J. Immunol. Meth.*, 1983, 62:1-13, incorporated by reference in its entirety). Protein G is useful for all mouse isotypes and for human $\gamma 3$ (Guss *et al.*, *EMBO J.*, 1986, 5:1567-1575, incorporated by reference in its entirety).

[00461] The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_{H3} domain, the BakerBond ABX[®] resin is useful for purification.

[00462] Other techniques for protein purification, such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin Sepharose[®], chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available, and can be applied by one of skill in the art.

[00463] Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5 to about 4.5, generally performed at low salt concentrations (e.g., from about 0 to about 0.25 M salt).

7. Assays

[00464] A variety of assays known in the art may be used to identify and characterize anti-TF antibodies provided herein.

7.1. Binding, Competition, and Epitope Mapping Assays

[00465] Specific antigen-binding activity of the antibodies provided herein may be evaluated by any suitable method, including using SPR, BLI, RIA and MSD-SET, as described elsewhere in this disclosure. Additionally, antigen-binding activity may be evaluated by ELISA assays and Western blot assays.

[00466] Assays for measuring competition between two antibodies, or an antibody and another molecule (e.g., one or more ligands of TF) are described elsewhere in this disclosure and, for example, in Harlow and Lane, *Antibodies: A Laboratory Manual* ch.14, 1988, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., incorporated by reference in its entirety.

[00467] Assays for mapping the epitopes to which the antibodies provided herein bind are described, for example, in Morris "Epitope Mapping Protocols," in *Methods in Molecular Biology* vol. 66, 1996, Humana Press, Totowa, N.J., incorporated by reference in its entirety. In some embodiments, the epitope is determined by peptide competition. In some embodiments, the epitope is determined by mass spectrometry. In some embodiments, the epitope is determined by crystallography.

7.2. Thrombin Generation, FXa Conversion, and TF Signaling Assays

[00468] Thrombin generation in the presence of the antibodies provided herein can be determined by the Thrombin Generation Assay (TGA), as described elsewhere in this disclosure.

[00469] Assays for measuring FXa conversion in the presence of the antibodies provided herein are described elsewhere in this disclosure.

[00470] Inhibition of TF signaling can be determined by measuring the production of a cytokine regulated by the TF signaling, such as IL8 and GM-CSF. Assays for determining the IL8 and/or GM-CSF level are provided elsewhere in this disclosure and, for example, in Hjortoe *et al.*, *Blood*, 2004, 103:3029-3037.

7.3. Assays for Effector Functions

[00471] Effector function following treatment with the antibodies provided herein may be evaluated using a variety of *in vitro* and *in vivo* assays known in the art, including those described in Ravetch and Kinet, *Annu. Rev. Immunol.*, 1991, 9:457-492; U.S. Pat. Nos. 5,500,362, 5,821,337; Hellstrom *et al.*, *Proc. Nat'l Acad. Sci. USA*, 1986, 83:7059-7063; Hellstrom *et al.*, *Proc. Nat'l Acad. Sci. USA*, 1985, 82:1499-1502; Bruggemann *et al.*, *J. Exp. Med.*, 1987, 166:1351-1361; Clynes *et al.*, *Proc. Nat'l Acad. Sci. USA*, 1998, 95:652-656; WO 2006/029879; WO 2005/100402; Gazzano-Santoro *et al.*, *J. Immunol. Methods*, 1996, 202:163-171; Cragg *et al.*, *Blood*, 2003, 101:1045-1052; Cragg *et al.* *Blood*, 2004, 103:2738-2743; and Petkova *et al.*, *Int'l. Immunol.*, 2006, 18:1759-1769; each of which is incorporated by reference in its entirety.

7.4. Immunohistochemistry (IHC) Assays

[00472] Immunohistochemistry (IHC) assays can be used to evaluate expression and localization of TF and of proinflammatory markers in patient or animal samples. IHC assays for examining animal tissue (*e.g.* ocular tissue) are known in the art. For example, IHC can be used to evaluate the TF expression in patient or animal samples as described in PCT application PCT/US2019/012427 or US utility application number 16/959,652, each of which is incorporated by reference in its entirety. Methods for conducting IHC on ocular tissue (*e.g.*, retinal tissue) are also provided in Zalis MC *et al.*, *J Histochem Cytochem.* 2017;65(4):223-239 and Fitch, J.M. and Linsenmayer T.F., *Developmental biology* 95.1 (1983): 137-153., each of which are incorporated by reference in their entirety. Fixation strategies to improve cell shape preservation and quality of immunostains are provided in Stradleigh, T.W. and Ishida A.T., *Progress in retinal and eye research* 48 (2015): 181-202, incorporated by reference in its entirety.

7.5. Chimeric Construct Mapping and Epitope Binning Assays

[00473] Epitope binding differences between the anti-human TF antibodies provided herein can be determined by the chimeric TF construct mapping experiments and the epitope binning assays, as described elsewhere in this disclosure.

8. Pharmaceutical Compositions

[00474] The antibodies provided herein can be formulated in any appropriate pharmaceutical composition and administered by any suitable route of administration. Suitable routes of administration include, but are not limited to, the intravitreal, subretinal, suprachoroidal, intraarterial, intradermal, intramuscular, intraperitoneal, intravenous, nasal, parenteral, topical, pulmonary, and subcutaneous routes.

[00475] The pharmaceutical composition may comprise one or more pharmaceutical excipients. Any suitable pharmaceutical excipient may be used, and one of ordinary skill in the art is capable of selecting suitable pharmaceutical excipients. Accordingly, the pharmaceutical excipients provided below are intended to be illustrative, and not limiting. Additional pharmaceutical excipients include, for example, those described in the *Handbook of Pharmaceutical Excipients*, Rowe *et al.* (Eds.) 6th Ed. (2009), incorporated by reference in its entirety.

8.1. Parenteral Dosage Forms

[00476] In certain embodiments, the antibodies provided herein are formulated as parenteral dosage forms. Parenteral dosage forms can be administered to subjects by various routes including, but not limited to, subcutaneous, intravenous (including infusions and bolus injections), intramuscular, and intraarterial. Because their administration typically bypasses subjects' natural defenses against contaminants, parenteral dosage forms are typically, sterile or capable of being sterilized prior to administration to a subject. Examples of parenteral dosage forms include, but are not limited to, solutions ready for injection, dry (*e.g.*, lyophilized) products ready to be dissolved or suspended in a pharmaceutically acceptable vehicle for injection, suspensions ready for injection, and emulsions.

9. Dosage and Unit Dosage Forms

[00477] In human therapeutics, the doctor will determine the posology which he considers most appropriate according to a preventive or curative treatment and according to the age, weight, condition and other factors specific to the subject to be treated.

[00478] In certain embodiments, a composition provided herein is a pharmaceutical composition or a single unit dosage form. Pharmaceutical compositions and single unit dosage forms provided herein comprise a prophylactically or therapeutically effective amount of one or more prophylactic or therapeutic antibodies.

[00479] The amount of the antibody or composition which will be effective in the prevention or treatment of a disorder or one or more symptoms thereof can vary with the nature and severity of the disease or condition, and the route by which the antibody is administered. The frequency and dosage can also vary according to factors specific for each subject depending on the specific therapy (*e.g.*, therapeutic or prophylactic agents) administered, the severity of the disorder, disease, or condition, the route of administration, as well as age, body, weight, response, and the past medical history of the subject. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

[00480] Different therapeutically effective amounts may be applicable for different diseases and conditions, as will be readily known by those of ordinary skill in the art. Similarly, amounts sufficient to prevent, manage, treat or ameliorate such disorders, but insufficient to cause, or sufficient to reduce, adverse effects associated with the antibodies provided herein are also encompassed by the dosage amounts and dose frequency schedules provided herein. Further, when a subject is administered multiple dosages of a composition provided herein, not all of the dosages need be the same. For example, the dosage administered to the subject may be increased to improve the prophylactic or therapeutic effect of the composition or it may be decreased to reduce one or more side effects that a particular subject is experiencing.

[00481] As discussed in more detail elsewhere in this disclosure, an antibody provided herein may optionally be administered with one or more additional agents useful to prevent or treat a disease or disorder. The effective amount of such additional agents may depend on the amount of antibody present in the formulation, the type of disorder or treatment, and the other factors known in the art or described herein.

[00482] In certain embodiments, the antibodies provided herein are administered by intravitreal injection and are administered once every month, two months, three months, four months or more. In preferred embodiments, when the antibodies provided herein are administered by intravitreal injection, they are administered no more than once a month.

10. Therapeutic Applications

[00483] For therapeutic applications, the antibodies of the invention are administered to a subject, generally a mammal, generally a human, in a pharmaceutically acceptable dosage form such as those known in the art and those discussed above. For example, the antibodies of the invention may be administered to a subject intravenously as a bolus or by continuous infusion over a period of time, by intravitreal, subretinal, suprachoroidal, intramuscular, intraperitoneal, intra-cerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, intratumoral, or topical routes. In preferred methods, the antibodies are administered to a subject by intravitreal routes (*e.g.*, intravitreal injection). In certain embodiments, administration is via intravenous, intramuscular, intratumoral, subcutaneous, intrasynovial, intraocular, intraplaque, or intradermal injection of the antibody or of an expression vector having cDNA encoding the antibody. The vector can be a replication-deficient adenoviral vector, retroviral vector or other viral vectors carrying a cDNA encoding the antibody.

[00484] In some embodiments, the patient is treated by intravenous or intratumoral injection of an effective amount of one or more replication-deficient adenoviral vectors, or one or more adeno-associated vectors carrying cDNA encoding the antibody.

[00485] An intravitreal injection is a type of intraocular injection. It is a procedure to place a medication directly into the space in the back of the eye called the vitreous cavity, which is filled with a jelly-like fluid called the vitreous humor gel. The procedure is typically performed by a medical profession (*e.g.*, physician or trained retina specialist) in an office setting.

[00486] The intravitreal administration route presents challenges for preparing suitable compositions (*e.g.*, compositions that include acceptable levels of active agent and contaminant(s)) due to the sensitive nature of the eye. There are also a limited collection of excipients acceptable for intravitreal administration compared with other delivery routes. In most cases, it is recommended that injection volume is limited to less than 0.10 mL per eye.

[00487] The antibodies provided herein may be useful for the treatment of ocular diseases involving TF. As used the term “ocular disease” refers broadly to any disease, disorder, injury or condition that impairs normal function of the eye(s). “Ocular disease” also encompasses the state of an eye when it exhibits symptoms including, but not limited to, inflammation, impairment of visual acuity, decreased photoreceptor function, decreased night vision, leakiness, macular edema, abnormal leukocyte infiltration, vitreous detachment, hyperplasia in the ocular tissue (*e.g.*, retina or cornea), blurred vision, eye pain, eye redness, light sensitivity, discoloration of the cornea, irritation in or around the eye, double vision, abnormal tearing, tunnel vision, seeing shadowy areas in your central vision or experiencing

unusually fuzzy or distorted vision, loss of central vision, straight lines appearing wavy, blindness, retinal neovascularization, angiogenesis in ocular tissue, vascular leakage, retinal thickening within 500 μm of the foveal center, hard yellow exudates within 500 μm of the foveal center (optionally with adjacent retinal thickening), fog vision, floaters, or loss of contrast. In some embodiments, the an ocular disease is a disease, disorder, injury or condition that can benefit from treatment by an antibody of the present disclosure.

[00488] Any suitable ocular disease can be treated with the antibodies provided herein. Non-limiting examples of diseases that are contemplated for treatment in the present disclosure include intraocular neovascular syndromes such as neovascular AMD, wet AMD, dry AMD, diabetic retinopathy, proliferative diabetic retinopathy (PDR), non-proliferative diabetic retinopathy (NPDR), diabetic macular edema, retinopathy of prematurity, neovascularization glaucoma, retinal vein occlusion, central retinal vein occlusion, macular degeneration, retinitis pigmentosa, retinal angiomatous hyperplasia, macular telangiectasia, ischemic retinopathy, macular neovascularization (choroidal neovascularization), iris neovascularization, eye neovascularization, corneal neovascularization, retinal neovascularization, retinal degeneration, inherited retinal degeneration (such as retinitis pigmentosa, retinal detachment or injury), and retinopathies. In some embodiments, the ocular disease is selected from the group consisting of: wet AMD, dry AMD, diabetic macular edema (DME), macula cystic edema (CME), NPDR, PDR, cystoid macular edema, vasculitis (e.g., central retinal vein occlusion), papilledema, retinitis, conjunctivitis, uveitis, choroiditis, multifocal choroiditis, ocular mytoplasmosis, orbital inflammation, dry eye (Hugh Glenn) Disease, Sjögren's disease, and other eye diseases associated with ocular neovascularization, vascular leakage, and/or retinal edema.

[00489] In some embodiments, the disease or condition that can benefit from treatment with an anti-TF antibody is a disease or condition involving neovascularization. In certain embodiments, the disease or condition is AMD, wet AMD or dry AMD. In certain embodiments, the disease or condition involving neovascularization is diabetic retinopathy, PDR or NPDR.

[00490] In some embodiments, provided herein is a method of treating an ocular disease, for example, a disease or condition involving neovascularization in a subject in need thereof by administering an effective amount of an anti-TF antibody provided herein to the subject. In certain embodiments, the disease or condition involving neovascularization is AMD, wet AMD or dry AMD. In certain embodiments, the disease or condition involving neovascularization is diabetic retinopathy, PDR or NPDR.

[00491] In some embodiments, provided herein is a method of preventing the onset of an ocular disease, for example, a disease or condition involving neovascularization in a subject in need thereof by administering an effective amount of an antibody provided herein to the subject.

[00492] In some embodiments, provided herein is a method of delaying the onset of age-related macular degeneration (AMD) in a subject in need thereof by administering an effective amount of an antibody provided herein to the subject.

[00493] In some embodiments, provided herein is a method of preventing the onset of age-related macular degeneration (AMD) in a subject in need thereof by administering an effective amount of an antibody provided herein to the subject.

[00494] In some embodiments, provided herein is a method of delaying the onset of diabetic retinopathy in a subject in need thereof by administering an effective amount of an antibody provided herein to the subject.

[00495] In some embodiments, provided herein is a method of preventing the onset of diabetic retinopathy in a subject in need thereof by administering an effective amount of an antibody provided herein to the subject.

11. Combination Therapies

[00496] In some embodiments, an antibody provided herein is administered with at least one additional therapeutic agent. Any suitable additional therapeutic agent may be administered with an antibody provided herein. In some aspects, the additional therapeutic agent is selected from radiation, an ophthalmologic agent, a cytotoxic agent, a chemotherapeutic agent, a cytostatic agent, an anti-hormonal agent, an immunostimulatory agent, an anti-angiogenic agent, and combinations thereof.

[00497] In certain embodiments, the antibodies provided herein are combined with the comparator drugs described herein. For example, an antibody provided herein (*e.g.* 25G9, 25A5-T) may be combined with any one of: aflibercept (Eylea), ranibizumab, brolucizumab, pegaptanib, bevacizumab, abicipar pegol, Faricimab (RG7716), KSI-301, hI-con1 (also referred to as “ICON1” or “ICON-1”) and M1593

[00498] The additional therapeutic agent may be administered by any suitable means. In some embodiments, an antibody provided herein and the additional therapeutic agent are included in the same pharmaceutical composition. In some embodiments, an antibody provided herein and the additional therapeutic agent are included in different pharmaceutical compositions.

[00499] In embodiments where an antibody provided herein and the additional therapeutic agent are included in different pharmaceutical compositions, administration of the antibody can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent.

12. Diagnostic Methods

[00500] Also provided are methods for detecting the presence of TF on cells from a subject. Such methods may be used, for example, to predict and evaluate responsiveness to treatment with an antibody provided herein.

[00501] In some embodiments, the method can be used to detect TF in a subject having or suspected of having an ocular disease as described herein. In some embodiments, the methods comprise (a) receiving a sample from the subject; and (b) detecting the presence or the level of TF in the sample by contacting the sample with the antibody provided herein. In some embodiments, the methods comprise (a) administering to the subject the antibody provided herein; and (b) detecting the presence or the level of TF in the subject. In certain embodiments, the ocular disease involves neovascularization, and optionally is age-related macular degeneration (AMD). In certain embodiments, the disease or condition involving neovascularization is diabetic retinopathy, PDR or NPDR.

[00502] In some embodiments, the methods comprise (a) administering to the subject the antibody provided herein; and (b) detecting the presence or the level of TF in the subject. In some embodiments, the disease or condition is an ocular disease as described herein.

[00503] In some embodiments, the antibody provided herein is conjugated with a fluorescent label. In some embodiments, the antibody provided herein is conjugated with a radioactive label. In some embodiments, the antibody provided herein is conjugated with an enzyme label.

[00504] In some embodiments, the relative amount of TF expressed by such cells is determined. The fraction of cells expressing TF and the relative amount of TF expressed by such cells can be determined by any suitable method. In some embodiments, flow cytometry is used to make such measurements. In some embodiments, fluorescence assisted cell sorting (FACS) is used to make such measurement.

13. Kits

[00505] Also provided are kits comprising the antibodies provided herein. The kits may be used for the treatment, prevention, and/or diagnosis of a disease or disorder, as described herein.

[00506] In some embodiments, the kit comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, and IV solution bags. The containers may be formed from a variety of materials, such as glass or plastic. The container holds a composition that is by itself, or when combined with another composition, effective for treating, preventing and/or diagnosing a disease or disorder. The container may have a sterile access port. For example, if the container is an intravenous solution bag or a vial, it may have a port that can be pierced by a needle. At least one active agent in the composition is an antibody provided herein. The label or package insert indicates that the composition is used for treating the selected condition.

[00507] In some embodiments, the kit comprises (a) a first container with a first composition contained therein, wherein the first composition comprises an antibody provided herein; and (b) a second container with a second composition contained therein, wherein the second composition comprises a further therapeutic agent. The kit in this embodiment of the invention may further comprise a package insert indicating that the compositions can be used to treat a particular condition.

[00508] Alternatively, or additionally, the kit may further comprise a second (or third) container comprising a pharmaceutically-acceptable excipient. In some aspects, the excipient is a buffer. The kit may further include other materials desirable from a commercial and user standpoint, including filters, needles, and syringes.

EXAMPLES

[00509] The following are examples of methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided herein.

[00510] Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

[00511] The practice of the present invention will employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and

pharmacology, within the skill of the art. Such techniques are explained fully in the literature. *See, e.g.,* T.E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Freeman and Company, 1993); A.L. Lehninger, *Biochemistry* (Worth Publishers, Inc., current addition); Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); Carey and Sundberg *Advanced Organic Chemistry 3rd Ed.* (Plenum Press) Vols A and B(1992).

Example 1: Generation of TF Antibodies

[00512] Human, cynomolgus monkey, and mouse TF extracellular domain (ECD) fragments were expressed as C-terminal His or Fc γ fragment fusions. Expi293 cells (ThermoFisher Scientific, Waltham, MA, USA) were transiently transfected as recommended by the manufacturer with pcDNA3.1V5-HisA (ThermoFisher Scientific) encoding human, cynomolgus, or mouse TF ECD–His6 (TF-His; SEQ ID NOs:811, 815, and 819, respectively) or pFUSE-hIgG1-Fc (Invivogen, San Diego, CA, USA) encoding human, cynomolgus or mouse TF ECD–Fc (TF-Fc; SEQ ID NOs:812, 816, and 820, respectively). For the His-tagged proteins, cell culture supernatants cleared from cells by centrifugation were preconditioned with 330 mM sodium chloride and 13.3 mM imidazole. Using recommended procedures, the TF-His6 and TF-Fc proteins were purified by affinity chromatography with a HisTrap HP and MabSelect SuRe column (GE Healthcare Bio-Sciences, Marlborough, MA, USA), respectively. FVII-Fc expressed in Expi293 was purified by affinity chromatography with a MabSelect SuRe column, followed by size exclusion chromatography. The TF-His6 and TF-Fc proteins were biotinylated with a 15x molar excess of Sulfo-NHS-SS-biotin as recommended (ThermoFisher Scientific). The non-labeled and biotinylated proteins were further purified by size exclusion chromatography using a Superdex 200 Increase 10/300 column (GE Healthcare Bio-Sciences).

[00513] Human antibodies against human TF were generated by Adimab™ yeast-based antibody presentation using the biotinylated recombinant TF proteins as screening antigens, as described below. All antibodies against human TF were evaluated for cross-reactivity with cynomolgus monkey and mouse TF. The binding activity of the antibodies to human, cynomolgus monkey, and mouse TF is shown in **Table 5**.

[00514] I. Library interrogation and selection methodology for isolation of anti-TF antibodies

[00515] *Naive library selections*

[00516] Eight naïve human synthetic yeast libraries each of $\sim 10^9$ diversity were designed, generated, and propagated as described previously (*see, e.g.*, WO2009036379; WO2010105256; WO2012009568; Xu *et al.*, *Protein Eng Des Sel.*, 2013, 26(10):663-70). Eight parallel selections were performed, using the eight naïve libraries for monomeric human TF selections.

[00517] For the first two rounds of selection, a magnetic bead sorting technique utilizing the Miltenyi MACS system was performed, essentially as described (Siegel *et al.*, *J Immunol Methods*, 2004, 286(1-2):141-53). Briefly, yeast cells ($\sim 10^{10}$ cells/library) were incubated with 10 nM of biotinylated human TF Fc-fusion antigen for 15 min at room temperature in FACS wash buffer PBS with 0.1% BSA. After washing once with 50 mL ice-cold wash buffer, the cell pellet was resuspended in 40 mL wash buffer, and 500 μ L Streptavidin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany; Cat # 130-048-101) were added to the yeast and incubated for 15 min at 4°C. Next, the yeast were pelleted, resuspended in 5 mL wash buffer, and loaded onto a MACS LS column (Miltenyi Biotec, Bergisch Gladbach, Germany; Cat.# 130-042-401). After the 5 mL was loaded, the column was washed 3 times with 3 mL FACS wash buffer. The column was then removed from the magnetic field, and the yeast were eluted with 5 mL of growth media and then grown overnight.

[00518] Subsequent to the two rounds of MACS, the following four rounds of sorting were performed using flow cytometry (FACS). For the first round of FACS, approximately 5×10^7 yeast were pelleted, washed three times with wash buffer, and incubated with 10 nM of each the biotinylated Fc-fusion proteins of mouse and/or cynomolgus TF antigen for 10-15 min at room temperature. Yeast were then washed twice and stained with LC-FITC diluted 1:100 (Southern Biotech, Birmingham, Alabama; Cat# 2062-02) and either SA-633 (Life Technologies, Grand Island, NY; Cat # S21375) diluted 1:500, or EA-PE (Sigma-Aldrich, St Louis; Cat # E4011) diluted 1:50, secondary reagents for 15 min at 4°C. After washing twice with ice-cold wash buffer, the cell pellets were resuspended in 0.4 mL wash buffer and transferred to strainer-capped sort tubes. Sorting was performed using a FACS ARIA sorter (BD Biosciences), and sort gates were determined to select for TF binding. The mouse- and cyno-selected populations from the first round of FACS were grown out and expanded through sub-culturing in selective media. The second, third, and fourth rounds of FACS involved positive sorts to enrich for TF binders and/or negative sorts to decrease the number of non-specific binders using soluble membrane proteins from CHO cells (*see, e.g.*,

WO2014179363 and Xu et al., *PEDS*, 2013, 26(10):663-70). After the final round of sorting, yeast were plated and sequenced.

[00519] *Affinity maturation of clones identified in naïve selections*

[00520] Heavy chains from the naïve outputs (described above) were used to prepare light chain diversification libraries, which were then used for additional selection rounds. In particular, heavy chain variable regions were extracted from the fourth naïve selection round outputs and transformed into a light chain library with a diversity of 1×10^6 .

[00521] The first of selection round utilized Miltenyi MACS beads and 10 nM biotinylated human TF Fc-fusion as antigen. Subsequent to the MACS bead selections, three rounds of FACS sorting were performed as described above using cynomolgus and mouse Fc-fusion TF at 10 nM oreither biotinylated Fc-fusion TF antigens or biotinylated monomeric HIS-forms of human, mouse or cynomolgus TF. Individual colonies from each FACS selection round were sequenced.

[00522] *Optimization of leads identified from naïve or light chain diversification selections*

[00523] Optimization of lead clones was carried out utilizing three maturation strategies: diversification of CDR-H1 and CDR-H2; diversification of CDR-H3 following CDR-H1 and CDR-H2 diversity pool optimization; and diversification of CDR-L3 within selected CDR-L1 and CDR-L2 diversity pools.

[00524] CDR-H1 and CDR-H2 selection: The CDR-H3s from clones selected from either naïve or light chain diversification procedure were recombined into a premade library with CDR-H1 and CDR-H2 variants of a diversity of 1×10^8 and selections were performed using biotinylated Fc-fusion cynomolgus TF antigen, biotinylated cynomolgus HIS-TF antigen, and/or biotinylated human HIS-TF. Affinity pressures were applied by using decreasing concentrations of biotinylated HIS-TF antigens (down to 1 nM) under equilibrium conditions at room temperature.

[00525] CDR-H3/CDR-H1/CDR-H2 selections: Oligos were ordered from IDT which comprised the CDR-H3 as well as a homologous flanking region on either side of the CDR-H3. Amino acid positions in the CDR-H3 were variegated via NNK diversity at two positions per oligo across the entire CDR-H3. The CDR-H3 oligos were double-stranded using primers which annealed to the flanking region of the CDR-H3. The remaining FR1 to FR3 of the heavy chain variable region was amplified from pools of antibodies with improved affinity that were isolated from the CDR-H1 and CDR-H2 diversities selected above. The library was then created by transforming the double stranded CDR-H3 oligo, the FR1 to FR3 pooled fragments, and the heavy chain expression vector into yeast already containing the light chain

of the parent. Selections were performed as during previous cycles using FACS sorting. FACS rounds assessed non-specific binding, species cross-reactivity, and affinity pressure, and sorting was performed to obtain populations with the desired characteristics. Affinity pressures for these selections were performed as described above in the CDR-H1 and CDR-H2 selection.

[00526] CDR-L3/CDR-L1/CDR-L2 selections: Oligos were ordered from IDT which comprised the CDR-L3 as well as a homologous flanking region on either side of the CDR-L3. Amino acid positions in the CDR-L3 were variegated via NNK diversity at one position per oligo across the entire CDR-L3. The CDR-L3 oligos were double-stranded using primers which annealed to the flanking region of the CDR-L3. The remaining FR1 to FR3 of the light chain variable region was amplified from pools of antibodies with improved affinity that were isolated from the CDR-L1 and CDR-L2 diversities selected above. The library was then created by transforming the double stranded CDR-L3 oligo, the FR1 to FR3 pooled fragments, and the light chain expression vector into yeast already containing the heavy chain of the parent. Selections were performed as during previous cycles using FACS sorting. FACS rounds assessed non-specific binding, species cross-reactivity, and affinity pressure, and sorting was performed to obtain populations with the desired characteristics. Affinity pressures included titrations as well as incorporation of the parental Fab in antigen pre-complexation.

[00527] II. IgG and Fab production and purification

[00528] In order to produce sufficient amounts of selected antibodies for further characterization, the yeast clones were grown to saturation and then induced for 48 h at 30°C with shaking. After induction, yeast cells were pelleted and the supernatants were harvested for purification. IgGs were purified using a Protein A column and eluted with acetic acid, pH 2.0. Fab fragments were generated by papain digestion and purified over CaptureSelect IgG-CH1 affinity matrix (LifeTechnologies, Cat # 1943200250).

Example 2: Efficacy Study in Swine CNV Model

[00529] An efficacy study in a swine choroidal neovascularization (CNV) model was performed to determine the effect of 4 different anti-TF antibodies in reducing lesion size (or leakiness area).

[00530] 10-12 week old animals (Swine/Hampshire Cross) underwent bilateral laser using an 810 nm Diode laser delivered through an indirect ophthalmoscope to create approximately 6 single laser spots between retinal veins in each eye of each animal. For efficacy

assessments, animals were treated intravitreally on day 7 post-laser treatment with 0.6 mg of ICON-1, 2 mg of each anti-TF antibody, 25G9, 43D8, 1G, and 29D, respectively, or 2 mg of adalimumab ($n = 5/\text{group}$). A vehicle control group was also included in the study.

[00531] Fluorescein Angiography (FA) was performed on day 7 (baseline), day 14, and day 28 to determine total lesion fluorescence. FA was evaluated using a Corrected Total Lesion Fluorescence (CTLF) measurement for each individual lesion. The perimeter of the lesion was traced, and an integrated density value was obtained. CTLF was then calculated by subtracting the mean fluorescence background adjacent to the lesion from the integrated density measurement. The CTLF was representative of the total leakiness area or total lesion size. Percent change in CTLF between the vehicle and the anti-TF antibody groups from day 7 to day 14 and from day 7 to day 28 are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety. The mean CTLF over time for all groups and percent of CTLF relative to day 7 (baseline) for all groups are shown in **FIG. 2A** and **FIG. 2B**, respectively.

[00532] The data indicated that from day 7 to day 14, anti-hTF antibodies from groups 25 and 43, 25G9 and 43D8, reduced lesion size by greater than 20%. By day 14, ICON-1 reduced lesion size by greater than 10% relative to baseline but did not exhibit as much lesion size reduction as the 25 and 43 group antibodies within the same period. By day 28, anti-hTF antibody 25G9 showed the greatest lesion size reduction of all groups relative to baseline with greater than 40% lesion size reduction relative to baseline. Adalimumab and ICON-1 also showed significant lesion size reduction relative to the vehicle control, however, neither therapies resulted in as much lesion size reduction as the 25 group antibody. There was no significant difference in the % CTLF at 14 days and 28 days relative to baseline, when the 43 family antibody was administered. Throughout the study, the anti-hTF antibodies 1G and 29D did not reduce lesion size significantly as compared to the vehicle control group (**FIG. 2B**).

[00533] These data indicate that antibodies from groups 25 and 43 (*e.g.*, 25G9 and 43D8), are effective in reducing lesion size.

Example 3: Efficacy of 25-Group Antibodies in Swine CNV Model

[00534] An efficacy study in a swine choroidal neovascularization (CNV) model was performed to determine the effect of various 25-group anti-TF antibodies in reducing lesion size (or leakiness area).

[00535] 10-12 week old animals (Swine/Hampshire Cross) underwent bilateral laser using an 810 nm Diode laser delivered through an indirect ophthalmoscope to create approximately 6 single laser spots between retinal veins in each eye of each animal. For efficacy assessments, animals were treated intravitreally on day 7 post-laser treatment with 4 mg of 25A, 25A5-T, or 25G9, 5 mg of 25G1, or 2 mg of Eylea. Another group of animals received the combination of 2 mg of Eylea and 4 mg of 25G9. A vehicle control group was also included in the study ($n = 5/\text{group}$).

[00536] Fluorescein Angiography (FA) was performed on day 7 (baseline), day 14 and day 28 to determine total lesion fluorescence. FA was evaluated using a Corrected Total Lesion Fluorescence (CTLF) measurement for each individual lesion. The perimeter of the lesion was traced, and an integrated density value was obtained. CTLF was then calculated by subtracting the mean fluorescence background adjacent to the lesion from the integrated density measurement. The CTLF was representative of the total leakiness area or total lesion size. The mean CTLF over time for all groups and percent of CTLF for all groups relative to day 7 (baseline) are shown in **FIG. 3** and **FIG. 4**, respectively.

[00537] The data revealed that by day 14, anti-hTF antibodies 25A5-T and 25G9 reduced lesion size by greater than 20% relative to baseline but by a comparable amount to the vehicle control. By day 14, 25A and 25G1 reduced lesion size by greater than 30% relative to baseline, exceeding the lesion size reduction in the vehicle control group over the same time period. By day 28, groups treated with 25A, 25A5-T, and 25G1 reported less lesion size reduction than the vehicle control group, while groups treated with Eylea or the combination therapy reported significantly more lesion size reduction than the vehicle control and relative to baseline levels. The greatest lesion size reduction throughout the study was observed in animals treated with Eylea, which exhibited over 80% lesion size reduction by day 28 relative to baseline levels (**FIG. 4**).

[00538] These results indicate that 14 days post-laser treatment, 25A and 25G1 antibodies are effective in reducing lesion size (leakiness area) in swine CNV model, though Eylea is more effective. The moderate (or absence of) lesion size reduction observed in groups treated solely with 25-group anti-TF antibodies 28 days post-laser treatment, may have been attributable to inflammation, which was observed in all groups, including the vehicle control group. There was also more inflammation observed at day 28 than day 14.

Example 4: Dose-Dependent Response of 25G9 in Swine CNV Model

[00539] An efficacy study in a swine choroidal neovascularization (CNV) model was performed to compare different doses of anti-TF antibody 25G9 for their ability to reduce lesion size (or leakiness area).

[00540] 10-12 week old animals (Swine/Hampshire Cross) underwent bilateral laser using an 810 nm Diode laser delivered through an indirect ophthalmoscope to create approximately 6 single laser spots between retinal veins in each eye. For efficacy assessments 600 µg, 2 mg or 4 mg of anti-TF antibody 25G9 were administered intravitreally on Day 7 post-laser treatment ($n = 5/\text{group}$). A vehicle control group was also included in the study.

[00541] Fluorescein Angiography (FA) was performed on day 7 (baseline) and day 28 to determine total lesion fluorescence. FA was evaluated using a Corrected Total Lesion Fluorescence (CTLF) measurement for each individual lesion. The perimeter of the lesion was traced, and an integrated density value was obtained. CTLF was then calculated by subtracting the mean fluorescence background adjacent to the lesion from the integrated density measurement. The CTLF was representative of the total leakiness area or total lesion size. Percent changes in lesion size from day 7 to day 28 are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety.

[00542] From day 7 to day 28, anti-hTF antibody 25G9 reduced lesion size in a dose-dependent manner. 25G9 reduced lesion size by greater than 50% at 4 mg. This data indicates that antibody 25G9 was effective in reducing lesion size in the swine CNV model in a dose-dependent manner.

Example 5: Efficacy of Wild-type vs. Mutated 25G9 in Swine CNV Model

[00543] An efficacy study in a swine choroidal neovascularization (CNV) model was performed to compare different doses of anti-TF antibody wild type 25G9, 25G9DE, ICON1, and anti-TF antibody M1593 for their ability to reduce lesion size (or leakiness area). The wild-type 25G9 (25G9WT) is the unmutated form of 25G9 as disclosed herein. Anti-TF antibody 25G9DE is the 25G9 antibody having two mutations in the human IgG1 Fc region (S239D and I332E, as determined by Kabat numbering) that result in enhanced Fc effector function (particularly, ADCC function). (See, for example, Lazar *et al. Proceedings of the National Academy of Sciences*, 103.11 (2006): 4005-4010, which is incorporated by reference in its entirety).

[00544] 10-12 week old animals (Swine/Hampshire Cross) underwent bilateral laser using an 810 nm Diode laser delivered through an indirect ophthalmoscope to create approximately 6 single laser spots between retinal veins in each eye. For efficacy assessments, animals were treated intravitreally on Day 7 post-laser treatment with one of the following:

- 600 µg of ICON-1;
- 600 µg, 2 mg or 4 mg of anti-TF antibody 25G9WT (wild-type 25G9)
- 600 µg or 4 mg of anti-TF antibody 25G9DE
- 600 µg of anti-TF antibody M1593

A vehicle control group was also included in the study ($n = 5/\text{group}$).

[00545] Fluorescein Angiography (FA) was performed on day 7 (baseline) and day 28 to determine total lesion fluorescence. FA was evaluated using a Corrected Total Lesion Fluorescence (CTLF) measurement for each individual lesion. The perimeter of the lesion was traced, and an integrated density value was obtained. CTLF was then calculated by subtracting the mean fluorescence background adjacent to the lesion from the integrated density measurement. The CTLF was representative of the total leakiness area or total lesion size. The mean CTLF for all groups and the percent CTLF for all groups relative to baseline are shown in **FIG. 5** and **FIG. 6**.

[00546] The data revealed that from day 7 to day 28, anti-hTF antibody 25G9WT reduced lesion size in a dose-dependent manner. By day 14, animals treated with 600 µg and 2 mg of 25G9WT exhibited greater than 10% lesion size reduction relative to baseline. By day 28, animals treated with 4 mg of 25G9WT exhibited greater than 30% lesion size reduction relative to baseline. By day 14, animals treated with 600 µg of 25G9DE exhibited significantly less lesion size reduction relative to baseline than those treated with the same dose of 25G9WT. However, at the high dose of 4 mg, the 25G9DE group showed more lesion size reduction relative to baseline than the counterpart 25G9WT group (**FIG. 6**).

[00547] By day 28, animals treated with 600 µg and 4 mg of 25G9WT exhibited greater than 10% and greater than 50% lesion size reduction relative to baseline, respectively. The difference in lesion size reduction for groups treated with 25G9DE over 28 days post-laser treatment was more pronounced than the 25G9WT counterparts. For example, by day 28, 600 µg of 25G9DE resulted in over 60% lesion size reduction relative to baseline—the greatest reduction for across all groups (**FIG. 6**).

[00548] These data indicate that 25 group antibodies, *e.g.* 25G9, are effective in reducing lesion size in a dose-dependent manner and the addition of Fc region mutations to enhance effector function can amplify lesion size reduction.

Example 6: Effectiveness of 25G9 with Effector Function Mutations in Swine CNV Model

[00549] An efficacy study in a swine choroidal neovascularization (CNV) model was performed to determine the effect of 25G9 anti-TF antibodies with various effector function mutations in reducing lesion size (or leakiness area).

[00550] 10-12 week old animals (Swine/Hampshire Cross) underwent bilateral laser using an 810 nm Diode laser delivered through an indirect ophthalmoscope to create approximately 6 single laser spots between retinal veins in each eye. For efficacy assessments, animals were treated intravitreally on Day 7 post-laser treatment with one of the following:

- 2 mg of Eylea
- 600 µg of 25G9
- Combination therapy: 600 µg of 25G9 + 2 mg of Eylea
- 600 µg of 25G9DE
- Combination therapy: 600 µg of 25G9DE + 2 mg of Eylea
- 600 µg of 25G9-LALAPG
- 600 µg of 25G9 FAB

[00551] A vehicle control group was also included in the study ($n = 5/\text{group}$). Anti-TF antibody 25G9 was the unmutated form of the 25G9 antibody as disclosed herein. Anti-TF antibody 25G9DE was the 25G9 antibody having two mutations in the Fc region (S239D and I332E according to Kabat numbering) that result in enhanced effector function (particularly ADCC function). Anti-TF antibody 25G9-LALAPG was the 25G9 antibody having the combination of L₂₃₄A, L₂₃₅A, and P₃₂₉A (LALA-PG) substitutions in the human IgG1 heavy chain constant region (according to Kabat numbering) that have been shown to effectively silence the effector function of human IgG1 antibodies. (*See, for example, Schlothauer et al. Protein Engineering, Design and Selection* 29.10 (2016): 457-466, which is incorporated by reference in its entirety). The antigen-binding fragment (Fab) for the 25G9 antibody was identified as 25G9 FAB (*i.e.*, this is an Fc null version of the antibody having a single FAB domain).

[00552] Smaller antibody fragments such as Fab or Fab (2) are often used for intravitreal injection treatment of ocular diseases because of their low serum half-life and low risk of

systemic toxicity. However, this smaller fragment also typically has a lower intravitreal half-life (*e.g.*, due to faster diffusion into the serum) and usually must be administered more frequently.

[00553] Fluorescein Angiography (FA) was performed on day 7 (baseline) and day 28 to determine total lesion fluorescence. FA was evaluated using a Corrected Total Lesion Fluorescence (CTLF) measurement for each individual lesion. The perimeter of the lesion was traced, and an integrated density value was obtained. CTLF was then calculated by subtracting the mean fluorescence background adjacent to the lesion from the integrated density measurement. The CTLF was representative of the total leakiness area or total lesion size. The mean CTLF for all groups and the percent CTLF for all groups relative to day 7 (baseline) are shown in **FIG. 7** and **FIG. 8**, respectively.

[00554] The data revealed that from day 7 to day 14, all groups, with the exception of the 25G9 FAB group, exhibited significantly more lesion size reduction than the vehicle control. By day 14, all groups, with the exception of the 25G9 FAB group, exhibited greater than 40% lesion size reduction relative to baseline. By day 28, all groups, with the exception of the 25G9 FAB group, exhibited greater than 50% lesion size reduction relative to baseline and demonstrated effectiveness relative to the vehicle control. By day 28, the most effective treatments were 600 µg of 25G9, 2 mg of Eylea, and Combination therapy: 600 µg of 25G9 + 2 mg of Eylea (**FIG. 8**).

[00555] These results showed that unmutated 25G9 antibody was more effective in reducing lesion size than the 25G9 counterparts having the Fc region mutations utilized in this study. These data also indicate that the presence of Fc contributes to the effectiveness of 25G9 in lesion size reduction in the swine CNV model.

Example 7: Effectiveness Comparison of 25A5-T to Eylea in Swine CNV Model

[00556] An efficacy study in a swine choroidal neovascularization (CNV) model was performed to compare the effects of anti-TF antibody 25A5-T and Eylea in reducing lesion size (or leakiness area).

[00557] 10-12 week old animals (Swine/Hampshire Cross) underwent bilateral laser using an 810 nm Diode laser delivered through an indirect ophthalmoscope to create approximately 6 single laser spots between retinal veins in each eye of each animal. For efficacy assessments, animals were treated intravitreally on day 7 post-laser treatment with 400 µg of 25A5-T, 4 mg of 25A5-T, 200 µg of Eylea or 2 mg of Eylea. A vehicle control group was also included in the study ($n = 5/\text{group}$).

[00558] Fluorescein Angiography (FA) was performed on day 7 (baseline), day 14, and day 28 to determine total lesion fluorescence. FA was evaluated using a Corrected Total Lesion Fluorescence (CTLF) measurement for each individual lesion. The perimeter of the lesion was traced, and an integrated density value was obtained. CTLF was then calculated by subtracting the mean fluorescence background adjacent to the lesion from the integrated density measurement. The CTLF was representative of the total leakiness area or total lesion size. The mean CTLF over time for all groups and percent of CTLF for all groups relative to baseline are shown in **FIG. 9** and **FIG. 10**, respectively.

[00559] By day 14, both Eylea groups showed significant reduction in lesion size relative to baseline and relative to the vehicle control. By day 14, 200 µg of Eylea resulted in about 50% lesion size reduction relative to baseline, and 2 mg of Eylea resulted in greater than 40% lesion size reduction relative to baseline. By day 28, the 200 µg and 2 mg Eylea treatments resulted in over 60% and over 70% lesion size reduction, respectively. With regard to the 25A5-T groups, by day 14, there was not a significant reduction in lesion size relative to baseline and relative to the vehicle control. By day 28, the lesion size reduction in the 400 µg 25A5-T group was greater than 50% relative to baseline and greater than the vehicle control (**FIG. 10**).

[00560] The data in this study indicate that Eylea is more effective in reducing lesion size or leakiness than 25A5-T in swine CNV model. Inflammation was mostly observed in the 25A5-T high-dose group by day 21. The moderate effectiveness by the 25A5-T treatment may also have been attributable to the affinity of the antibody to pig TF. 25A5-T shows a higher affinity to human TF than to pig TF. In comparison, 25G9 shows a higher affinity for pig TF than 25A5-T (*see Examples below*), which may explain greater lesions size reduction when using 25G9 in swine CNV model in some experiments than 25A5-T.

Example 8: Ocular Pharmacokinetics Study in Rabbits

[00561] A pharmacokinetics (PK) study was conducted to evaluate the ocular pharmacokinetics of 25A5-T intravitreal (IVT) injection in rabbits. Briefly, rabbits were treated by IVT injection with either a single dose of 25A5-T or a two doses of 25A5-T, as indicated in **Table 46**. A naive group of rabbits served as the negative control—they did not receive any 25A5-T dose, and were sacrificed on day 0 for eye tissue collection, sampling, and analysis.

Table 46: Experimental design for ocular PK study in rabbits.

Group	# Animals (Male/Female)	Conc. (mg/mL)	Dose Level	Dose Volume	Frequency (Day)
Control	12/7	N/A	N/A	N/A	N/A
1	12/12	6	600 µg/eye	OU: 1x 100µL	1
2	12/12	36	3600 µg/eye	OU: 1x 100µL	1
4	2/2	6	600 µg/eye	OU: 1x 100µL	1 and 14
5	2/2	36	3600 µg/eye	OU: 1x 100µL	1 and 14

[00562] All experimental groups received an ocular exam on the day of treatment (baseline), and days 2, 4, 8, 12, and 14. Blood (serum) samples were collected immediately following treatment, 8 h following treatment, and on days 2, 3, 4, 6, 8, 12, and 16.

[00563] For groups 1 and 2, three animals were euthanized per time point, wherein the timepoints were 8 h (day 1), 24 h (day 2), and days 3, 4, 6, 8, 12, and 16. An anti-drug antibody (ADA) assay was performed on predose and day 16 serum samples and day 16 ocular matrices. The samples were analyzed for antibodies for 25A5-T using an ECL bridging assay.

[00564] For groups 3 and 4, all four animals were euthanized on day 28 of the study. An anti-drug antibody (ADA) assay was performed on predose, day 14, and day 18 serum samples and day 28 ocular matrices. The samples were analyzed for antibodies for 25A5-T using an ECL bridging assay.

[00565] For the IVT injections, 45 mg/mL 25A5-T in PBS was diluted with PBS to get to the desired concentrations. One drop of topical proparacaine HCl 0.5% and phenylephrine HCl 10% was applied to the eye prior to injection. IVT injections were administered to sedated rabbits using a 30-gauge needle.

[00566] Following euthanasia, eye tissue samples were collected from the retina, vitreous humor, and aqueous humor. Following enucleation, aqueous humor was collected using a 30-gauge syringe and snap frozen in liquid nitrogen. Eye tissues were dissected into vitreous and retina while frozen. ADA was quantified in vitreous samples. All tissue samples were analyzed by ELISA to determine the concentration of 25A5-T (LOQ = 3.5 ng/mL). Vitreous PK data were analyzed using a 1-compartment model using WinNonlin. The mean concentration profiles and results for 25A5-T in rabbit vitreous humor are shown in **FIG. 11**

and **Table 47**. These results revealed linear pharmacokinetics of the 25A5-T in the vitreous humor. The mean concentration profiles and results for 25A5-T were compared to existing ICON-1 0.6 mg dose results determined using the same methods (**Table 47**).

Table 47: PK results for 25A5-T and ICON-1 in rabbit vitreous humor—single IVT administration. *The AUC was determined using non-compartmental methods.

Group	Molecule	Dose (mg)	CL (mL/days)	Vc (mL)	t _{1/2} (days)	MRT (days)	AUC _{inf} (µg*day/mL)
1	25A5-T	0.6	0.132 (0.0300)	1.24 (0.158)	6.52 (1.91)	9.40 (2.76)	4540 (1030)
2	25A5-T	3.6	0.114 (0.0138)	0.978 (0.0747)	5.93 (0.965)	8.55 (1.39)	31500 (3780)
3	ICON-1	0.6	0.275 (0.0344)	2.50 (0.120)	6.31 (0.985)	9.10 (1.42)	2180 (273)

[00567] The mean concentration profiles and results for 25A5-T in all serum and tissue samples from rabbits in groups 1 and 2 are shown in **FIG. 12A** and **FIG. 12B**. The results showed that the concentration of 25A5-T in the retina was approximately half of that in the vitreous humor. Following single IVT administration, the 25A5-T disposition was dose linear with a vitreous half-life of about 6 days. Concentrations in other ocular compartments declined in parallel with those in the vitreous. The results also showed significantly less 25A5-T in the serum samples than in the ocular tissues. These results indicate higher antibody exposure in the target ocular tissues than in the serum. The mean concentration profiles and results for ICON-1 in all plasma and tissue samples from rabbits in group 3 are shown in **FIG. 13**.

[00568] Vitreous PK data were analyzed using a non-compartmental method and are shown in **Table 65**. The non-compartmental vitreous parameter measurements were similar for groups 1 and 2 and were consistent with those estimated using the 1-compartmental model.

Table 65: Vitreous 25A5-T PK results following single IVT administration of 600 (G1) and 3600 (G2) µg/eye (Non-Compartmental Method)

Group (dose/eye)	Parameters						
	C _{max} (µg/mL)	t _{max} (days)	t _{1/2} (days)	AUC _{0-t} (µg*day/mL)	AUC _{inf} (µg*day/mL)	CL (mL/days)	V _{ss} (mL)
1 (600 µg)	559	1.00	5.41	3700	4300	0.140	1.11
2 (3600 µg)	3520	2.00	5.13	26400	29800	0.121	0.906

[00569] Retinal and aqueous PK data were also analyzed using a non-compartmental method and are shown in **Tables 66 and 67**, respectively.

Table 66: Retinal 25A5-T PK data following IVT Administration (Non-Compartmental Method)

Group (dose/eye)	Parameters				
	C _{max} (ng/mg)	t _{max} (days)	t _{1/2} (days)	AUC _{0-t} (ng*day/mg)	AUC _{inf} (ng*day/mg)
1 (600 µg)	272	1.00	4.83	1830	2060
2 (3600 µg)	885	4.00	7.20	10100	12500

Table 67: Aqueous 25A5-T PK data following IVT Administration (Non-Compartmental Method)

Group (dose/eye)	Parameters				
	C _{max} (ng/mg)	t _{max} (days)	t _{1/2} (days)	AUC _{0-t} (ng*day/mg)	AUC _{inf} (ng*day/mg)
1 (600 µg)	41.6	3	5.22	321	376
2 (3600 µg)	300	3	4.64	2360	2600

[00570] The results from the non-compartmental analysis revealed that concentrations of 25A5-T in the retina and aqueous humor declined generally in parallel with those in the vitreous. The highest concentrations were found in the vitreous humor, followed by the retina, and then the aqueous humor.

[00571] Serum PK data were analyzed using a non-compartmental method are shown in Table 68 and the serum concentration-time profile is shown in **FIG. 23**.

Table 68: Serum 25A5-T PK data following IVT administration (Non-Compartmental Method)

Group (dose, frequency)	Parameters				
	C _{max} (ng/mL)	t _{max} (days)	t _{1/2} (days)	AUC ₀₋₇ (ng*day/mL)	AUC _{inf} (ng*day/mL)
1 (600 µg/eye, once)	1110	3	2.16	5730	7320
2 (3600 µg/eye, once)	7340	3	3.23	40600	59900
4 (600 µg/eye, twice)	1040	5	NC	5450	NC
5 (3600 µg/eye, twice)	8010	5	1.60	43700	83200

[00572] The 25A5-T serum concentrations increased approximately linearly with increasing dose and were much lower than those in the vitreous humor. Due to the presence of ADA on D16, the 25A5-T serum concentrations might be underestimated at later times

Antidrug Antibody Observations

[00573] At Day 16, vitreous samples from G1 and G2 were all negative for ADA. On Day 29, 8/8 vitreous samples were ADA positive in G4 and 6/8 samples were ADA positive in G5.

[00574] On Day 16, 2/3 and 1/3 animals were serum ADA positive for G1 and G2, respectively. For G4, 3/3 animals were serum ADA positive on Day 14 and 4/4 on at Day 29. For G5, 3/4 animals were serum ADA positive on Day 14 and on Day 28. Emergence of ADA is expected when using human antibodies in any toxicology species.

Example 9: Ocular Pharmacokinetics Study in Cynomolgus Monkeys

[00575] A pharmacokinetics (PK) study was conducted to evaluate the ocular pharmacokinetics of 25A5-T intravitreal (IVT) injection in cynomolgus (cyno) monkeys (Charles River Research Models, Houston, Texas, USA). Briefly, 8 male cyno monkeys were treated intravitreally with 100 μ L/eye of 6 mg/mL 25A5-T (dose level = 0.6 mg/eye). Another 8 male cyno monkeys were treated intravitreally with 100 μ L/eye of 120 mg/mL 25A5-T (dose level = 12 mg/eye). In both groups, the 100 μ L/eye was administered as two 50 μ L injections per eye, administered 10-15 minutes apart.

[00576] For dose administration, a topical antibiotic (tobramycin) was applied to both eyes, twice on the day before and twice on the day after each injection. Prior to dosing, the animals were sedated by an intramuscular injection of ketamine and dexmedetomidine. The conjunctivae were flushed with diluted benzalkonium chloride. 1% tropicamide was applied to each eye, as needed.

[00577] For both groups, two animals were sacrificed at each of the following time points relative to the IVT administration and ocular tissues were collected: Day 5, Day 10, Day 14 and Day 21. Following enucleation, aqueous humor was collected using a 30-gauge syringe and snap frozen by immersion in liquid nitrogen. Eye tissues were dissected into vitreous and retinal tissue while frozen. 25A5-T concentrations were quantified in vitreous, aqueous and retina samples. ADA was analyzed in vitreous samples.

[00578] Blood samples were collected up to 21 days and processed into serum. Serum samples were used for measurement of 25A5-T concentrations and ADA.

[00579] Serum and ocular tissue (retina, vitreous humor, and aqueous humor) samples were collected over 21 days following treatment. All samples were analyzed by MSD-ECLA to determine the concentration of 25A5-T (LOQ = 20 ng/mL). Ocular and serum concentration-time data were analyzed by non-compartmental method, while vitreous concentration-time data were analyzed using a 1-compartment model.

[00580] The vitreous humor PK results and mean concentration profiles are shown in **Table 60** and **FIG. 14**.

Table 60: PK results for 25A5-T in cyno vitreous humor (1-compartment model)

Dose Group (mg)	Parameters (SE of estimate)				
	CL (mL/days)	V _c (mL)	t _{1/2} (days)	MRT (days)	C _{max} (ug/mL)
0.6	0.569 (0.0257)	2.55 (0.312)	3.10 (0.287)	4.48 (0.414)	235 (28.9)
12	0.463 (0.00197)	2.08 (0.0241)	3.12 (0.0271)	4.50 (0.0392)	5757 (66.3)

Table 69: Vitreous 25A5-T PK Data following single IVT administration of 0.6 and 12 mg/eye (Non-Compartmental Method)

Dose (mg/eye)	Parameters							
	C ₀ (ug/mL)	C _{max} (ug/mL)	t _{max} (days)	t _{1/2} (days)	AUC _{0-t} (ug*day/mL)	AUC _{inf} (ug*day/mL)	CL (mL/days)	V _{ss} (mL)
0.6	255	77.4	5	4.42	1210	1250	0.479	2.00
12	5690	1890	5	3.05	28100	28300	0.424	1.56

[00581] As shown in **Tables 60 and 69**, the vitreous CL and V_c/V_{ss} were similar for both dose levels and consistent with those estimated by compartmental method.

[00582] The results revealed that 25A5-T, administered at both dose levels, has linear pharmacokinetics in cyno vitreous humor with vitreous half-life of ~3 days. The central volume (V_c) reported in this experiment (~2 mL) was consistent with that found in physiological vitreous humor of non-human primates (typically ~1.5-3.0 mL). The results also revealed that the 25A5-T half-time in cyno vitreous humor was twice as short as compared to that observed in rabbit vitreous humor (examples above).

[00583] The non-compartmental parameter measurements for retina and aqueous humor are shown in **Tables 70 and 71**.

Table 70: Retinal 25A5-T PK data following IVT administration of 0.6 and 12 mg/eye 25A5-T (Non-Compartmental Method)

Dose (mg/eye)	Parameters					
	C ₀ (ug/mg)	C _{max} (ug/mg)	t _{max} (days)	t _{1/2} (days)	AUC _{0-t} (ug*day/mg)	AUC _{inf} (ug*day/mg)
0.6	0.993	0.732	5	4.95	9.92	10.6
12	71.3	36.3	5	3.13	487	492

Table 71: Aqueous 25A5-T PK data following IVT administration of 0.6 and 12 mg/eye 25A5-T (Non- Compartmental Method)

Dose (mg/eye)	Parameters					
	C ₀	C _{max}	t _{max}	t _{1/2}	AUC _{0-t}	AUC _{inf}

	($\mu\text{g}/\text{mg}$)	($\mu\text{g}/\text{mg}$)	(days)	(days)	($\mu\text{g} \cdot \text{day}/\text{mg}$)	($\mu\text{g} \cdot \text{day}/\text{mg}$)
0.6	3.60	1.15	5	3.63	17.2	17.5
12	825	85.1	5	2.14	2540	2540

[00584] In contrast with the vitreous parameter data, 25A5-T concentrations increased more than proportionally in both the retina and the aqueous humor, while the $t_{1/2}$ were similar in both compartments. The highest concentrations were observed in the vitreous humor followed by the retina and aqueous humor, which were similar.

[00585] The mean concentration profiles for 25A5-T in all serum and tissue samples from cyno monkeys are shown in **FIG. 15A** and **FIG. 15B**. The concentration-time profiles for 25A5-T in serum after a single IVT dose of 0.6 and 12 mg/eye are shown in **FIG. 16**.

[00586] The non-compartmental parameter measurements for serum are shown in Table 72.

Table 72: Serum 25A5-T PK data following IVT administration (Non-Compartmental Method)

Dose (mg/eye)	Parameters				
	$C_{\max}$ ($\mu\text{g}/\text{mL}$)	$t_{\max}$ (days)	$t_{1/2}$ (days)	AUC_{0-t} ($\mu\text{g} \cdot \text{day}/\text{mL}$)	AUC_{inf} ($\mu\text{g} \cdot \text{day}/\text{mL}$)
0.6	1.78	2	1.61	8.65	8.88
12	67.50	5	5.52	735	798

[00587] The results revealed that the concentration of 25A5-T in the retina was approximately 0.05 of that in the vitreous humor. The concentration of 25A5-T in aqueous humor was approximately 0.02-0.1 of that in the vitreous humor. The results also showed significantly less 25A5-T in the serum samples than in the ocular tissues. These results indicate higher antibody exposure in the target ocular tissues (particularly the vitreous humor) than in the serum.

[00588] Following IVT administration, the serum concentrations were much lower than those measured in the ocular compartments. Serum concentrations increased more than dose proportionally. Due to the presence of ADA, 25A5-T serum concentrations might be underestimated.

Antidrug Antibody

[00589] All vitreous samples were negative for ADAs. In serum prior to 25A5-T administration, 6/8 of the G1 (0.6 mg/eye group) animals were ADA positive with titers ranging from 1–4, while 4/8 of the G2 (12 mg/eye group) animals were ADA positive with

titers ranging from 2–3. Following 25A5-T administration, ADA titers ranged from 2–254 for G1 and from 1–3 for the G2 animals.

Example 10: Pilot Toxicology Study in Non-Human Primates (NHP)

[00590] The objective of this study was to assess the ocular toxicity parameters of anti-TF antibody 25A5-T in cynomolgus monkeys and to evaluate the potential reversibility of any findings following a 28-day observation period. Nine male cynomolgus monkeys were received from Charles River Research Models, Houston, Texas, USA, for intravitreal injection dose initiation per the Experimental Design shown in **Table 61** and **FIG. 17**. At dosing onset, the animals were 2 to 4 years old and weighed between 2.7 and 3.4 kg.

Table 61: Experimental design for the pilot toxicology study in cyno monkeys

Group No.	Test Material	Dose Level (mg/eye) ^a	Dose Volume (μL/eye)	Dose Concentration (mg/mL)	Animal Numbers
					Males
1	Formulation Buffer	0	100	0	1001 to 1003
2	25A5-T	4	100	40	2001 to 2003
3	25A5-T	11.9	100	119.2	3001 to 3003

^a Animals were dosed on Days 1 and 15, kept for a 28-day observation, and euthanized on Day 44.

[00591] Before dosing initiation, male monkeys were weighed and assigned to 3 groups consisting of 3 animals per group, using a computer-based randomization procedure. Animals were identified using a subcutaneously implanted electronic identification chip and were socially housed (3 animals of the same dosing group together, with the exception of times when they were separated for designated study procedures/activities) in stainless steel cages. The disposition of all animals was documented throughout the study.

[00592] All animals were fed twice daily, except during designated procedures. The chow was provided in amounts appropriate for the size and age of the animals. For psychological/environmental enrichment, animals were provided with items such as perches, floor enrichment devices, foraging devices and/or suspended devices, music, natural sounds and/or color video films. Municipal tap water after treatment by reverse osmosis and ultraviolet irradiation was freely available to each animal via an automatic watering system. Target temperatures of 20°C to 26°C with a relative target humidity of 30% to 70% were maintained. A 12-hour light/12-hour dark cycle was maintained (except during designated procedures).

[00593] Formulation buffer, which was administered to group 1, served as the negative control (vehicle control) and comprised of 20 mM Sodium Acetate, 8% Trehalose, and 0.02%

(v/v) Polysorbate 20 (pH 5.5). The anti-TF antibody, 25A5-T, was stored in a freezer set to maintain -80°C and thawed on ice prior to use. The 25A5-T antibody was withdrawn from the original vial using a filter needle with 5 micron filter and then transferred into a new sterile vial, mixed well and allowed to warm to ambient temperature prior to dosing. The anti-TF antibody was formulated at appropriate concentrations to meet dose level requirements. The dosing formulations were prepared on each dosing day.

[00594] The dosing formulations were transferred immediately for dosing after preparation. On Days 1 and 15, dose formulation samples (duplicate 100 µL samples for analysis) were collected from the dosing container of all groups and were stored in a freezer set to maintain -80°C until shipment to the test site for concentration verification.

[00595] For dose administration, a topical antibiotic (tobramycin) was applied to both eyes twice on the day before and twice on the day after each injection. Prior to dosing, and following an appropriate fasting period, animals received an intramuscular injection of a sedative cocktail of ketamine (5 mg/kg) and dexmedetomidine (0.01 mg/kg). Following completion of the dosing procedure, animals received an intramuscular injection of 0.1 mg/kg atipamezole, a reversal agent for dexmedetomidine. The conjunctivae were flushed with diluted benzalkonium chloride (Zephiran™). Mydriatic drops (1% tropicamide) were applied to each eye as needed. The respective test materials were administered bilaterally by intravitreal injection on Days 1 and 15, using a ½ cc syringe with attached 29-gauge, ½ inch needle. The dose volume in all group was 100 µL/eye, administered as two 50 µL injections, 10 to 15 minutes apart. Intravitreal injections were performed by a board-certified veterinary ophthalmologist. Both eyes were examined by slit lamp biomicroscopy and/or indirect ophthalmoscopy following completion of each treatment to document any abnormalities (especially to the lens, vitreous and retina) caused by the administration procedure.

[00596] **Table 62** details the parameters that were measured in this pilot tox study.

Table 62: Parameters and measurement frequency for the cyno pilot toxicology study.

Parameter	Frequency
Mortality/Moribundity	Twice daily, once in the morning and once in the afternoon, throughout the study, the animals were observed for general health/mortality and moribundity. The animals were not removed from cage during observation, unless necessary for identification or confirmation of possible findings.
Detailed Examinations	At least every 2 weeks during the pretreatment (on Days -24, -17 and -1) and once every 2 weeks during the treatment and observation periods (on Days 14, 28 and 42). Animals were removed from the cage for examination.
Body Weights	At least every 2 weeks during the pretreatment (on Days -29, -21, -17 and -1) and once every 2 weeks during treatment and observation periods (on Days 14, 28 and 42), body weight was measured

Parameter	Frequency
Food Evaluation	Once daily; from Week -1 (pretreatment) and throughout the study. Individual qualitative food evaluation was assessed by visual inspection for overall appetite.
Ophthalmic Examinations/Ocular Exams	Once pretreatment and again on Days 3, 7, 14, 17, 29, and 43. All animals were subjected to funduscopy (direct and indirect ophthalmoscopy) and biomicroscopic (slit lamp) examinations by a vet ophthalmologist to provide a qualitative description of the front and back of the eye. The mydriatic used was 1% tropicamide. A sedative, Ketamine® HCl for Injection, U.S.P., was administered by intramuscular injection following an appropriate fasting period. Ocular inflammation was also determined using a funduscopy (direct and indirect ophthalmoscopy) and biomicroscopic (slit lamp) examinations by a vet ophthalmologist
Tonometry	Performed during ophthalmic examinations. Intraocular pressure (IOP) measurements were made using a TonoVet® rebound tonometer. Measurements were performed following administration of the mydriatic agent. Animals were sedated at the time of IOP measurement.
Serum Bioanalysis	On Days 1 and 15 (at pre-dose and at 8, 24, 168 and 336 [on Day 15 only] hours post dose). Blood samples were centrifuged at 2400 g for 10 minutes at 4°C and the resultant serum was separated, transferred to duplicate uniquely labeled clear polypropylene tubes, frozen immediately over dry ice and transferred in a freezer set to maintain -80°C. Serum samples were shipped following each collection occasion in two batches (each with one set of aliquots) on dry ice to the testing facility. Samples were stored at the bioanalytical laboratory at -80°C until analysis. Serum samples were analyzed for concentration of 25A5-T using MSD-ECLA Assay.
Anti-drug Antibody (ADA)	On Day 1 (pre-dose) and on Days 14, 21, 28, and 44. Blood samples were centrifuged at 2400 g for 10 minutes at 4°C and the resultant serum was separated, transferred to duplicate uniquely labeled polypropylene tubes, and frozen immediately over dry ice or in a freezer set to maintain -80°C. Serum samples were shipped following each collection occasion in two batches (each with one set of aliquots) on dry ice to the testing facility. Samples were stored at the bioanalytical laboratory at -80°C until analysis. Serum samples were analyzed for antibodies against 25A5-T by an ECL bridging assay.
Ocular Tissue Collection	Following scheduled euthanasia (Day 44), the right eye and optic nerve was collected and preserved in Davidson's Fixative. The left eye was enucleated and dissected. The following tissues were collected and weighed: retina and vitreous humor into cryotubes and aqueous humor into appropriately sized sterile polypropylene tubes. The tubes were frozen in liquid nitrogen prior to storage in a freezer set to maintain -80°C. Retina, vitreous and aqueous humor samples were shipped on dry ice to a testing facility for bioanalysis of drug tissue concentration and/or ADA analysis.
Tissue Drug Concentration and ADA Analysis	Tissue samples (retina, vitreous humor, and aqueous humor) were analyzed for concentration of 25A5-T. The vitreous humor was analyzed for antibodies against 25A5-T. 25A5-T concentration analysis was conducted using an MSD-ECLA Assay and the ADA analysis was conducted using an ECL bridging assay. Statistical analyses including regression analysis and descriptive statistics including arithmetic means and standard deviations, accuracy and precision were performed.

Parameter	Frequency
Histology and Microscopic Evaluation	<p>Five transverse sections of the right eye were prepared for all animals.</p> <p>Tissues (eyes and optic nerves) were evaluated histopathologically by a board-certified veterinary pathologist.</p> <p>Animal No. 3003 (administered 11.9 mg/eye), exhibiting ophthalmic examination findings that were indicative of severe ocular inflammation on Day 29 (despite a single Kenalog injection and ongoing treatments), was sent for terminal euthanasia on Day 30. This animal underwent terminal main necropsy procedures and tissue collection for microscopic examination, as well as analysis for tissue drug concentration and ADA.</p>

[00597] For the ADA assay, the drug was labeled with biotin as the capture reagent and with Sulfo-TAG™ as the detection reagent. During the acid dissociation/neutralization step, the serum or vitreous humor sample was incubated with the capture and detection reagents. Anti-25A5-T antibodies present in the sample formed a bridge between the biotin-labeled drug and the Sulfo-TAG-labeled drug. The complex was then captured onto a Streptavidin coated MSD plate. For detection, an electrical potential causes light emission as ECL from the SULFO-TAG™ labels that are bound. The assay provided a relative anti-25A5-T (titer) antibody concentration. The positive control (PC) antibody used for the screening and titer assays consisted of an affinity purified rabbit polyclonal raised against the Fab portion of 25A3.

[00598] Serum 25A5-T concentrations were highly variable, and the serum concentrations increased more than proportionally between the 4 and the 11.9 mg/eye dose groups. At Day 44, 25A5-T vitreous concentrations were quantifiable in all eyes with concentrations ranging from 38.3 ng/g (4 mg/eye animal) to 74572 ng/g (11.9 mg/eye animal). All three 4 mg/eye animals were serum ADA-positive after 25A5-T administration and 2/3 11.9 mg/eye dose group animals developed ADA following 25A5-T administration. The presence of serum ADA was generally associated with lower 25A5-T serum concentrations. All vitreous samples were ADA-positive and there was no relationship between ADA titers and 25A5-T vitreous concentrations.

Additional Veterinary Care for Specific Animals

[00599] *Animal No. 3001 (Administered 11.9 mg/eye):* Due to moderate ocular inflammation observed on Day 17 ophthalmic examinations, a mydriatic agent (atropine) was applied topically to both eyes from Days 17 to 19.

[00600] *Animal No. 3003 (Administered 11.9 mg/eye):* Due to moderate ocular inflammation observed on Day 17 ophthalmic examinations, a mydriatic agent (atropine) was applied topically to both eyes from Days 17 to 19. A brief ophthalmology examination was

performed on Day 23 and revealed ocular inflammation that was considered secondary to an immune response. As such, the animal received one subconjunctival injection (100 µL) of Kenalog®-40 (Triamcinolone acetonide, 40 mg/mL) in both eyes on Day 24, an analgesic (Buprenorphine slow-release) on Days 23, 26, and 29, and a topical anti-inflammatory (1% Prednisolone) three times daily from Day 23 to 29.

[00601] *Animal No. 2002 (Administered 4 mg/eye):* On Day 24, due to signs of pain and discomfort (blepharospasm) noted, a brief ophthalmology examination was performed on the animal and revealed similar inflammation to that seen in high dose Animal No. 3003 on Day 23. Therefore Animal No. 2002 received one subconjunctival injection (100 µL) of Kenalog®-40 (Triamcinolone acetonide, 40 mg/mL) in both eyes on Day 24, a mydriatic agent (atropine) topically in both eyes on Days 24 to 26, 31 to 32, 35 to 36, and 39 to 42, an analgesic (Buprenorphine slow-release) on Days 24, 27, 34, 37, 40, and 43, and a topical anti-inflammatory (1% Prednisolone) three times daily on Days 24 to 43.

RESULTS

[00602] *Dose Formulation Analyses*

[00603] The mean concentrations for all 25A5-T samples analyzed on Days 1 and 15 ranged from 93.5% to 100.3% of the theoretical concentrations (individual values were 92.3% to 103.5% of the theoretical concentrations). Therefore, the dose formulation concentrations were within the acceptance criteria of $\pm 20\%$ ($\pm 20\%$ for individual values) of their theoretical concentrations. 25A5-T was not detected in the control samples.

[00604] *Mortality*

[00605] One male (Animal No. 3003) given 11.9 mg/eye was euthanized on Day 30 due to severe ocular inflammation. 25A5-T-related microscopic findings were noted in the right eye and included marked global retinal degeneration and moderate retinal detachment associated with retinal necrosis and hemorrhage as well as hypertrophy of the retinal pigmented epithelium. Moderate to marked mononuclear cell infiltration was also noted in the retina (diffuse), choroid, vitreous body, iris, ciliary body, limbus and conjunctiva. These infiltrations were associated with the presence of multinucleated giant cells in the retina, accumulation of eosinophilic material in the vitreous body, hypertrophy of the ciliary body epithelium and extended into the optic disk and sclera.

[00606] *Clinical Observations*

[00607] There were no 25A5-T-related clinical signs observed during the study, with the exception of ocular inflammation in Animals 2002 and 3003, which was considered secondary to an immune response. Any other clinical observations were considered unrelated

to 25A5-T based upon their low and/or sporadic incidence, and/or similarity to observations in controls and/or during the pretreatment period.

[00608] *Body Weights and Body Weight Gains*

[00609] There were no 25A5-T-related changes in body weights or body weight gains observed during the study. Any differences noted were attributed to the fluctuations (increases and decreases) in individual body weight seen during the study and considered not related to the administration of the test item due to the lack of dose-related pattern, direction of change, and/or general overlap and variability in individual values.

[00610] *Food Evaluation*

[00611] There were no 25A5-T-related changes in appetite observed during the study.

[00612] *Ophthalmic Examinations (Prestudy and Days 3, 7, 14, 17, 29 and 43)*

[00613] Intravitreal administration of 25A5-T was performed on Day 1 and Day 15 and most noteworthy changes were seen subsequent to the second injection. Following the first injection, animals were examined three times and the two most common findings were anterior chamber cells and vitreal cells. Anterior chamber cells are regularly noted following intravitreal administration and usually resolve rapidly if procedure-related. In this study, a dose-dependent response was observed when looking at the presence of those cells as an increased incidence of eyes graded moderate on Day 3 was observed (2 eyes in the 4 mg/eye group and 4 eyes in the 11.9 mg/eye group). By Day 14, anterior chamber cells should have resolved if procedure-related, however, they were observed in most eyes in the high-dose group (5/6 eyes and most of them graded very slight) and none were seen in the low-dose group. Vitreal cells were the most common observation and were noted in nearly all eyes on this study (by Day 14, all were graded very slight except for one reference item animal that was graded slight).

[00614] The second injection was performed on Day 15 and caused more inflammation in the two treated groups. The incidence and severity of ocular changes were higher in eyes treated with 11.9 mg/eye, supporting a dose-dependent effect. Two out of three animals in the low- and high-dose groups developed more prominent inflammation. Some observations included anterior chamber cells, flare and fibrin, keratic precipitates/corneal endothelial cells, cellular deposits on the anterior lens capsule and incomplete pupil dilation. Changes at the level of the posterior segment (behind the lens) included retinal sheathing (perivascular retinal opacities), vitreal haze and cells, retina/choroid hemorrhage and fundus hazy view.

[00615] Due to the severe inflammation of both eyes in one high-dose animal (Animal No. 3003), it was sacrificed early on Day 30 and its last examination was on Day 29. Moderate to

severe ocular changes included anterior chamber cells, flare, cells on corneal endothelium and lens capsule, vitreous haze and cells, and most importantly, retinal sheathing. Slight chorio-retinal hemorrhages were also noted. Despite improvement at the level of the anterior chamber, the second high-dose animal with more inflammation (Animal No. 3002) developed retinal sheathing. The remaining high-dose animal (Animal No. 3001) had procedure-related changes (transient anterior chamber and flare post dosing) and by the end of the observation period, only minor vitreal cells were noted along with some vitreous air bubbles in the superior vitreal chamber.

[00616] In the low-dose group, Animal No. 2001 was comparable to Animal No. 3001 with minor and transient ocular changes associated with the dosing procedure and vitreal cells still present at the end of the study. Animal No. 2003 did not develop significant anterior segment inflammation; however, retinal sheathing was observed (graded very slight) during the last two occasions. The most inflamed low-dose treated eyes were seen in Animal No. 2002 in which anterior segment inflammation was mostly observed following the second dosing but had resolved by the end of the study. This animal had retinal sheathing that was graded from slight to moderate and both eyes were graded slight on the last exam supporting improvement over time.

[00617] The control group did not develop significant inflammation. The most common finding was the presence of vitreal cells seen in 4/6 eyes on the last exam and in all 25A5-T treated animals.

[00618] *Tonometry (Intraocular Pressure, IOP)*

[00619] There were no 25A5-T-related effects on intraocular pressure observed at any dose level. Intraocular pressures measured on Days 3, 7, 14, 17, 29, and 43 were generally within normal ranges in all animals.

[00620] In Animal No. 3003 (given 11.9 mg/eye), a notably low IOP (3 mmHg) was measured in both eyes on Day 29, and was considered secondary to the severe ocular inflammation.

[00621] *Serum and Tissue 25A5-T Concentration Analysis*

[00622] 25A5-T concentrations in serum, vitreous humor, retina and aqueous humor samples were below the lower limit of quantitation (LLOQ; 20 ng/mL) for all control samples (Group 1) and Day 1 predose serum samples from 25A5-T-treated Groups 2 and 3.

[00623] Serum concentrations of 25A5-T were highly variable, were quantifiable at all time points in samples collected from 25A5-T-treated animals on Day 1 and were generally quantifiable up to 168 hours postdose on Day 15.

[00624] Mean serum concentrations increased with dose and in a greater than dose-proportional manner. At the timepoints evaluated, peak mean serum concentrations occurred at 24 hours postdose in both Days 1 and 15 (**FIG. 18** and **FIG. 19**).

[00625] Vitreous humor, retina and aqueous humor concentrations of 25A5-T were quantifiable on Day 44 in all 25A5-T-treated animals with the exception of Animal No. 2002 (all tissue samples below LLOQ) and Animal No. 3002 (below LLOQ for aqueous humor samples only). Despite lower serum concentrations from Day 14 onward compared to other animals given 11.9 mg/eye, Animal No. 3003 (that was euthanized on Day 30 due to severe ocular inflammation), had markedly higher 25A5-T concentrations measured in the vitreous humor, retina and aqueous humor samples on Day 30 compared to the concentrations measured in animals of the same dose group on Day 44, with the greatest concentrations noted in the vitreous humor and retina.

[00626] *Anti-25A5-T-Antibody (Predose and Days 14, 21, 28 and 44)*

[00627] All 3 control animals from Group 1 were screened negative for anti-drug antibodies (ADAs) in serum throughout the time points. One of the 3 animals (Animal No. 1001) was screened positive for ADAs in its vitreous humor (titer of 1).

[00628] For the 4 mg/eye and 11.9 mg/eye groups, all 3 animals screened positive for ADAs in the vitreous humor on Day 44 with no apparent effect on vitreous humor, aqueous humor, or retinal concentrations of 25A5-T associated with the ADA titer. The ADA titers in the positive samples were relatively low and tended to increase over time. There was no association with systemic or vitreous humor titers and the level of ocular inflammation.

[00629] *Microscopic Findings- Terminal Euthanasia Animals (Day 44)*

[00630] 25A5-T-related microscopic findings were noted in the right eye and are summarized in **Table 63**.

Table 63: Summary of Microscopic Findings – Scheduled Euthanasia (Day 44)

	Males		
	Group 1	Group 2	Group 3
	Dose (mg/eye) 0	4	11.9
No. Animals per Group	3	3	3
Eye, right (No. Examined)	3	3	2
Infiltration, mononuclear cell; retina	(0) ^a	(3)	(1)
Minimal	-	3	0
Mild	-	0	1
Infiltration, mononuclear cell; vitreous body	(0)	(3)	(1)
Minimal	-	3	1
Infiltration, mononuclear cell; iris; ciliary body	(0)	(2)	(1)
Minimal	-	2	0
Mild	-	0	1
Infiltration, mononuclear cell; limbus; conjunctiva	(0)	(1)	(1)
Minimal	-	1	1

		Males		
Group	1	2	3	
Dose (mg/eye)	0	4	11.9	
No. Animals per Group	3	3	3	

^a Numbers in parentheses represent the number of animals with the finding.

[00631] In the right eye of animals administered ≥ 4 mg/eye, there was minimal to mild mononuclear cell infiltration in the retina, vitreous body, iris/ciliary body and/or limbus/conjunctiva (extending into the sclera) with a minor dose-related increase in severity. In the retina, these infiltrations, mainly perivascular, extended into the optic disk and sometimes were associated with rare single cell necrosis in the photoreceptor layer.

[00632] Other microscopic findings observed were considered incidental, of the nature commonly observed in this strain and age of monkeys, and, therefore, were considered unrelated to administration of 25A5-T.

DISCUSSION

[00633] Intravitreal administration of 25A5-T was performed on Day 1 and Day 15. Dose-related ocular inflammation was observed at both 4 and 11.9 mg/eye and the findings were more severe following the second dose administration.

[00634] Following the first injection, anterior and posterior segment changes were observed at both 4 and 11.9 mg/eye with the two most common findings being anterior chamber cells and vitreal cells. Following the second injection, the eyes of animals given ≥ 4 mg/eye developed inflammation with a stronger response at 11.9 mg/eye. The ocular inflammation was severe enough to require the early euthanasia of 1/3 animals receiving 11.9 mg/eye despite local steroid treatment.

[00635] Perivascular retinal opacities are associated with the movement of inflammatory cells from the circulation into the retinal tissue. Both dose groups had 2/3 animals (4/6 eyes) that exhibited this change on Day 29 (2 weeks following the second dose). By Day 43, perivascular retinal opacities were still observed in 2/3 animals at 4 mg/eye, but with improvement of the findings in 1 of the 2 animals. At 11.9 mg/eye, perivascular retinal opacities were only observed in 1/3 animals and there was improvement in the severity of the findings.

[00636] Ocular changes were also more severe following the second injection (on Day 15) in both treated groups, indicating the development of an immune response (as supported by the general positive serum ADA results on Days 21, 28 and 44) following sensitization associated with the first injection.

[00637] Overall, the presence of anti-drug antibodies (ADAs) had an impact on serum concentrations in 25A5-T-treated animals, as seen by the decreased serum 25A5-T concentrations at TK timepoints that were preceded by a positive ADA serum result. There were no degenerative ocular changes observed microscopically.

CONCLUSION

[00638] In conclusion, administration of 25A5-T to cynomolgus monkeys by bilateral intravitreal injections on Day 1 and 15 at dose levels of 4 or 11.9 mg/eye resulted in varied ocular inflammation in both treated groups, with high individual variation in response to treatment during the in-life phase at each dose level (little to no inflammation to significant inflammatory response). In general, the inflammatory response was dose-related in severity and resulted in early termination of one 11.9 mg/eye animal 2 weeks following the second dose. There were no degenerative ocular changes observed microscopically. Immunogenicity screening confirmed a general positive ADA result in both the serum and vitreous humor as early as Day 14 in the serum for 1/3 animals and mostly following the second intravitreal injection for the remainder.

Example 11: Effect of 25A5-T on Ocular and Systemic Multidose Administration in Cyno Monkeys

[00639] Cynomolgus monkeys were selected as the species for *in vivo* toxicology, partly because 25A5-T affinity for monkey and human TF is similar. 25A5-T pharmacokinetics in cynomolgus monkeys is more relevant to 25A5-T disposition in humans. To evaluate the effect of 25A5-T ocular and systemic disposition after multiple doses 40 cynomolgus divided into 4 groups and received IVT administration of 100 uL of 25A5-T, once monthly for a total of 3 doses. The experimental design is provided in **Table 73**.

Table 73: Experimental design for 25A5-T Multi-Dose TK study

Group No.	Test Material	Dose Level (mg/eye) ^a	Dose Volume (μL/eye) ^b	Dose Concentration (mg/mL)	No. of Animals			
					Main Study		Recovery Study	
					Males	Females	Males	Females
1	Reference Item	0	100	0	4	4	2	2
2	25A5-T	0.6	100	6	4	4	-	-
3	25A5-T	2.0	100	20	4	4	-	-
4	25A5-T	6.0	100	60	4	4	2	2

^a Animals were dosed on Days 1, 28 and 56, Main Study animals were euthanized on Day 85 and the Recovery cohort on Day 113.

^a The dose volume was 100 μL/eye, administered as two 50 μL injections, 10 to 15 minutes apart.

- = Not applicable.

[00640] As shown in **Table 73**, the main study monkeys were sacrificed ~ 28 days after the 3rd dose (Day 85), while the recovery animals were sacrificed ~56 days following administration of the 3rd dose (Day 113). Due to the presence of intraocular inflammation, some animals received topical steroids following administration of the 2nd dose and all 25A5-T-treated animals received subconjunctival triamcinolone prior to administration of the 3rd 25A5-T dose. Due to the inflammation, 7 eyes did not receive all three bilateral doses as in the experimental design.

[00641] Blood samples were collected during each dosing interval for quantification of 25A5-T concentrations and ADA in serum samples. The left eye was collected from all animals on Day 85 (main study) or 113 (recovery animals). Vitreous, aqueous, and retinal samples were analyzed for 25A5-T concentrations, while vitreous was also analyzed for ADA. Serum concentration-time profiles were analyzed by non-compartmental methods. Ocular concentrations at Day 85 and Day 113 were reported individually.

[00642] The serum concentration-time profiles at Day 1 are shown in **FIGS. 24A-24B** and those for Day 56 are shown in **FIGS. 25A-25B**. Following the first administration, 25A5-T serum concentrations increased with the dose in both males and females and were dose proportional for the two lower doses and more than proportional for the 6 mg/eye dose. There were no significant differences observed between male and female monkeys. Following the 2nd and the 3rd administration, 25A5-T serum concentrations were generally lower than following the first administration and could not be quantitated in most animals, therefore no TK parameters were calculated after the 1st dose. The decrease in 25A5-T concentration over time can most likely be attributed to the development of ADA.

[00643] Approximately 28 days after administration of the last dose, vitreous 25A5-T concentrations were < LLOQ *i.e.* < 20 ng/mL for the 0.6 mg/eye dose group and were quantifiable in 3/8 eyes only with concentrations ranging from 37.2–134 ng/g for the animals receiving 2 mg/eye. Approximately 56 days after the last 25A5-T administration of 6 mg/eye dose, vitreous concentrations were quantifiable in a total of 4/8 animals with concentrations of 33.2 ng/g and 34.5 ng/g in the two ADA positive animals, and concentrations of 1650 ng/g and 261 ng/g for the two ADA negative animals. Due to the presence of ADA, 25A5-T vitreous concentrations are most likely underestimated.

[00644] Human vitreous volume is ~ 4.5 mL, and the cynomolgus vitreous volume is ~ 2 mL, thus for any given IVT dose, the maximum vitreous concentrations in humans are expected to be 2.25 lower than in monkeys.

Example 12: Effect of 25A5-T on Inflammatory Cytokine Inhibition

[00645] To evaluate the effect of 25A5-T on the inhibition of relevant inflammatory cytokine (*e.g.*, IL-8 and GM-CSF), the inflammatory cytokine protein levels are measured as described previously in Hjortoe *et al.*, *Blood*, 2004, 103:3029-3037. For example, TF-positive ocular cells undergo a 2 hr serum starvation with Leibovitz's L-15 medium and are incubated with an 8-point 1:2.5 titration starting at 100 nM of anti-TF antibody 25A5-T. After 30 min in the presence of FVII at 37°C, FVIIa (NovoSeven RT, Novo Nordisk, Bagsvaerd, Denmark) is added to the cells at a final concentration of 20 nM. 5 hr later cell culture supernatants is harvested and analyzed by ELISA or an Olink custom panel for the inflammatory cytokines as recommended in manufacturer instructions.

[00646] The following inflammatory cytokines are measured: IL-1 beta, IL-6, IL-8, IL-9, IL-10, IL-12, MCP-1 (CCL2), MCP-3 (CCL7), VEGF, TNF, MMP9. A standard curve using each of the recombinant inflammatory cytokines (R&D Biosystems, Minneapolis, MN, USA) is used in Prism to calculate cytokine concentrations in the cell culture supernatants. Percent of each of the inflammatory cytokines at reported antibody concentration is calculated relative to a no-antibody control. The results show a significant decrease in measured inflammatory cytokine(s) in the cell culture supernatant relative to the no-antibody control group.

Example 13: Binding Affinity Assay

[00647] Kinetic measurements for the anti-TF antibodies were conducted on an Octet QK384 (Pall ForteBio, Fremont, CA, USA) or a Biacore (GE Healthcare Bio-Sciences).

[00648] ForteBio affinity measurements were performed generally as previously described (Estep *et al.*, *MAbs*. 2013 Mar-Apr;5(2):270-8). Briefly, ForteBio affinity measurements were performed by loading IgGs on-line onto AHC sensors. Sensors were equilibrated off-line in assay buffer for 30 min and then monitored on-line for 60 seconds for baseline establishment. Sensors with loaded IgGs were exposed to 100 nM antigen (human, cynomolgus, or mouse TF) for 3 min, afterwards they were transferred to assay buffer for 3 min for off-rate measurement. Alternatively, binding measurements were obtained by loading biotinylated TF monomer on SA sensors followed by exposure to 100 nM antibody Fab in solution. Kinetic data was analyzed and fitted using a 1:1 Langmuir binding model and the

K_D was calculated by dividing the k_{off} by the k_{on} . The K_D values of the TF antibodies measured by the Octet-based experiments are shown in **Table 5**.

[00649] For the Biacore-based measurements, the antibody was covalently coupled to a CM5 or C1 chip using an amine-coupling kit (GE Healthcare Bio-Sciences). Association between the anti-TF antibodies and a five-point three-fold titration of TF-His starting at 25 to 500 nM was measured for 300 sec. Subsequently, dissociation between the anti-TF antibody and TF-His was measured for up to 1800 sec. Kinetic data was analyzed and fitted globally using a 1:1 binding model. The K_D values of the TF antibodies measured by the Biacore-based experiments are shown in **Table 5**.

[00650] As shown in **Table 5**, the affinity of the antibodies for hTF, as indicated by K_D , is between 10^{-7} M and 10^{-11} M. All anti-hTF antibodies are cross-reactive with cTF. In addition, all anti-hTF antibodies from groups 25 and 43 exhibit binding activity to mTF. The anti-hTF antibodies 25G, 25G1, 25G9, and 43D8 are cross-reactive with mTF. There are no other reported human or humanized anti-hTF monoclonal antibodies that exhibit binding activity and cross-reactivity to mouse TF, indicating that the antibodies from groups 25 and 43 bind to a novel TF epitope.

Table 5: Antibody Kinetics

Ab	Human K_D (nM) [Biacore]	Cynomolgus K_D (nM) [Biacore]	Mouse K_D (nM) [Biacore]	Human K_D (nM) [ForteBio]	Cynomolgus K_D (nM) [ForteBio]	Mouse K_D (nM) [ForteBio]
1F	0.31	0.26	nd*	1.28	1.43	no binding*
1G	nd*	nd*	nd*	2.20	2.70	nd*
25A	6.20	5.42	nd*	8.45	7.65	263
25A3	0.36	0.21	nd*	1.67	1.36	131
25A5	0.08	0.04	nd*	0.64	0.76	188
25G	23.0	18.0	nd*	21.9	17.5	114
25G1	0.94	0.78	5.4	3.97	4.99	34.2
25G9	13.3	16.4	2.9	35.8	42.9	9.16
29D	nd*	nd*	nd*	3.30	12.0	nd
29E	0.47	5.06	nd*	2.32	15.0	no binding*
39A	0.09	0.08	nd*	0.83	0.57	no binding*
43B	1.75	5.64	nd*	2.40	3.40	161
43B1	0.07	0.12	nd*	0.96	1.05	72.1
43B7	0.14	0.24	nd*	0.86	0.94	360
43D	2.09	5.66	nd*	3.84	4.12	281
43D7	0.06	0.12	21	1.02	1.11	41.4

Ab	Human K _D (nM) [Biacore]	Cynomolgus K _D (nM) [Biacore]	Mouse K _D (nM) [Biacore]	Human K _D (nM) [ForteBio]	Cynomolgus K _D (nM) [ForteBio]	Mouse K _D (nM) [ForteBio]
43D8	0.15	0.39	2.4	1.61	1.96	6.12
43E	1.46	5.69	nd*	2.52	4.07	121
43Ea	1.60	6.42	nd*	2.28	2.71	140
54E	0.42	1.83	nd*	1.59	4.16	no binding*

no binding*: no to weak binding, with no reportable K_D

nd*: not determined

Example 14: Cell-Based Binding Assay

[00651] HCT116 cells with endogenous expression of human TF were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) and were maintained as recommended. Flp-In-CHO cells expressing mouse TF were generated by transfection of Flp-In-CHO cells as recommended with a pcDNA5/FRT vector (ThermoFisher Scientific) encoding full-length mouse TF with a C-terminal FLAG tag. A mouse TF-positive CHO clone was isolated by limiting dilution in tissue culture-treated 96-well plates.

[00652] Cell-based antibody binding was assessed as previously described in Liao-Chan *et al.*, *PLoS One*, 2015, 10:e0124708, which is incorporated by reference in its entirety. 1.2×10^5 cells collected with Cellstripper (Mediatech, Manassas, VA, USA) were incubated with a twelve-point 1:3 dilution titration of anti-human TF IgG1 or Fab antibody starting at 250 nM or 100 nM for 2 hr on ice. After 2 washes, cells labeled with IgG1 or Fab were incubated for 30 min on ice with 150 nM of Goat Phycoerythrin (PE) F(ab')₂ fragment goat anti-human IgG, Fcγ fragment specific (Jackson ImmunoResearch, West Grove, PA, USA) or FITC-labeled F(ab')₂ fragment goat anti-human kappa (SouthernBiotech, Birmingham, AL, USA), respectively. After 2 washes, dead cells were labeled with TO-PRO-3 Iodide (ThermoFisher Scientific) and samples were analyzed on a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) or Novocyte flow cytometer (ACEA Biosciences, San Diego, CA, USA). The median fluorescence intensities (MFIs) at each dilution were plotted and cell EC₅₀'s were derived using a 4-parameter binding model in Prism (GraphPad, La Jolla, CA, USA). The results of binding of anti-TF antibodies to human TF-positive HCT-116 cells are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety. The results of binding of anti-TF antibodies to CHO cells expressing mouse TF are shown in international PCT application

PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety.

[00653] All anti-hTF antibodies are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652 exhibit high affinity to human TF-positive HCT-116 cells with an EC_{50} ranging from about 687 pM to about 39 pM. Antibodies from groups 25 and 43 exhibit binding to CHO cells expressing mouse TF with an EC_{50} ranging from about 455 nM to about 2.9 nM, are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety. The binding activity to mouse TF is a unique property of the anti-hTF antibodies from groups 25 and 43. This is advantageous for pre-clinical studies of these antibodies with mouse models.

Example 15: Thrombin Generation Assay (TGA)

[00654] The TGA assay was performed using the calibrated-automated-thrombogram (CAT) instrument manufactured and distributed by STAGO. The test method design was equivalent to a standard CAT assay measurement, except that the plasma source was normal pooled plasma (NPP) in citrate/CTI. The anti-TF antibodies were titrated at 0, 10, 50 and 100 nM and mixed with NPP collected in 11 mM citrate supplemented with 100 microgram/mL of corn trypsin inhibitor (citrate/CTI). Relipidated TF was added to a 96-well assay plate, followed by addition of the antibody/NPP mixture. After a 10-min incubation or directly after combining the relipidated TF with antibody/NPP, thrombin generation was initiated by the addition of calcium and the thrombin substrate. The STAGO software was used to report the following parameters: Peak IIa (highest thrombin concentration generated [nM]); Lag Time (time to IIa generation [min]); ETP (endogenous thrombin potential, area under the curve [nM x min]); and ttPeak (time to Peak IIa [min]). Percent peak thrombin generation (% Peak IIa) and percent endogenous thrombin potential (% ETP) in the presence of each antibody relative to a no antibody plasma control on the same plate were also reported.

[00655] The Peak IIa, Lag Time, ETP, ttPeak, % Peak IIa, and % ETP in the presence of each antibody selected from 1F, 25A, 25A3, 25G1, 29E, 39A, 43B1, 43D7, 43Ea, and 54E without antibody incubation prior to addition of calcium and thrombin substrate are shown in **Table 6**. The Peak IIa, Lag Time, ETP, ttPeak, % Peak IIa, and % ETP in the presence of each antibody selected from 1F, 25A, 25A3, 25G1, 29E, 39A, 43B1, 43D7, 43Ea, and 54E with 10 min antibody incubation prior to addition of calcium and thrombin substrate are shown in **Table 7**. The % Peak IIa in the presence of titrations of anti-TF antibodies without

antibody incubation prior to addition of calcium and thrombin substrate are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety. The % Peak IIa in the presence of titrations of anti-TF antibodies with 10 min antibody incubation prior to addition of calcium and thrombin substrate are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety.

[00656] The % Peak IIa is greater than 90% in the presence of antibodies from group 25, including 25A, 25A3, and 25G1. The % ETP is greater than 100% in the presence of antibodies from group 25, including 25A, 25A3, and 25G1. The % Peak IIa is greater than 40% in the presence of antibodies from group 43, including 43B1, 43D7, and 43Ea. The % ETP is greater than 90% in the presence of antibodies from group 43, including 43B1, 43D7, and 43Ea.

[00657] This data indicates that antibodies from groups 25 and 43 allow normal thrombin generation, and therefore are not inhibitors of thrombin generation.

Table 6: Thrombin Generation Assay without Antibody Pre-Incubation

Plate	Antibody	Ab conc. (nM)	Peak IIa (nM)	Lag Time (min)	ETP (nM•min)	ttPeak (min)	% Peak IIa	% ETP
1	1F	100	29	25.9	*	37.9	7	*
		50	32	27.2	*	36.8	8	*
		10	83	12.1	1395	19.8	21	58
1	25A	100	398	4.4	2610	7.1	99	108
		50	399	4.2	2621	7.1	99	108
		10	403	4.1	2555	6.8	100	106
1	25A3	100	405	3.9	2493	6.5	100	103
		50	404	3.9	2495	6.6	100	103
		10	401	4.2	2550	7.3	99	106
1	25G1	100	416	4.5	2626	7.1	103	109
		50	416	4.5	2680	7.1	103	111
		10	417	4.5	2635	7.0	103	109
1	29E	100	99	17.3	*	26.4	25	*
		50	107	14.4	1747	22.7	26	72
		10	266	5.7	2189	10.0	66	91
1	39A	100	26	28.9	*	40.1	6	*
		50	30	30.5	*	40.0	7	*
		10	82	12.1	1330	19.6	20	55
1	Plasma ctrl.	NA	403	4.1	2417	6.8	100	100
2	43B1	100	221	5.2	2167	10.6	64	100
		50	232	5.2	2195	10.3	67	101
		10	299	4.9	2288	8.9	87	105
2	43D7	100	179	5.4	2094	11.8	52	96
		50	202	5.3	2116	11.1	58	97

Plate	Antibody	Ab conc. (nM)	Peak IIa (nM)	Lag Time (min)	ETP (nM•min)	ttPeak (min)	% Peak IIa	% ETP
		10	287	5.0	2263	9.0	83	104
2	43Ea	100	300	4.6	2219	8.1	87	102
		50	307	4.6	2234	8.1	89	103
		10	328	5.0	2329	8.3	95	107
		100	68	14.8	1175	23.9	20	54
2	54E	50	154	8.9	2019	15.9	44	93
		10	307	5.7	2307	9.6	89	106
		100	348	5.0	2415	8.3	101	111
2	Isotype	50	347	5.0	2360	8.0	101	109
		10	346	4.3	2260	7.6	100	104
2	Plasma ctrl.	NA	345	4.7	2171	7.8	100	100

* Groups with "No Tail Found" Errors when the software cannot calculate the ETP.

Table 7: Thrombin Generation Assay with 10 min Antibody Pre-Incubation

Plate	Antibody	Ab conc. (nM)	Peak IIa (nM)	Lag Time (min)	ETP (nM•min)	ttPeak (min)	% Peak IIa	% ETP
1	1F	100	17	30.3	*	42.0	7	*
		50	20	27.6	*	38.9	7	*
		10	27	18.8	540	28.6	10	31
1	25A	100	285	3.3	1898	6.7	108	110
		50	284	3.3	1887	6.6	107	110
		10	277	3.3	1842	6.7	105	107
1	25A3	100	277	3.1	1785	6.3	105	104
		50	275	3.2	1824	6.4	104	106
		10	278	3.2	1827	6.6	105	106
1	25G1	100	293	3.3	1827	6.4	111	106
		50	301	3.3	1853	6.3	114	108
		10	302	3.3	1891	6.3	114	110
1	29E	100	68	15.1	1098	25.3	26	64
		50	70	14.2	1168	24.3	27	68
		10	78	10.4	1254	20.2	30	73
1	39A	100	17	28.0	*	40.2	7	*
		50	17	28.4	346	38.9	7	20
		10	25	20.8	482	30.7	9	28
1	Plasma ctrl.	NA	264	3.3	1720	6.8	100	100
2	43B1	100	152	3.2	1712	9.3	58	98
		50	163	3.2	1797	9.0	62	103
		10	200	3.2	1788	8.1	76	103
2	43D7	100	124	3.6	1656	10.3	47	95
		50	128	3.6	1677	10.3	49	96
		10	178	3.6	1745	8.8	68	100
2	43Ea	100	239	2.9	1820	6.9	91	104
		50	227	2.9	1791	7.1	87	103
		10	247	3.2	1825	7.0	94	105
2	54E	100	29	22.1	580	32.3	11	33
		50	35	18.3	680	28.4	13	39

Plate	Antibody	Ab conc. (nM)	Peak IIa (nM)	Lag Time (min)	ETP (nM•min)	ttPeak (min)	% Peak IIa	% ETP
		10	112	6.1	1530	13.4	43	88
		100	288	3.2	1888	6.6	110	108
2	Isotype	50	285	3.2	1879	6.6	109	108
		10	273	3.2	1804	6.6	104	104
2	Plasma ctrl.	NA	262	3.2	1742	6.9	100	100

* Groups with "No Tail Found" Errors when the software cannot calculate the ETP.

Example 16: FXa Conversion Assay

[00658] To evaluate the ability of TF:FVIIa to convert FX into FXa in the presence of human antibodies against TF, 5×10^4 MDA-MB-231 cells (ATCC, Manassas, VA, USA) were plated into tissue culture-treated black 96-well plates (Greiner Bio-One, Monroe, NC, USA). After removal of the cell culture media and addition of a final concentration of 200 nM of FX in a HEPES buffer with 1.5 mM CaCl_2 , cells were incubated with a titration of the antibodies for 15 min at 37°C. Upon reconstitution of the binary TF:FVIIa complex with a final concentration of 20 nM of FVIIa, cells were incubated for 5 min at 37°C. After quenching the reaction with ethylenediaminetetraacetic acid (EDTA), generated FXa was measured with 50 μM of SN-7 6-amino-1-naphthalenesulfonamide-based fluorogenic substrate (Haematologic Technologies, Essex Junction, VT, USA) on an Envision plate reader equipped with an Umbelliferone 355 excitation filter, an Umbelliferone 460 emission filter, and a LANCE/DELTA top mirror (Perkin Elmer, Waltham, MA, USA). FXa conversion percentages (% FXa) in the presence of an anti-TF antibody titration relative to a no-antibody control are summarized in **Table 8** and plotted in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety.

[00659] The FXa conversion percentage ranges from about 78% to about 120% in presence of different concentrations of antibodies from groups 25 and 43, including 25A, 25A3, 25G, 25G1, 25G5, 25G9, 43B, 43B1, 43B7, 43D, 43D7, 43D8, 43E, and 43Ea.

[00660] This data indicates that anti-TF antibodies from groups 25 and 43 do not inhibit TF:FVIIa mediated FXa conversion from FX. This data also indicates that anti-TF antibodies from groups 25 and 43 have a human TF binding site that is distinct from the human TF binding site bound by FX.

Table 8: % FXa conversion

Antibody	% FXa			
	12.5 nM	25 nM	50 nM	100 nM
1F	49	40	37	38
1G	55	48	41	41
25A	87	81	94	89
25A3	89	89	93	96
25A5	82	85	78	89
25G	99	109	102	116
25G1	101	96	99	108
25G9	98	97	104	117
29D	85	77	75	75
29E	81	68	63	66
39A	39	38	37	39
43B	113	109	105	105
43B1	106	108	108	112
43B7	113	104	108	112
43D	115	109	104	106
43D7	110	103	102	103
43D8	120	112	107	111
43E	85	89	97	98
43Ea	108	103	106	101
54E	53	44	41	42
5G9	37	33	30	30
Isotype ctrl	93	95	89	97

Example 17: FVIIa Competition Assay

[00661] FVII-Fc conjugates were generated using Alexa Fluor 488 5-sulfo-dichlorophenol esters (ThermoFisher Scientific). Excess Alexa Fluor dye was removed from the conjugate preparations by gel filtration (ThermoFisher Scientific).

[00662] To evaluate competition between FVIIa and the human antibodies against TF, TF-positive MDA-MB-231 cells (ATCC, Manassas, VA, USA) were first incubated for 1 hr on ice with a titration of the human antibodies against TF. Subsequently, a final concentration of 20 nM of FVII-Fc conjugated to Alexa488 was added to the antibody cell mixture. After another 1 hr incubation on ice, cells were washed, stained with a viability dye, and analyzed by flow cytometry. The Alexa488 fluorescence data from viable cells was summarized using median fluorescence intensity. FVII-Fc binding was summarized with % FVII-Fc binding = $[\text{MFI}_{\text{antibody labeled cells}} - \text{MFI}_{\text{unstained cells}}] / [\text{MFI}_{\text{IgG1 control labeled cells}} - \text{MFI}_{\text{unstained cells}}]$.

Percentage of FVIIa binding (% FVIIa) in the presence of an anti-TF antibody titration relative to a no-antibody control is summarized in **Table 9** and are shown in international

PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety.

[00663] The FVIIa binding percentage ranges from about 76% to about 102% in the presence of antibodies of different concentrations from groups 25 and 43, including 25A, 25A3, 25G, 25G1, 25G5, 25G9, 43B, 43B1, 43B7, 43D, 43D7, 43D8, 43E, and 43Ea.

[00664] This data indicates that anti-TF antibodies from groups 25 and 43 do not compete for binding to human TF with FVIIa. This data also indicates that anti-TF antibodies from groups 25 and 43 have a human TF binding site that is distinct from the human TF binding site bound by FVIIa.

Table 9: Competition of Anti-TF Antibody with FVIIa

Antibody	% FVIIa			
	9.25 nM	28 nM	83 nM	250 nM
1F	7	7	7	6
1G	7	7	7	6
25A	100	101	97	98
25A3	90	87	88	87
25A5	76	79	77	80
25G	97	96	93	92
25G1	97	93	94	95
25G9	93	93	91	89
29D	6	4	3	3
29E	5	3	2	2
39A	2	2	2	2
43B	99	95	93	91
43B1	97	95	93	91
43B7	98	98	97	97
43D	102	100	98	94
43D7	101	102	100	101
43D8	100	99	98	96
43E	95	92	91	89
43Ea	93	91	92	89
54E	11	3	3	2
Isotype	99	98	97	99

Example 18: TF Signaling Assay

[00665] IL-8 and GM-CSF protein levels were measured as described previously in Hjortoe *et al.*, *Blood*, 2004, 103:3029-3037. TF-positive MDA-MB-231 cells (ATCC, Manassas, VA, USA) that underwent a 2 hr serum starvation with Leibovitz's L-15 medium were incubated with an 8-point 1:2.5 titration starting at 100 nM of anti-TF antibody. After

30 min in the presence of FVII at 37°C, FVIIa (NovoSeven RT, Novo Nordisk, Bagsvaerd, Denmark) was added to the cells at a final concentration of 20 nM. 5 hr later cell culture supernatants were harvested and analyzed by ELISA for IL8 or GM-CSF as recommended (R&D Biosystems, Minneapolis, MN, USA). A standard curve using recombinant IL8 or GM-CSF (R&D Biosystems, Minneapolis, MN, USA) was used in Prism to calculate cytokine concentration in the cell culture supernatants. Percent IL8 and GM-CSF (% IL8 and % GM-CSF) at reported antibody concentration were calculated relative to a no antibody control. The concentration of IL8 with the anti-TF antibody titration are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety and the % IL8 at different antibodies concentrations are shown in **Table 10**. The concentration of GM-CSF with the anti-TF antibody titration is shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety and the % IL8 at different antibodies concentrations are shown in **Table 11**.

[00666] IL8 concentrations were reduced by more than 75% in the presence of the anti-TF antibodies at concentrations greater than or equal to 6.4 nM. GM-CSF concentrations were reduced by more than 60% in the presence of the anti-TF antibodies at concentrations greater than or equal to 6.4 nM.

[00667] This data indicates that all tested anti-TF antibodies inhibit FVIIa-dependent TF signaling.

Table 10: Inhibition of IL8

Antibody	% IL8				
	100 nM	40 nM	16 nM	6.4 nM	2.56 nM
1F	2	2	2	3	18
1G	2	2	3	4	26
25A	9	8	10	11	43
25A3	8	8	8	9	47
25A5	6	7	7	14	70
25G	9	10	16	22	60
25G1	9	8	9	12	46
25G9	13	14	15	22	51
29D	1	2	2	6	27
29E	2	2	2	5	33
39A	3	2	2	6	52
43B	4	4	5	11	50
43B1	5	5	6	12	56
43B7	4	4	8	15	55
43D	5	5	7	21	58
43D7	5	4	5	11	48
43D8	5	5	5	21	67

Antibody	% IL8				
	100 nM	40 nM	16 nM	6.4 nM	2.56 nM
43E	5	5	6	15	49
43Ea	6	6	6	14	52
54E	2	2	3	8	48
Control	106	108	84	88	90

Table 11: Inhibition of GM-CSF

Antibody	% GM-CSF				
	100 nM	40 nM	16 nM	6.4 nM	2.56 nM
1F	6	6	6	8	27
1G	7	7	7	9	34
25A	22	19	22	24	57
25A3	20	19	19	20	59
25A5	12	15	14	18	72
25G	19	18	32	39	77
25G1	17	16	17	18	48
25G9	25	26	26	34	60
29D	5	6	7	15	38
29E	6	6	5	9	33
39A	7	5	5	8	42
43B	14	13	12	21	59
43B1	11	11	13	16	50
43B7	11	11	13	17	50
43D	12	11	13	24	56
43D7	10	10	9	15	45
43D8	12	11	11	24	57
43E	14	15	15	21	61
43Ea	14	15	14	21	65
54E	5	5	5	10	38
Control	105	111	94	86	88

Example 19: Antibody Competition Assay

[00668] Alexa Fluor antibodies were generated using Alexa Fluor 488 5-sulfo-dichlorophenol esters (ThermoFisher Scientific). Excess Alexa Fluor dye was removed from the antibody dye conjugate preparations by gel filtration (ThermoFisher Scientific).

[00669] To evaluate competition between a first human antibody against TF and 25A, TF-positive A431 cells (ATCC, Manassas, VA, USA) were first incubated for 1 hr on ice with a titration of the first human antibody against TF. Subsequently, a final concentration of 20 nM of 25A conjugated to Alexa488 was added to the antibody cell mixture. After another 1 hr incubation on ice, cells were washed, stained with a viability dye, and analyzed by flow cytometry. The Alexa488 fluorescence data from viable cells was summarized using median fluorescence intensity. 25A binding was summarized with % 25A binding = $[\text{MFI}_{\text{antibody labeled cells}} - \text{MFI}_{\text{unstained cells}}] / [\text{MFI}_{\text{IgG1 control labeled cells}} - \text{MFI}_{\text{unstained cells}}]$.

[00670] To evaluate competition between a first human antibody against TF and 43Ea, TF-positive A431 cells (ATCC, Manassas, VA, USA) were first incubated for 1 hr on ice with a titration of the first human antibody against TF. Subsequently, a final concentration of 20 nM of 43Ea conjugated to Alexa488 was added to the antibody cell mixture. After another 1 hr incubation on ice, cells were washed, stained with a viability dye, and analyzed by flow cytometry. The Alexa488 fluorescence data from viable cells was summarized using median fluorescence intensity. 43Ea binding was summarized with % 43Ea binding = $[\text{MFI}_{\text{antibody labeled cells}} - \text{MFI}_{\text{unstained cells}}] / [\text{MFI}_{\text{IgG1 control labeled cells}} - \text{MFI}_{\text{unstained cells}}]$.

[00671] % 25A binding and % 43Ea binding are shown in **Table 12**. Antibodies from group 25 and group 43 reduced the % 25A binding and % 43Ea binding to less than 10%.

[00672] This data indicates that antibodies of group 25 and antibodies of group 43 compete with each other for binding to human TF, and may bind the same or an overlapping epitope of human TF.

Table 12: Competition of Anti-TF Antibody with Antibody Clone 25A or 43Ea

Antibody (100 nM)	% 25A binding	% 43Ea binding
1F	95	77
1G	75	58
25A	3	1
25G	7	3
29D	70	64
29E	96	85
39A	99	96
43B	0	0
43D	0	0
43E	0	0
54E	99	96
Isotype	100	100

Example 20: Cell Viability Assay

[00673] To evaluate internalization of the anti-TF antibodies, a cytotoxicity assay was conducted. Briefly, cells were plated in 384-well plates (Greiner Bio-One, Monroe, NC, USA) at 4×10^3 cells per well in 40 μl of media. Antibodies and secondary anti-human Fc antibodies conjugated to the tubulin inhibitor mono-methyl auristatin F (MMAF) (Moradec, San Diego, CA, USA) were serially diluted starting at 5 and 30 nM, respectively. Plates were incubated for 3 days, followed by lysis in CellTiter-Glo (CTG) assay reagent (Promega, Madison, WI, USA). CTG luminescence was measured on an Envision plate reader and the mean and standard deviation of 4 replicates graphed in Prism. For each anti-TF antibody, the IC_{50} and its associated 95% confidence interval were calculated in Prism using a 4-parameter binding model.

[00674] The cell viability as indicated by the level of luminescence and the calculated IC₅₀ is shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety.

[00675] This data indicates that all anti-TF antibodies tested from groups 1, 25, 29, 39, 43, and 54 were effective in reducing the viability of TF-positive A431 cells.

Example 21: Thrombin Generation Assay (TGA)

[00676] The TGA assay was performed using the calibrated-automated-thrombogram (CAT) instrument manufactured and distributed by STAGO. The test method design was equivalent to a standard CAT assay measurement, except that the plasma source was normal pooled plasma (NPP) in citrate supplemented with corn trypsin inhibitor (citrate/CTI). The anti-TF antibodies were titrated at 0, 10, 50 and 100 nM and mixed with normal pooled plasma (NPP) collected in 11 mM citrate supplemented with 100 microgram/mL of corn trypsin inhibitor (citrate/CTI). Relipidated TF was added to a 96-well assay plate, followed by addition of the antibody/NPP mixture. After a 10-min incubation or directly after combining the relipidated TF with antibody/NPP, thrombin generation was initiated by the addition of calcium and the thrombin substrate. The STAGO software was used to report the following parameters: Peak IIa (highest thrombin concentration generated [nM]); Lag Time (time to IIa generation [min]); ETP (endogenous thrombin potential, area under the curve [nM x min]); and ttPeak (time to Peak IIa [min]). Percent peak thrombin generation (% Peak IIa) and percent endogenous thrombin potential (% ETP) in the presence of each antibody relative to a no antibody plasma control on the same plate were also reported.

[00677] The Peak IIa, Lag Time, ETP, ttPeak, % Peak IIa, and % ETP in the presence of each antibody selected from 25A, 25A3, 25A5, 39A, 43B1, 43D7, 43Ea, and M1593 without antibody incubation prior to addition of calcium and thrombin substrate are shown in **Table 37**. The Peak IIa, Lag Time, ETP, ttPeak, % Peak IIa, and % ETP in the presence of each antibody selected from 25A, 25A3, 25A5, 39A, 43B1, 43D7, 43Ea, and M1593 with 10 min antibody incubation prior to addition of calcium and thrombin substrate are shown in **Table 38**. The % Peak IIa in the presence of titrations of anti-TF antibodies without antibody incubation prior to addition of calcium and thrombin substrate are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety. The % Peak IIa in the presence of titrations of anti-TF antibodies with 10 min antibody incubation prior to addition of calcium and thrombin substrate is shown in international PCT application PCT/US2019/012427 and US

utility application number 16/959,652, incorporated herein by reference in their entirety. The M1593 antibody has a VH sequence of SEQ ID NO:821 and VL sequence of SEQ ID NO:822.

[00678] The % Peak IIa is 95% or greater in the presence of antibodies from group 25, including 25A, 25A3, and 25A5 without antibody pre-incubation. The % Peak IIa is 100% or greater in the presence of antibodies from group 25, including 25A, 25A3, and 25A5 with 10 min antibody pre-incubation. The % ETP is 99% or greater in the presence of the tested antibodies from group 25.

[00679] The % Peak IIa is greater than 50% but equal to or less than 96% in the presence of antibodies from group 43, including 43B1, 43D7, and 43Ea and anti-TF antibody M1593 without antibody pre-incubation. The % Peak IIa is greater than 40% but equal to or less than 93% in the presence of antibodies from group 43, including 43B1, 43D7, and 43Ea and anti-TF antibody M1593 with 10 min antibody pre-incubation. The % ETP is 92% or greater in the presence of the tested antibodies from group 43 and M1593 antibody.

[00680] This data indicates that antibodies from groups 25 and 43 allow normal thrombin generation, and therefore are not inhibitors of thrombin generation. The percent peak thrombin generation (% Peak IIa) is greater in the presence of antibodies of group 25 compared to antibodies of group 43 and M1593 antibody.

Table 37: Thrombin Generation Assay without Antibody Pre-Incubation

Plate	Antibody	Ab conc. (nM)	Peak IIa (nM)	Lag Time (min)	ETP (nM•min)	ttPeak (min)	% Peak IIa	% ETP
3	25A	100	334	5.0	2390	8.7	96	105
		50	335	5.0	2380	8.7	96	104
		10	333	5.0	2387	8.6	95	104
3	25A3	100	343	5.0	2405	8.4	98	105
		50	349	5.0	2433	8.4	100	106
		10	350	5.0	2426	8.0	100	106
3	25A5	100	342	5.1	2393	8.5	98	105
		50	344	4.8	2317	8.1	98	101
		10	343	4.7	2270	8.0	98	99
3	39A	100	22	38.1	*	48.3	6	*
		50	29	33.1	*	43.2	8	*
		10	84	12.4	1332	20.7	24	58
3	43B1	100	223	4.8	2111	10.0	64	92
		50	239	4.9	2134	9.9	68	93
		10	303	5.1	2318	9.1	87	101
3	43D7	100	186	5.6	2105	12.2	53	92
		50	216	5.5	2183	11.3	62	96
		10	301	5.4	2338	9.3	86	102
3	43Ea	100	302	5.1	2347	9.1	87	103
		50	308	5.1	2392	8.8	88	105

Plate	Antibody	Ab conc. (nM)	Peak IIa (nM)	Lag Time (min)	ETP (nM•min)	ttPeak (min)	% Peak IIa	% ETP
		10	336	4.5	2305	7.8	96	101
3	M1593	100	242	5.1	2235	10.4	69	98
		50	270	5.1	2282	9.8	77	100
		10	322	5.1	2368	8.8	92	104
		100	347	5.0	2319	8.1	99	101
3	Isotype	50	348	5.0	2324	8.1	100	102
		10	348	5.0	2326	8.3	100	102
		NA	349	4.7	2285	7.7	100	100

* Groups with "No Tail Found" Errors when the software cannot calculate the ETP.

Table 38: Thrombin Generation Assay with 10 min Antibody Pre-Incubation

Plate	Antibody	Ab conc. (nM)	Peak IIa (nM)	Lag Time (min)	ETP (nM•min)	ttPeak (min)	% Peak IIa	% ETP
3	25A	100	274	3.3	1879	7.0	103	106
		50	279	3.3	1876	7.0	105	106
		10	280	3.6	1872	7.0	105	106
3	25A3	100	290	3.4	1906	6.8	109	108
		50	291	3.6	1925	6.8	109	109
		10	287	3.3	1886	6.8	108	107
3	25A5	100	286	3.7	1883	7.0	107	107
		50	277	3.7	1803	7.0	104	102
		10	278	3.7	1808	7.0	104	102
3	39A	100	17	32.1	*	43.2	6	*
		50	21	29.0	*	39.7	8	*
		10	30	20.9	*	30.8	11	*
3	43B1	100	156	3.6	1701	9.3	58	96
		50	148	3.3	1667	9.6	55	94
		10	203	3.7	1776	8.2	76	101
3	43D7	100	120	3.7	1633	10.8	45	92
		50	131	3.7	1724	10.4	49	98
		10	197	3.7	1784	8.8	74	101
3	43Ea	100	244	3.3	1817	7.3	91	103
		50	246	3.3	1833	7.3	92	104
		10	247	3.3	1779	7.1	93	101
3	M1593	100	160	3.7	1737	9.4	60	98
		50	165	3.7	1739	9.3	62	99
		10	224	3.7	1807	8.0	84	102
3	Isotype	100	279	3.7	1829	7.2	105	104
		50	283	3.7	1839	7.0	106	104
		10	279	3.7	1814	7.1	105	103
3	Plasma ctrl.	NA	267	3.7	1766	7.2	100	100

* Groups with "No Tail Found" Errors when the software cannot calculate the ETP.

Example 22: Binding Affinity Assay For Pig TF

[00681] The ability of certain antibodies was tested for binding to pig TF. For pig TF Biacore-based measurements, a given anti-TF antibody was captured by an anti-human IgG antibody covalently coupled to a CM5 chip (GE Healthcare Bio-Sciences). Association between the anti-TF antibodies and a five-point three-fold titration of pig TF-His starting at 100 nM was measured for 180 to 240 sec. Subsequently, dissociation between the anti-TF antibody and TF-His was measured for 1800 sec. Kinetic data was analyzed and fitted globally using a 1:1 binding model. The K_D values of the indicated TF antibodies measured by the Biacore-based experiments are shown in **Table 40**.

[00682] As shown in **Table 40**, anti-hTF antibodies from groups 25 and 43, 25G9 and 43D8, exhibit binding activity and cross-reactivity to pig TF.

Table 40: Antibody kinetics for pig TF

Ab	Pig K_D (nM) [standard deviation]
1G	no binding*
29D	no binding*
25G9	3.31 [0.08]
43D8	12.9 [0.03]

no binding*: no binding to weak binding, with no reportable K_D

Example 23: Cell-Based Binding Assay

[00683] Human TF-positive cancer cell lines A431 and MDA-MB-231 and *Macaca mulatta* TF-positive cell line RF/6A were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) and were maintained as recommended.

[00684] Cell-based antibody binding was assessed as previously described in Liao-Chan *et al.*, *PLoS One*, 2015, 10:e0124708, which is incorporated by reference in its entirety. 1.2×10^5 cells collected with Cellstripper (Mediatech, Manassas, VA, USA) were incubated with a twelve-point 1:3 dilution titration of anti-human TF IgG1 antibody starting at 250 nM or 100 nM for 2 hr on ice. After 2 washes, cells labeled with IgG1 antibody were incubated for 30 min on ice with 150 nM of Goat Phycoerythrin (PE) F(ab')₂ fragment goat anti-human IgG, Fcγ fragment specific (Jackson ImmunoResearch, West Grove, PA, USA) or FITC-labeled F(ab')₂ fragment goat anti-human kappa (SouthernBiotech, Birmingham, AL, USA), respectively. After 2 washes, dead cells were labeled with TO-PRO-3 Iodide (ThermoFisher Scientific) and samples were analyzed on a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) or Novocyte flow cytometer (ACEA Biosciences, San Diego, CA, USA). The median fluorescence intensities (MFIs) at each dilution were plotted and cell EC₅₀'s were

derived using a 4-parameter binding model in Prism (GraphPad, La Jolla, CA, USA).

Antibodies that does not substantially affect FX conversion (*i.e.* 25A, 25A3, 25G1, 43B1, 43D7 and 43Ea) and antibodies that inhibited FX conversion by more than 50 % (*i.e.* 1F, 29E, 39A and 54E) were included in the assay. The results of binding of anti-TF antibodies to human TF-positive A431 cells are shown in international PCT application

PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety. The results of binding of anti-TF antibodies to human TF-positive MDA-MB-231 cells are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety.

[00685] All tested anti-hTF antibodies in Figure 12A of PCT application PCT/US2019/012427 and US utility application number 16/959,652 exhibit high affinity to human TF-positive A431 cells with an EC₅₀ ranging from about 1.50 nM to about 0.34 nM. An IgG1 isotype control did not bind A431 cells (no binding, nb). All tested anti-hTF antibodies in Figure 12B of international PCT application PCT/US2019/012427 and US utility application number 16/959,652 exhibit high affinity to human TF-positive MDA-MB-231 cells with an EC₅₀ ranging from about 1.50 nM to about 0.06 nM. An IgG1 isotype control did not bind MDA-MB-231 cells (no binding, nb).

[00686] As described in Example 13 and shown in **Table 5**, the binding affinity of anti-hTF antibodies was evaluated on TF from cynomolgus monkey (*Macaca fascicularis*). The protein sequences of *Macaca fascicularis* TF and *Macaca mulatta* TF are identical. The binding of the TF-specific antibodies to cynomolgus monkey was confirmed using the *Macaca mulatta* RF/6A cell line as shown in **Table 42**. All tested anti-hTF antibodies exhibit high affinity to TF-positive *Macaca mulatta* RF/6A cells with an EC₅₀ ranging from about 1.28 nM to about 0.17 nM. The ability of the anti-TF antibodies to bind to cynomolgus monkey is advantageous for toxicology studies of these antibodies with nonhuman primate models.

Table 42: Binding of anti-TF antibodies to *Macaca mulatta* RF/6A cells

Ab	RF/6A EC ₅₀ (nM)	RF/6A 95% CI
1F	0.17	0.14 to 0.21
25A	0.43	0.37 to 0.50
25A3	0.27	0.24 to 0.30
25G1	0.27	0.23 to 0.32
29E	0.53	0.46 to 0.61
39A	0.27	0.23 to 0.32

Ab	RF/6A EC50 (nM)	RF/6A 95% CI
43B1	0.47	0.40 to 0.55
43D7	0.41	0.35 to 0.49
43Ea	0.92	0.83 to 1.01
54E	1.28	1.16 to 1.41

Example 24: Binding Assay to *E. Coli*-Derived TF

[00687] *E. coli*-derived TF was expressed as a fusion between the OmpA signal sequence and TF ECD-His6, and purified by affinity and anion exchange chromatography. The binding of anti-TF antibodies 1F, 25A, 25A3, 25G1, 29E, 39A, 43B1, 43D7, 43Ea, and 54E to Expi293- or *E. coli*-derived TF was determined by protein ELISA studies. Plates coated with Expi293- or *E. coli*-derived TF-His were incubated with increasing concentrations of antibodies. After incubation with an HRP-conjugated secondary antibody (Jackson ImmunoResearch), luminescence data were obtained and used to calculate an EC₅₀ with 95 % confidence intervals using Prism. The EC₅₀'s and 95% confidence intervals of the antibodies are listed in **Table 43**.

Table 43: Binding of anti-TF antibodies to Expi293- or *E. coli*-derived TF

Ab	Expi293-derived TF protein EC50 (nM)	Expi293-derived TF protein 95% CI	<i>E.coli</i> -derived TF protein EC50 (nM)	<i>E.coli</i> -derived TF protein 95% CI
1F	0.41	0.37 to 0.46	0.32	0.30 to 0.34
25A	0.54	0.49 to 0.60	0.35	0.30 to 0.41
25A3	0.47	0.39 to 0.56	0.36	0.31 to 0.42
25G1	0.42	0.36 to 0.47	0.31	0.29 to 0.33
29E	0.98	0.78 to 1.24	0.68	0.39 to 1.26
39A	0.45	0.39 to 0.53	0.34	0.28 to 0.40
43B1	0.57	0.53 to 0.61	0.39	0.34 to 0.44
43D7	0.71	0.62 to 0.80	0.43	0.35 to 0.53
43Ea	0.74	0.68 to 0.81	0.46	0.40 to 0.53
54E	0.96	0.73 to 1.29	0.38	0.22 to 0.62

[00688] All tested anti-hTF antibodies exhibit high affinity to *E. coli*-derived TF with an EC₅₀ ranging from about 0.68 nM to about 0.31 nM, which is comparable to the binding affinity of the antibodies to Expi293-derived TF (about 0.98 nM to 0.41 nM). These results indicate that although the anti-TF antibodies were selected against glycosylated TF from a

human cell line, the antibodies can bind to *E. coli*-derived TF with similar affinity when measured by protein ELISA.

Example 25: Thrombin Generation Assay (TGA)

[00689] TGA assay was performed using the calibrated-automated-thrombogram (CAT) instrument manufactured and distributed by STAGO (Diagnostica Stago SAS, Asnières sur Seine, France). *See Samama et al., Thromb Res*, 2012, 129:e77-82, which is incorporated by reference in its entirety. The test method design was equivalent to a standard CAT assay measurement, except that the plasma source was normal pooled plasma (NPP) collected in 11 mM citrate supplemented with 100 µg/mL of corn trypsin inhibitor (citrate/CTI). The anti-TF antibodies were titrated at 0, 10, 50 and 100 nM and mixed with NPP in citrate/CTI. Relipidated TF was added to a 96-well assay plate, followed by addition of the antibody/NPP mixture. After a 10-min incubation or directly after combining the relipidated TF with antibody/NPP, thrombin generation was initiated by the addition of calcium and the thrombin substrate. The STAGO software was used to report the following parameters: Peak IIa (highest thrombin concentration generated on the thrombin generation curve [nM]); Lag Time (time from assay start to the moment 10 nM of thrombin is formed [min]); ETP (endogenous thrombin potential, area under the curve [nM x min]); and ttPeak (time from assay start to Peak IIa [min]). Percent peak thrombin generation (% Peak IIa), percent endogenous thrombin potential (% ETP), and percent ttPeak (% ttPeak) in the presence of each antibody relative to a no-antibody plasma control on the same plate were also reported. As used herein, the term “thrombin generation assay” (TGA) refers to the TGA used in this example.

[00690] The Peak IIa, Lag Time, ETP, ttPeak, % Peak IIa, % ETP, and % ttPeak in the presence of each antibody selected from 1F, 25A, 25A3, 25G1, 29E, 39A, 43B1, 43D7, 43Ea, 54E, TF-011, 5G9, and 10H10 without antibody incubation prior to addition of calcium and thrombin substrate are shown in **Table 44**. The Peak IIa, Lag Time, ETP, ttPeak, % Peak IIa, % ETP, and % ttPeak in the presence of each antibody selected from 1F, 25A, 25A3, 25G1, 29E, 39A, 43B1, 43D7, 43Ea, 54E, TF-011, 5G9, and 10H10 with 10 min antibody incubation prior to addition of calcium and thrombin substrate are shown in **Table 45**. The thrombin generation curve in the presence of 100 nM anti-TF antibody without antibody pre-incubation are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety. The Peak thrombin concentration in the presence of titrations of anti-TF antibodies without antibody

pre-incubation are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety.

[00691] As shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652 (incorporated herein by reference in their entirety), under the conditions without antibody pre-incubation, at the 100 nM antibody concentration, 1F, 29E, 39A, 54E diminished the peak Ila concentration by 92, 76, 91 and 70 %, respectively. Similarly, 100 nM of 5G9 and TF-011 inhibited peak Ila concentration by 92 % and 91 %, respectively. Severely reduced thrombin generation in the presence of the two highest concentrations of 1F, 39A, 5G9 and TF-011 hampered endogenous thrombin generation (ETP) calculations and increased time to Peak Ila/thrombin generation (ttPeak) by at least 284 % and 353 % at 50 nM and 100 nM respectively. In contrast, antibodies from group 25 did not impact the peak Ila concentration or ttPeak by more than 9 %. Group 43 antibodies and 10H10 exhibited mild interference with the peak Ila concentration: 100 nM of 43B1, 43D7, 43Ea and 10H10 reduced the peak Ila concentration by 33, 44, 13 and 34 %, respectively. In addition, 100 nM of 43B1, 43D7 and 10H10 showed at least a 29 % increase in ttPeak. However, the observed decline in peak Ila concentration and delayed ttPeak for group 43 antibodies and 10H10 did not result in more than a 10 % decline in the ETP.

[00692] Similar results are shown in **Table 45** under the conditions with 10 min antibody pre-incubation. At the 100 nM antibody concentration, 1F, 29E, 39A, 54E diminished the peak Ila concentration by 93, 72, 93 and 87 %, respectively. Similarly, 100 nM of 5G9 and TF-011 inhibited peak Ila concentration by 92 % and 91 %, respectively. Severely reduced thrombin generation in the presence of the two highest concentrations of 1F, 39A, 54E and TF-011 and all tested concentrations of 5G9 hampered endogenous thrombin generation (ETP) calculations and increased time to Peak Ila/thrombin generation (ttPeak) by at least 303 % and 371 % at 50 nM and 100 nM respectively. In contrast, antibodies from group 25 did not decrease the peak Ila concentration or increase ttPeak. Group 43 antibodies and 10H10 exhibited mild interference with the peak Ila concentration: 100 nM of 43B1, 43D7, 43Ea and 10H10 reduced the peak Ila concentration by 41, 56, 13 and 48 %, respectively. In addition, 100 nM of 43B1, 43D7 and 10H10 showed at least a 33 % increase in ttPeak. However, the observed decline in peak Ila concentration and delayed ttPeak for group 43 antibodies and 10H10 did not result in more than an 11 % decline in the ETP.

[00693] Overall, these results indicate that group 25 antibodies are completely inert in the penultimate step of the coagulation cascade when all three TGA parameters (ETP, Peak Ila concentration and ttPeak) are taken into consideration.

Table 44: Thrombin Generation Assay without Antibody Pre-Incubation

Plate	Sample	Ab conc. (nM)	Peak IIa [nM] (SD)	Lag Time [min] (SD)	ETP [nM•min] (SD)	ttPeak [min] (SD)	% Peak IIa	% ETP	% ttPeak
1	1F	100	25 (1)	31 (1)	*	41 (0.7)	8	*	419
		50	31 (0)	25.6 (0.3)	*	35.3 (0.3)	9	*	347
		10	155 (1)	8.2 (0.2)	1738 (25)	14.9 (0.2)	47	86	89
1	25A	100	317 (6)	5.2 (0.2)	2134 (28)	8.6 (0.2)	95	105	9
		50	317 (2)	5.2 (0.2)	2122 (30)	8.6 (0.2)	95	105	9
		10	322 (2)	5 (0)	2108 (29)	8.2 (0.2)	97	104	4
1	25A3	100	323 (1)	4.6 (0.2)	2031 (19)	7.9 (0.2)	97	100	0
		50	328 (2)	4.7 (0)	2080 (23)	8 (0)	98	103	1
		10	326 (4)	5.3 (0)	2152 (14)	8.4 (0.2)	98	106	6
1	25G1	100	340 (3)	5.3 (0)	2160 (27)	8.3 (0)	102	107	5
		50	346 (6)	5.1 (0.2)	2221 (40)	8.2 (0.2)	104	110	4
		10	337 (1)	4.7 (0)	2061 (34)	7.8 (0.2)	101	102	-1
1	29E	100	81 (0)	17.1 (0.2)	1257 (18)	26.2 (0.2)	24	62	232
		50	95 (1)	14.1 (0.2)	1365 (26)	22.6 (0.4)	29	67	186
		10	235 (3)	7 (0)	1926 (9)	11.7 (0)	71	95	48
1	Isotype	100	326 (3)	5.3 (0)	2132 (13)	8.6 (0.2)	98	105	9
		50	331 (3)	5.3 (0)	2177 (19)	8.3 (0)	99	108	5
		10	328 (4)	5.3 (0)	2129 (26)	8.4 (0.2)	98	105	6
1	TF-011	100	30 (1)	26 (0.3)	*	35.8 (0.2)	9	*	353
		50	39 (3)	21.3 (0.5)	*	30.3 (1.1)	12	*	284
		10	156 (7)	8 (0)	1714 (41)	14.7 (0.5)	47	85	86
1	5G9	100	27 (1)	29.9 (0.4)	*	39.6 (0.4)	8	*	401
		50	28 (0)	25.1 (0.4)	*	34.6 (0.2)	8	*	338
		10	79 (1)	10.4 (0.2)	1176 (16)	18.6 (0.2)	24	58	135
1	10H10	100	221 (4)	5.2 (0.2)	1945 (37)	10.2 (0.2)	66	96	29
		50	248 (3)	5.2 (0.2)	1978 (32)	9.8 (0.3)	74	98	24
		10	310 (2)	5.2 (0.2)	2036 (33)	8.6 (0.2)	93	101	9
1	Plasma ctrl.	NA	333 (0)	4.7 (0)	2023 (30)	7.9 (0.2)	100	100	0
2	39A	100	29 (0)	34.7 (0)	*	44.6 (0.2)	9	*	465
		50	36 (1)	29.8 (0.7)	*	39.3 (0.7)	11	*	397
		10	122 (3)	10.8 (0.3)	1694 (57)	18.6 (0.2)	37	84	135
2	43B1	100	238 (4)	5.3 (0)	2300 (32)	10.8 (0.2)	67	99	37
		50	258 (5)	5.2 (0.2)	2301 (29)	10.2 (0.2)	72	99	29

		10	317 (1)	5 (0)	2341 (34)	8.6 (0.2)	89	101	9
2	43D7	100	199 (6)	5.1 (0.2)	2124 (27)	11.2 (0.2)	56	91	42
		50	234 (1)	5 (0)	2190 (15)	10.3 (0)	66	94	30
		10	312 (3)	5 (0)	2343 (49)	8.9 (0.2)	88	101	13
2	43Ea	100	308 (2)	5 (0)	2349 (9)	9 (0)	87	101	14
		50	316 (3)	5 (0)	2430 (69)	8.7 (0)	89	105	10
		10	337 (4)	5 (0)	2416 (82)	8.3 (0)	95	104	5
2	54E	100	108 (3)	12.2 (0.2)	1589 (13)	20.2 (0.2)	30	68	156
		50	191 (2)	8 (0)	2109 (51)	14.3 (0)	54	91	81
		10	311 (5)	5 (0)	2275 (41)	8.8 (0.2)	87	98	11
2	Isotype	100	351 (2)	4.7 (0)	2304 (14)	7.9 (0.2)	99	99	0
		50	353 (1)	5 (0)	2391 (29)	8.2 (0.2)	99	103	4
		10	348 (1)	5 (0)	2367 (9)	8.3 (0)	98	102	5
2	Plasma ctrl.	NA	356 (1)	4.9 (0.2)	2323 (76)	8.11 (0.3)	100	100	3

* Groups with "No Tail Found" Errors when the software cannot calculate the ETP.

Table 45: Thrombin Generation Assay with 10 min Antibody Pre-Incubation

Plate	Sample	Ab conc. (nM)	Peak IIa [nM] (SD)	Lag Time [min] (SD)	ETP [nM·min] (SD)	ttPeak [min] (SD)	% Peak IIa	% ETP	% ttPeak
1	1F	100	20 (1)	29.5 (0.2)	*	40.8 (0.6)	7	*	483
		50	23 (0)	26.5 (0.7)	*	37.3 (0.4)	8	*	433
		10	44 (2)	13.8 (0.5)	742 (23)	22.4 (0.4)	16	41	220
1	25A	100	291 (3)	3.3 (0.1)	1964 (36)	6.7 (0.1)	106	108	-4
		50	290 (0)	3.3 (0.1)	1972 (22)	6.8 (0)	106	108	-3
		10	284 (1)	3.3 (0.1)	1899 (21)	6.8 (0)	104	104	-3
1	25A3	100	290 (3)	3.1 (0)	1893 (28)	6.4 (0)	106	104	-9
		50	284 (4)	3.1 (0)	1875 (16)	6.4 (0)	104	103	-9
		10	288 (3)	3.1 (0)	1901 (26)	6.4 (0)	105	105	-9
1	25G1	100	311 (3)	3.1 (0)	1954 (20)	6.3 (0.1)	114	107	-10
		50	311 (1)	3.1 (0)	1951 (22)	6.1 (0)	114	107	-13
		10	302 (3)	3.1 (0)	1877 (33)	6.1 (0)	110	103	-13
1	29E	100	76 (1)	14.7 (0.1)	1201 (24)	24.3 (0.3)	28	66	247
		50	83 (1)	14.1 (0)	1300 (17)	23.6 (0.1)	30	72	237
		10	98 (1)	9.4 (0)	1408 (11)	18.1 (0)	36	77	159
1	Isotype	100	288 (2)	3.4 (0)	1922 (28)	6.8 (0)	105	106	-3
		50	292 (2)	3.4 (0)	1921 (25)	6.8 (0)	107	106	-3
		10	290 (3)	3.4 (0)	1926 (38)	6.8 (0)	106	106	-3
1	TF-011	100	26 (0)	23.8 (1.1)	*	34.2 (0.9)	9	*	389
		50	27 (1)	22.4 (0.1)	*	33 (0.1)	10	*	371
		10	46 (3)	13.5 (0.5)	792 (55)	22.5 (0.2)	17	44	221
1	5G9	100	22 (0)	26.7 (0.3)	*	37.5 (0.5)	8	*	436

		50	23 (3)	23.6 (2.2)	*	34 (2.4)	8	*	386
		10	30 (1)	19.3 (0.4)	*	29 (0.8)	11	*	314
1	10H10	100	169 (3)	3.4 (0)	1795 (36)	9.3 (0.1)	62	99	33
		50	175 (4)	3.4 (0)	1754 (20)	9.2 (0.1)	64	96	31
		10	235 (8)	3.4 (0)	1807 (42)	7.8 (0)	86	99	11
1	Plasma ctrl.	NA	274 (1)	3.4 (0)	1818 (24)	7 (0.1)	100	100	0
2	39A	100	19 (1)	33.6 (0.7)	*	44.6 (0.9)	7	*	537
		50	22 (0)	30.7 (0.1)	*	41.4 (0.1)	8	*	491
		10	36 (1)	19.6 (0.7)	*	29.3 (0.8)	13	0	319
2	43B1	100	167 (0)	4 (0)	1806 (15)	9.8 (0.1)	59	98	40
		50	174 (1)	3.8 (0.1)	1831 (22)	9.6 (0)	62	99	37
		10	222 (5)	3.7 (0.1)	1841 (37)	8.3 (0)	79	100	19
2	43D7	100	123 (2)	4 (0)	1673 (27)	11.5 (0.1)	44	91	64
		50	122 (1)	3.7 (0.1)	1639 (29)	11.3 (0)	43	89	61
		10	194 (5)	4 (0)	1796 (35)	8.8 (0.1)	69	97	26
2	43Ea	100	244 (2)	3.5 (0.1)	1857 (42)	7.5 (0.1)	87	101	7
		50	245 (0)	3.6 (0)	1851 (29)	7.6 (0)	87	100	9
		10	262 (1)	3.6 (0)	1877 (15)	7.3 (0)	93	102	4
2	54E	100	37 (1)	22.3 (0.2)	*	33 (0.5)	13	*	371
		50	44 (1)	18.3 (0.4)	*	28.2 (1)	16	*	303
		10	121 (4)	6.5 (0.1)	1523 (20)	13.7 (0.3)	43	83	96
2	Isotype	100	275 (2)	3.6 (0)	1862 (23)	7.3 (0)	98	101	4
		50	284 (0)	3.6 (0)	1899 (15)	7.2 (0.1)	101	103	3
		10	281 (3)	3.6 (0)	1877 (13)	7.3 (0)	100	102	4
2	Plasma ctrl.	NA	282 (2)	3.8 (0.1)	1845 (22)	7.3 (0)	100	100	4

* Groups with "No Tail Found" Errors when the software cannot calculate the ETP.

Example 26: FXa Conversion Assay and FVIIa Competition Assay with Previously Described Anti-TF Antibodies

[00694] The previously described TF-specific antibodies TF-011, 5G9 and 10H10 (Breij *et al.*, *Cancer Res*, 2014, 74:1214-1226; Versteeg *et al.*, *Blood*, 2008, 111:190-199; each of which is incorporated by reference in its entirety) were tested in FXa conversion assay and FVIIa competition assay.

[00695] To evaluate the ability of TF:FVIIa to convert FX into FXa in the presence of human antibodies against TF, a cell-based FX conversion assay was conducted as described in Larsen *et al.*, *J Biol Chem*, 2010, 285:19959-19966, which is incorporated by reference in its entirety. Briefly, 5x10⁴ MDA-MB-231 cells (ATCC, Manassas, VA, USA) were plated into tissue culture-treated black 96-well plates (Greiner Bio-One, Monroe, NC, USA) and cultured overnight. After removal of the cell culture media and addition of a final

concentration of 200 nM of FX in a HEPES buffer with 1.5 mM CaCl₂, cells were incubated with a titration of the antibodies for 15 min at 37°C. Upon reconstitution of the binary TF:FVIIa complex with a final concentration of 20 nM of FVIIa, cells were incubated for 5 min at 37°C. After quenching the reaction with ethylenediaminetetraacetic acid (EDTA) in a black 96-well plate, generated FXa was measured with 50 μM of SN-7 6-amino-1-naphthalenesulfonamide-based fluorogenic substrate (Haematologic Technologies, Essex Junction, VT, USA) on an Envision plate reader equipped with an Umbelliferone 355 excitation filter, an Umbelliferone 460 emission filter, and a LANCE/DELFI top mirror (Perkin Elmer, Waltham, MA, USA). FXa conversion percentages (% FXa) in the presence of an anti-TF antibody titration relative to a no antibody control are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety.

[00696] To evaluate competition between FVIIa and the human antibodies against TF, TF-positive MDA-MB-231 cells (ATCC, Manassas, VA, USA) were first incubated for 1 hr on ice with a titration of the human antibodies against TF or an isotype control. Subsequently, FVII-Fc conjugated to Alexa488 was added to the antibody-cell mixture at a final concentration of 20 nM. After another 1 hr incubation on ice, cells were washed, stained with a viability dye, and analyzed by flow cytometry. The Alexa488 fluorescence data from viable cells was summarized using median fluorescence intensity (MFI). FVII-Fc binding was summarized with % FVII-Fc binding = $\frac{[\text{MFI}_{\text{antibody labeled cells}} - \text{MFI}_{\text{unstained cells}}]}{[\text{MFI}_{\text{IgG1 control labeled cells}} - \text{MFI}_{\text{unstained cells}}]}$. Percentage of FVIIa binding (% FVIIa) in the presence of an anti-TF antibody titration relative to an isotype control is shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety.

[00697] As shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652 (incorporated herein by reference in their entirety), TF-011 and 5G9 inhibited FX conversion by 57-59 % and 67-70 % at concentrations of 25, 50, and 100 nM. 10H10 did not significantly inhibit FX conversion at these three concentrations.

[00698] As shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652 (incorporated herein by reference in their entirety), TF-011 effectively competed with FVII, whereas 5G9 and 10H10 showed less than 25 % and 10 % competition at the highest concentration of antibody, respectively.

[00699] These results indicate that 5G9 predominantly competes with substrate FX binding, resulting in the observed inhibition of FX conversion and thrombin generation. TF-011 inhibits thrombin generation by competing with FVIIa for binding to TF. However, 10H10 inhibits TF-FVIIa mediated signaling without substantially affecting binding of FVIIa to TF. These findings are consistent with previous observations described in Huang *et al.*, *J Mol Biol*, 1998, 275:873-894; Ruf *et al.*, *Biochem J*, 1991, 278:729-733; and Teplyakov *et al.*, *Cell Signal*, 2017, 36:139-144; each of which is incorporated by reference in its entirety.

Example 27: Antibody Competition Assay

[00700] Alexa Fluor antibodies were generated using Alexa Fluor 488 5-sulfo-dichlorophenol esters (ThermoFisher Scientific) following manufacturer's protocol. Excess Alexa Fluor dye was removed from the antibody dye conjugate preparations by gel filtration (ThermoFisher Scientific).

[00701] To evaluate competition between a first human antibody against TF and 25A3, TF-positive MDA-MB-231 cells (ATCC, Manassas, VA, USA) were first incubated for 1 hr on ice with a titration of the first human antibody against TF. Subsequently, a final concentration of 20 nM of 25A3 conjugated to Alexa488 was added to the antibody cell mixture. After another 1 hr incubation on ice, cells were washed, stained with a viability dye, and analyzed by flow cytometry. The Alexa488 fluorescence data from viable cells was summarized using median fluorescence intensity. 25A3 binding was summarized with % 25A3 binding = $[\text{MFI}_{\text{antibody labeled cells}} - \text{MFI}_{\text{unstained cells}}] / [\text{MFI}_{\text{IgG1 control labeled cells}} - \text{MFI}_{\text{unstained cells}}]$.

[00702] To evaluate competition between a first human antibody against TF and 43D7, TF-positive MDA-MB-231 cells (ATCC, Manassas, VA, USA) were first incubated for 1 hr on ice with a titration of the first human antibody against TF. Subsequently, a final concentration of 20 nM of 43D7 conjugated to Alexa488 was added to the antibody cell mixture. After another 1 hr incubation on ice, cells were washed, stained with a viability dye, and analyzed by flow cytometry. The Alexa488 fluorescence data from viable cells was summarized using median fluorescence intensity. 43D7 binding was summarized with % 43D7 binding = $[\text{MFI}_{\text{antibody labeled cells}} - \text{MFI}_{\text{unstained cells}}] / [\text{MFI}_{\text{IgG1 control labeled cells}} - \text{MFI}_{\text{unstained cells}}]$.

[00703] To evaluate competition between a first human antibody against TF and 39A, TF-positive MDA-MB-231 cells (ATCC, Manassas, VA, USA) were first incubated for 1 hr on ice with a titration of the first human antibody against TF. Subsequently, a final concentration

of 20 nM of 39A conjugated to Alexa488 was added to the antibody cell mixture. After another 1 hr incubation on ice, cells were washed, stained with a viability dye, and analyzed by flow cytometry. The Alexa488 fluorescence data from viable cells was summarized using median fluorescence intensity. 39A binding was summarized with % 39A binding = $[\text{MFI}_{\text{antibody labeled cells}} - \text{MFI}_{\text{unstained cells}}] / [\text{MFI}_{\text{IgG1 control labeled cells}} - \text{MFI}_{\text{unstained cells}}]$.

[00704] % 25A3 binding, % 43D7 binding, and % 39A binding are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety. Antibodies from groups 25 and 43, 5G9, and 10H10 reduced % 25A3 binding and % 43D7 binding and did not reduce % 39A binding. Antibodies from groups 1, 29, 39, and 54, and TF-011 reduced % 39A binding and did not reduce % 25A3 binding and % 43D7 binding.

[00705] While the antibody competition assay results indicate that groups 25 and 43 antibodies, 5G9, and 10H10 may bind to the same or an overlapping epitope of human TF or may affect the TF binding of each other through an allosteric mechanism, the chimeric TF construct mapping experiments as described elsewhere in this disclosure demonstrate that group 25 antibodies, group 43 antibodies, 5G9 and 10H10 bind distinct epitopes. In addition, while the antibody competition assay results indicate that antibodies of groups 1, 29, 39, and 54, and TF-011 may bind to the same or an overlapping epitope of human TF or may affect the TF binding of each other through an allosteric mechanism, the chimeric TF construct mapping experiments as described elsewhere in this disclosure demonstrate that the antibodies of groups 29, 39 and 54 bind epitopes distinct from TF-011's epitope.

Example 28: Anti-TF Antibody Internalization

[00706] To evaluate internalization of the anti-TF antibodies, a cytotoxicity assay was conducted as described in Liao-Chan *et al.*, *PLoS One*, 2015, 10:e0124708, which is incorporated by reference in its entirety. Briefly, cells were plated in 384-well plates (Greiner Bio-One, Monroe, NC, USA) at 4×10^3 cells per well in 40 μl of media. Antibodies and an anti-human Fc Fab conjugated to the tubulin inhibitor mono-methyl auristatin F (MMAF) (Moradec, San Diego, CA, USA) were serially diluted starting at 5 and 30 nM, respectively. The anti-human Fc Fab conjugated to MMAF consisted of a polyclonal antibody specific to the Fc region of human IgGs with a DAR of 1.2 to 1.5. Plates were incubated for 3 days, followed by lysis in CellTiter-Glo (CTG) assay reagent (Promega, Madison, WI, USA). CTG luminescence was measured on an Envision plate reader and the mean and standard deviation of 4 replicates graphed in Prism (GraphPad, La Jolla, CA, USA). For each anti-TF antibody,

the IC₅₀ and its associated 95% confidence interval were calculated in Prism using a 4-parameter binding model. The cell viability results after incubation with anti-TF antibodies and anti-TF antibody Fab:MMAF complexes are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety. The 95% confidence intervals for the IC₅₀ values are also shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety.

[00707] Internalization of the anti-TF antibodies was also evaluated by a quantitative assay based on internalized fluorescence and quenched surface-fluorescence. Cell surface fluorescence quenching was assessed as described in Liao-Chan *et al.*, *PLoS One*, 2015,10:e0124708. Briefly, 1.2x10⁵ MDA-MB-231 cells were pre-incubated with 100 nM of A488-conjugated antibodies in media for 2 hr on ice. After 2 washes, cells were resuspended in cold media and pulsed for up to 4 hr at 37°C. Cells were rapidly chilled and incubated with or without 300 nM of anti-A488 antibody (clone 19A) for 30 min on ice. After 2 washes, dead cells were labeled with DAPI and samples were analyzed on a Novocyte flow cytometer (ACEA Biosciences). The median fluorescence intensities (MFIs) at each anti-A488 mAb concentration were normalized against the isotype control to obtain a normalized MFI percentage. Internalized fluorescence was calculated from quenched and non-quenched sample data by correcting for incomplete surface quenching: $1 - (N_1 - Q_1) / (N_1 - (N_1 Q_0 / N_0))$ with N₁ = unquenched MFI at each time point (t₁); Q₁ = Quenched MFI at t₁; Q₀ = Quenched MFI for the sample kept on ice (t₀); N₀ = Unquenched MFI at t₀. Percent internalization of anti-TF antibodies conjugated to A488 is shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety.

[00708] Because Fab:MMAF binds the Fc region of the TF-specific antibodies, cellular uptake of these complexes can trigger cell death. While the TF-specific antibodies alone had no impact on cell viability in three-day cultures of TF-positive A431 cells, the TF-specific antibodies in complex with Fab:MMAF showed dose-dependent cell killing with IC₅₀ values ranging between 0.07 and 0.14 nM. (See international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety).

[00709] Cellular uptake was corroborated with fluorescently labeled TF-specific antibodies. In a quantitative assay based on internalized fluorescence and quenched surface-fluorescence, the TF-specific antibodies showed between 28 and 37 % internalization after a

4 h incubation. (See international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety).

[00710] These results indicate that the tested anti-TF antibodies can mediate internalization and toxin delivery into TF-positive cells.

Example 29: Cytotoxicity Assays and G₂/M arrest in HUVECs

[00711] To evaluate TF copy number on the cell surface of human umbilical vein endothelial cells (HUVECs), 1.2×10^5 HUVECs were harvested and incubated with 133 nM of anti-human TF antibody 5G9 on a mouse IgG2a backbone for 2 hr on ice. After 2 washes, QIFIKIT beads (Agilent) and cells labeled with anti-TF antibody were incubated for 30 min on ice with 150 nM of Goat Phycoerythrin (PE) F(ab')₂ fragment goat anti-mouse IgG, Fc-gamma fragment specific (Jackson ImmunoResearch). After 2 washes, dead cells were labeled with TO-PRO-3 Iodide (ThermoFisher Scientific) and samples were analyzed on a CytoFLEX flow cytometer (Beckman Coulter). After gating for single live cells, the MFI's were determined using FlowJo (Flowjo, Ashland, OR, USA). A standard curve using QIFIKIT beads was generated in Prism using a 5-parameter binding model to determine copy number. The lower limit of quantitation was 1.9×10^3 antibody binding sites (also referred to as copy number) and the upper limit of quantitation was 8.0×10^5 antibody binding sites.

[00712] In response to injury, inflammatory and angiogenic factors transiently increase expression of surface TF in the vasculature. See Holy *et al.*, *Adv Pharmacol*, 2010, 59:259-592, which is incorporated by reference in its entirety. The transient upregulation of TF in cell culture was mimicked by treating HUVECs with a combination of inflammatory cytokines (5 ng/mL IL1-beta, 25 ng/mL TNF-alpha and 50 ng/mL VEGF). As shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652 (incorporated herein by reference in their entirety), surface TF levels increased from 2.4×10^3 copies in the absence of inflammatory cytokines to 1.2×10^4 copies after 6 h of cytokine treatment. The surface TF was ~3-fold lower after 20 h of cytokine treatment relative to 6 h of treatment, which indicates that the cytokine-induced TF upregulation was transient.

Example 30: Erk Phosphorylation Assay

[00713] For assessment of Erk phosphorylation, A431 cells were plated in 6-well plates (Corning) in media overnight. The following day, cells were washed once and serum starved in serum-free media. After starvation, cells were preincubated with 100 nM of anti-TF antibodies for 30 min at 37 °C. FVIIa was spiked into the wells at 50 nM and incubated for 20

min at 37 °C for p-ERK induction. After induction, cells were lysed with RIPA Lysis and Extraction Buffer with Halt™ Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific). Western blot was performed with 20 µg of cell lysate using Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) and p44/42 MAPK (Erk1/2) (137F5) (Cell Signaling Technology) as primary antibodies and Peroxidase AffiniPure Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch) as a secondary antibody. Non-saturating band intensities for pErk and Erk were measured on an Amersham AI600 (GE Healthcare). Each pErk intensity was normalized against its respective Erk intensity and the no-antibody no-FVIIa sample intensity.

[00714] The Western blot results of pErk and Erk are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety. Treatment with FVIIa induced Erk phosphorylation by 5.2 fold in cell cultures without pretreatment of anti-TF antibodies. The induction of Erk phosphorylation was ablated by pretreatment with 1F, 39A and 54E (fold induction between 0.8 and 1.2) and attenuated by 29E and the members of groups 25 and 43 (fold induction between 2.0 and 3.4).

[00715] This data indicates that anti-TF antibodies inhibit FVIIa-dependent TF signaling when assessing Erk phosphorylation.

Example 31: Antibody-Dependent Cellular Cytotoxicity (ADCC) Assay

[00716] To evaluate ADCC activity, an ADCC Reporter Bioassay Core Kit (Promega) was used following the manufacturer's protocol. Briefly, A431 cells were plated on a microtiter plate (Corning). The following day, the cells were incubated with a ten-point 1:3 dilution titration of anti-TF antibodies starting at 50 nM. An ADCC effector-to-target cell ratio of 8:1 was added to each well and incubated for 6 h at 37 °C. Bio-Glo™ Luciferase Assay Reagent was added to each well to measure luminescence on an Envision plate reader (PerkinElmer, Waltham, MA, USA). The mean and standard deviation of 4 replicates were graphed in Prism. For each antibody, the EC₅₀ and its associated 95 % confidence interval were calculated in Prism using a 4-parameter binding model.

[00717] ADCC reporter luminescence after incubation with the reporter Jurkat cell line in the representative titrations of anti-TF antibodies is shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety. The ADCC reporter luminescence EC₅₀ values for each anti-TF

antibody are shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety.

[00718] All the tested TF-specific antibodies exerted induction of luciferase-dependent luminescence with EC₅₀ values ranging between 0.18 nM and 0.43 nM.

[00719] These data indicate that both the TF-specific antibodies can induce antibody-dependent cellular cytotoxicity (ADCC) via the IgG1 Fc domain of the antibody.

Example 32: Binding Affinity Assay For Pig TF and Rabbit TF

[00720] The ability of certain antibodies was tested for binding to pig TF. For pig TF Biacore-based measurements, a given anti-TF antibody was captured by an anti-human IgG antibody covalently coupled to a CM5 chip (GE Healthcare Bio-Sciences). Association between the anti-TF antibodies and a five-point three-fold titration of pig TF-His starting at 100 nM was measured for 180 to 240 sec. Subsequently, dissociation between the anti-TF antibody and TF-His was measured for 1800 sec. Kinetic data was analyzed and fitted globally using a 1:1 binding model. The K_D values of the indicated TF antibodies measured by the Biacore-based experiments are shown in **Table 48**.

[00721] The ability of certain antibodies was tested for binding to rabbit TF. For rabbit TF Biacore-based measurements, a given anti-TF antibody was captured by an anti-human IgG antibody covalently coupled to a CM5 chip (GE Healthcare Bio-Sciences). Association between the anti-TF antibodies and a five-point three-fold titration of rabbit TF-His starting at 100 nM was measured for 180 to 240 sec. Subsequently, dissociation between the anti-TF antibody and TF-His was measured for 1800 sec. Kinetic data was analyzed and fitted globally using a 1:1 binding model. The K_D values of the indicated TF antibodies measured by the Biacore-based experiments are shown in **Table 48**.

[00722] As shown in **Table 48**, anti-hTF antibodies from groups 25 and 43 exhibit binding activity and cross-reactivity to pig TF and rabbit TF. In contrast, antibodies from groups 1 and 29 show no binding activity to pig TF or rabbit TF.

Table 48: Antibody kinetics for pig and rabbit TF

Antibody	Pig K_D, nM	Rabbit K_D, nM
1G	no binding	no binding
25A	18.7	50.5
25A3	5.5	12.4
25A5	5.2	5.4
25A5-T	4.5	5.4

25G	26.0	75.5
25G1	2.6	3.6
25G9	3.3	4.2
29D	no binding	no binding
43D7	8.8	6.8
43D8	19.2	7.7

no binding*: no binding to weak binding, with no reportable K_D

Example 33: Epitope Binning of Anti-TF Antibodies

[00723] To establish epitope binding differences between the anti-human TF antibodies, chimeric TF construct mapping experiments were conducted. This mapping technique enables discrimination of antibody epitopes.

[00724] Because all the anti-human TF antibodies evaluated do not bind rat TF, the rat TF sequence was used for the construction of chimeric human-rat TF constructs. Chimeric human-rat construct design was guided by the N- and C-terminal domain of TF extracellular domain (amino acids 1 – 107 and 108 – 219 of the extracellular domain, respectively), with an alignment shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety. Based on the chimera mapping results using the constructs from Figure 36 of international PCT application PCT/US2019/012427 and US utility application number 16/959,652, rat amino acid segment 141 – 194 was replaced by the human sequence (amino acid 136 – 189 of hTF extracellular domain), with an alignment shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety. Design of three human TF constructs with either 1 or 2 human–rat substitutions (hTF_K68N, hTF_K149N and hTF_N171H_T197K) was based on reported contact residues K68, K149 and N171 and T197 for the 10H10 antibody (Teplyakov *et al.*, *Cell Signal.*, 2017, 36:139-144), with an alignment shown in international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety.

[00725] To establish binding of the anti-human TF antibodies to the various TF constructs, HEK293 cells were transfected with a DNA plasmid that co-expresses the TF construct and a green fluorescent protein marker. For a subset of the antibodies, an antibody titration (a 12-point 1:3 dilution series starting at 250 nM) was evaluated on select TF constructs (*See* international PCT application PCT/US2019/012427 and US utility application number

16/959,652, incorporated herein by reference in their entirety). These antibody titrations demonstrated that the antibody concentration of 15 µg/ml (100 nM) used in **Tables 51 and 52** was appropriate to establish “Percentage antibody binding to TF construct relative to hTF”. Two days after transfection, cells were collected from the tissue culture plate, stained with 15 µg/ml of the indicated anti-TF antibody, washed, stained with anti-human IgG-Fc Alexa Fluor 647 polyclonal antibody, washed, and stained with the viability dye 4',6-Diamidino-2-Phenylindole, Dihydrochloride. Upon acquisition of 80,000 live events on a flow cytometer, live cells marked with the fluorescent marker were analyzed for the degree of staining by the anti-TF antibody. The median fluorescence intensity values relative to an isotype control for each TF expression construct were divided by the median fluorescence intensity value relative to an isotype control for the hTF expression construct, and the resulting percentage listed as “Percentage antibody binding to TF construct relative to hTF” in **Tables 51 and 52**. As used herein, the term “live cell staining assay” refers to the antibody binding assay used in this example.

[00726] The assumption that all chimeric TF constructs were expressed on the cell surface at levels between 50% and 150% of the hTF control construct was met for all TF constructs for at least one anti-human TF antibody in the antibody collection, with the exception of the h1-107_r construct (human amino acid segment 1–107 replaced by rat sequence). Lack of binding of the anti-human TF antibodies to cell surface-expressed rat TF was expected. When “Percentage antibody binding to TF construct relative to hTF” in **Tables 51 and 52** was less than 50%, the antibody was considered a non-binder (0) in **Tables 53 and 54**. When “Percentage antibody binding to TF construct relative to hTF” in **Tables 51 and 52** was between 50% and 150%, the antibody was considered a binder (1) in **Tables 53 and 54**.

[00727] Each antibody was assigned to an epitope bin in **Table 55** based on the combination of unbound constructs from **Table 53**. The antibodies from Lineage 25 (25A, 25A3, 25A5-T, 25G1 and 25G9) bind a unique epitope, referred to as Epitope Bin 6 in **Table 55**. The antibodies from Lineage 43 (43B1, 43D7, 43D8 and 43Ea) also bind a unique epitope, referred to as Epitope Bin 7 in **Table 55**. The antibody from Lineage 29 (29E) binds a unique epitope, referred to as Epitope Bin 2 in **Table 55**. The antibodies from Lineage 39 and 54 (39A and 54E) bind a unique epitope, referred to as Epitope Bin 3 in **Table 55**.

[00728] Lineage 25 and 43 antibodies are the only antibodies in the antibody panel that bind r141-194_h, the chimeric construct in which rat amino acids 141–194 were replaced by human sequence (**Table 54**). Furthermore, while M1593 cannot bind hTF_K68N, all the other antibodies in the antibody panel bind hTF_K68N (**Table 54**). Only Lineage 25 and 43

antibodies cannot bind hTF_K149N (**Table 54**). Only Lineage 25 antibodies cannot bind hTF_N171H_T197K (**Table 54**). (See international PCT application PCT/US2019/012427 and US utility application number 16/959,652, incorporated herein by reference in their entirety).

[00729] In summary, these results indicate that lineage 25 antibodies bind a unique epitope on human TF compared to all other antibodies tested. Lineage 43 antibodies bind a unique epitope on human TF compared to all other antibodies tested. Lineage 25 and lineage 43 antibodies bind a different epitope on human TF from M1593.

Example 34: Binding Kinetics of 25A5-T to different TF antigens

[00730] The binding kinetics of 25A5-T antibody to human TF, cynomolgus monkey (cyno) TF, pig TF, rabbit TF, and mouse TF were determined. Briefly, the anti-TF antibody was captured by an anti-human IgG antibody covalently coupled to a CM5 chip (GE Healthcare Bio-Sciences). Association between the anti-TF antibodies and a five-point three-fold titration of TF protein starting at 100 nM was measured for 180 to 240 sec. The tested TF proteins are shown in **Table 74**. Subsequently, dissociation between the anti-TF antibody and TF protein was measured for 1800 sec. The experimental conditions for each antigen-antibody pair are listed in **Table 75**.

Table 74: The tested TF proteins—extracellular domain fragments (aa 1-219) with C-terminal His6 tag (SEQ ID NO: 943)

Name	Concentration [mg/mL]	Formulation Buffer	Monomer by SEC (%)
Human TF	2.78	PBS, pH 7.4	100.0
Cynomolgus monkey TF	1.43	PBS, pH 7.4	100.0
Rabbit TF	1.74	PBS, pH 7.4	95.0
Pig TF	4.11	PBS, pH 7.4	95.0
Mouse TF	3.02	PBS, pH 7.4	95.0

Table 75: Experimental Conditions for the 25A5T to TF proteins

Antigen	Concentration Range [nM]	Association Time [s]	Dissociation Time [s]	Flow Rate [ul/min]
human TF	0.4, 1.2, 3.7, 11, 33	180	900	30
cyno TF	0.4, 1.2, 3.7, 11, 33	180	1800	30
pig TF	1.2, 3.7, 11, 33, 100	180	600	30
rabbit TF	1.2, 3.7, 11, 33, 100	180	900	30
mouse TF	6.2, 18.5, 55.6, 166.7, 500	180	30	30

[00731] Kinetic data was obtained from the resulting analyte sensorgrams. The sensorgrams were fitted using a 1:1 Langmuir Binding Model, using Biacore X100 Evaluation software. R_{max} , t_c , k_a , and k_d were fit globally. Because samples were diluted more than 20-fold in running buffer, bulk refractive index contributions were set to zero. Each titration was fit individually as a replicate, and the resulting average and standard deviation of the three replicates is reported. The TF binding kinetics data is provided in **Table 76** and **FIG. 26**.

Table 76: TF Binding Kinetics to 25A5-T. Standard deviations are shown in parentheses.

	K_a (SD) [1/Ms]	K_d (SD) [1/s]	K_D (SD) [M]	R_{max} [RU]	χ^2 [RU ²]
Human TF	1.09E+06 (2.63E+04)	4.74E-04 (8.04E-06)	4.35E-10 (1.13E-11)	89.13	0.77
Cyno TF	9.13E+05 (3.88E+03)	2.14E-04 (1.09E-05)	2.35E-10 (1.29E-11)	69.29	0.81
Pig TF	5.31E+05 (1.29E+04)	2.38E-03 (1.33E-05)	4.49E-09 (8.48E-11)	42.74	0.22
Rabbit TF	4.66E+05 (1.10E+04)	2.51E-03 (2.07E-05)	5.39E-09 (8.90E-11)	76.58	0.25
Mouse TF	6.09E+05 (8.50E+04)	4.59E-02 (6.69E-03)	7.52E-08 (5.74E-10)	70.14	0.81

[00732] The results showed that 25A5T binds to human TF and cyno TF with similar high affinity. The binding affinity observed to rabbit TF and pig TF was approximately 10-fold lower, and was further reduced when tested against mouse TF. These results support the use of cynomolgus monkeys as the relevant toxicology species, based on similar binding characteristics as seen to the human target.

Example 35: Binding affinity of 25A5-T antibody vs. ICON-1

[00733] To evaluate the difference in affinity between 25A5-T antibody and ICON-1 (an immunoconjugate), Biacore measurements were taken of the 25A5-T antibody and compared to previously determined ICON-1 measurements obtained using the same methods. Briefly, the antibody was covalently coupled to a CM5 chip using an amine-coupling kit and a five-point titration of TF-His in triplicate was analyzed using recommended procedures (GE Healthcare Bio-Sciences). Association between the anti-TF antibodies and a five-point three-fold titration of TF-His starting at 25 to 500 nM was measured for 300 sec. Subsequently, dissociation between the anti-TF antibody and TF-His was measured for up to 1800 sec. Kinetic data was analyzed and fitted globally using a 1:1 binding model. The K_D value of the 25A5-T antibody was significantly lower than that of ICON-1, as previously determined, as measured by Biacore. See **Table 64**.

Table 64: The affinity of ICON-1 and 25A5-T to human TF, as measured by Biacore

Molecule	K _D for Human TF (nM)
ICON-1	2.8
25A5-T	0.4

Example 36: Thrombin Generation Assay for 25A-T antibody vs. ICON-1

[00734] A TGA assay was performed using the calibrated-automated-thrombogram (CAT) instrument manufactured and distributed by STAGO. The test method design was equivalent to a standard CAT assay measurement, except that the plasma source was normal pooled plasma (NPP) in citrate/CTI. The anti-TF antibodies were titrated at 0, 10, 50 and 100 nM and mixed with NPP collected in 11 mM citrate supplemented with 100 microgram/mL of corn trypsin inhibitor (citrate/CTI). Relipidated TF was added to a 96-well assay plate, followed by addition of the antibody/NPP mixture. Directly after combining the relipidated TF with antibody/NPP, thrombin generation was initiated by the addition of calcium and the thrombin substrate. The STAGO software was used to report Peak IIa (highest thrombin concentration generated on the thrombin generation curve, [nM]) and percent peak thrombin generation (% Peak IIa) in the presence of 25A5-T antibody relative to a no-antibody plasma control on the same plate. The data was compared to ICON-1 peak thrombin generation previously determined using the same methods. The percent Peak IIa results for ICON-1 and 25A5-T antibody are shown in **FIG. 20**. The Peak IIa% was 95% or greater in the presence of 25A5-T antibody, at all measured antibody concentrations (10 nM, 50 nM and 100 nM). In comparison to the 25A5-T Peak IIa measurement, the Peak IIa% for ICON-1 was significantly reduced at 50 nM and 100 nM of ICON-1 (**FIG. 20**).

Example 37: Effect of ICON-1 vs 25A5-T on Cytokine Secretion

[00735] IL-8 and GM-CSF protein levels were measured for 25A5-T antibody using the methods described in Example 18. Briefly, MDA-MB-231 cells were seeded at 2×10^5 cells/mL in L-15 media in 96-well plates overnight. After being serum starved for 2 hours, the indicated concentration of 25A5-T antibody was added to 20 nM human FVIIa and incubated for 5 hours at 37°C. IL-8 levels and GMCSF in culture supernatants were determined by ELISA. The GMCSF and IL-8 percent inhibition levels for 25A5-T antibody were compared to ICON-1 GMCSF and IL-8 percent inhibition levels previously determined using the same methods, and the results are shown in **FIGS. 21A and 21B**. The results showed that the 25A5-T antibody inhibited GMCSF (EC₅₀=0.9 nM) and IL-8 (EC₅₀=1.0

nM) at a significantly lower concentration than ICON-1 (which had a GMCSF EC50 and IL-8 EC50 of 33.5 nM and 32.9 nM respectively).

Example 38: ADCC and ADCP Assay for 25A-T antibody vs. ICON-1

[00736] The ADCC and ADCP for 25A5-T antibody and ICON-1 were determined using a Killing Immune-Lysis Reaction (KILR[®]) cytotoxicity assay. Briefly, KILR[®] MDA-MB-231 reporter cells were opsonized with a dose response of 25A5-T antibody, ICON-1 immunoconjugate or isotype control for 30 minutes followed by a 4 hour incubation with rested primary peripheral blood mononuclear cells (PBMCs) for ADCC at an effector-to-target cell ratio (E:T) of 40:1 or 24 hrs with M0 macrophages differentiated from monocytes to measure ADCP at an E:T of 8:1 followed by addition of KILR[®] detection reagent. The results are presented as mean % ADCC or ADCP values normalized to an isotype (no antibody) control wells in **FIGS. 22A and 22B**. As shown, the administration of 25A5T resulted in enhanced ADCC and ADCP relative to administration of ICON-1, as previously determined. The ADCP resulting from 25A5-T was about two-fold or more greater than that for ICON-1, as previously determined, at or above an antibody concentration of 10^{-2} ug/mL (**FIG. 22B**).

Table 51: Percent antibody binding to TF construct relative to hTF

Construct	Antibody															
	1F	29E	39A	54E	TF-011	5G9	M1593	25A	25A3	25A5-T	25G1	43B1	43D7	43D8	43Ea	
hTF	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
rTF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
h1-107_r(52)	0	0	0	0	0	41	0	32	36	36	37	28	33	35	31	
h1-77_r(25)	0	0	0	0	0	94	0	86	95	84	88	64	64	75	69	
h1-38_r(14)	91	87	100	102	104	100	104	101	104	93	101	88	97	106	104	
h39-77_r(11)	0	0	0	0	0	88	2	82	88	80	87	71	59	75	71	
h78-107_r(21)	0	8	81	68	32	114	74	108	116	103	113	108	113	114	114	
h78-107_r.v2(27)	0	0	76	62	23	101	59	95	96	91	94	93	97	100	101	
h78-93_r(18)	102	0	77	91	110	102	104	106	105	92	101	98	101	104	102	
h94-107_r(9)	1	82	85	89	27	91	46	82	86	78	83	77	84	92	89	
h108-219_r(46)	119	118	118	122	128	0	0	0	0	0	0	0	0	0	0	
h108-158_r(19)	98	101	107	108	108	63	4	1	0	0	11	22	0	1	0	
h108-132_r(10)	105	108	109	107	124	125	124	112	112	106	111	118	122	126	122	
h133-158_r(9)	113	122	119	130	134	91	0	0	0	0	2	3	0	4	1	
h133-145_r(4)	84	95	96	104	104	108	100	77	80	80	87	100	99	104	103	
h133-139_r(2)	82	90	95	103	102	104	103	88	89	88	91	86	94	101	97	
h140-145_r(2)	89	100	101	110	109	113	97	80	87	86	89	105	101	104	104	
h146-158_r(5)	115	122	125	134	134	91	133	2	17	18	17	0	3	20	10	
h146-151_r(1)	122	133	139	142	143	141	118	3	14	17	7	0	11	39	23	
h152-158_r(4)	110	121	128	127	136	82	132	110	116	112	116	111	119	134	129	
h159-219_r(27)	132	134	141	142	155	0	137	0	0	0	0	0	132	130	130	
h159-189_r(11)	94	101	104	110	112	0	105	0	0	0	0	0	100	106	104	
h159-174_r(6)	96	98	101	118	120	0	98	0	0	0	0	0	103	115	112	
h159-166_r(3)	89	93	96	100	98	104	100	93	95	87	91	88	99	106	105	
h167-174_r(3)	96	112	96	122	128	0	118	0	0	0	0	0	109	121	112	
h175-189_r(5)	97	113	112	118	123	119	114	86	95	99	100	86	109	118	114	
h190-219_r(16)	111	138	149	141	145	12	143	125	124	119	127	144	133	140	136	

Table 52: Percent antibody binding to TF construct relative to hTF

	Antibody															
Construct	1F	29E	39A	54E	TF-011	5G9	M1593	25A	25A3	25A5-T	25G1	25G9	43B1	43D7	43D8	43Ea
hTF	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
rTF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
r141-194_h*	0	0	0	0	0	32	0	65	89	88	83	108	90	102	95	81
hTF_K68N	105	115	119	118	111	132	0	93	124	126	115	103	107	116	119	118
hTF_K149N	115	117	131	127	132	145	111	2	12	13	7	0	10	29	20	1
hTF_N171H_T197K	83	98	94	89	109	102	113	1	4	7	1	0	98	101	103	118

*rat amino acid segment replaced by human segment, resulting in 20 amino acid changes

Table 53: Antibody binding to TF construct. 1=binding and 0=no binding.

Construct	Antibody													
	1F	29E	39A	54E	TF-011	5G9	M1593	25A	25A3	25A5-T	25G1	25G9	43B1	43D7
hTF	1	1	1	1	1	1	1	1	1	1	1	1	1	1
rTF	0	0	0	0	0	0	0	0	0	0	0	0	0	0
h1-107_r (52)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
h1-77_r (25)	0	0	0	0	0	1	0	1	1	1	1	1	1	1
h1-38_r (14)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
h39-77_r (11)	0	0	0	0	0	1	0	1	1	1	1	1	1	1
h78-107_r (21)	0	0	1	1	0	1	1	1	1	1	1	1	1	1
h78-107_r.v2 (27)	0	0	1	1	0	1	1	1	1	1	1	1	1	1
h78-93_r (18)	1	0	1	1	1	1	1	1	1	1	1	1	1	1
h94-107_r (9)	0	1	1	1	0	1	0	1	1	1	1	1	1	1
h108-219_r (46)	1	1	1	1	1	0	0	0	0	0	0	0	0	0
h108-158_r (19)	1	1	1	1	1	1	0	0	0	0	0	0	0	0
h108-132_r (10)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
h133-158_r (9)	1	1	1	1	1	1	0	0	0	0	0	0	0	0
h133-145_r (4)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
h133-139_r (2)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
h140-145_r (2)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
h146-158_r (5)	1	1	1	1	1	1	1	0	0	0	0	0	0	0
h146-151_r (1)	1	1	1	1	1	1	1	0	0	0	0	0	0	0
h152-158_r (4)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
h159-219_r (27)	1	1	1	1	1	0	1	0	0	0	0	0	1	1
h159-189_r (11)	1	1	1	1	1	0	1	0	0	0	0	0	1	1
h159-174_r (6)	1	1	1	1	1	0	1	0	0	0	0	0	1	1
h159-166_r (3)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
h167-174_r (3)	1	1	1	1	1	0	1	0	0	0	0	0	1	1
h175-189_r (5)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
h190-219_r (16)	1	1	1	1	1	0	1	1	1	1	1	1	1	1

Human amino acid segment replaced by rat segment
(in parentheses: number of amino acid changes relative to human TF)

Table 54: Antibody binding to TF construct. 1=binding and 0=no binding

Construct	Antibody													
	1F	29E	39A	54E	TF-011	5G9	M1593	25A	25A3	25A5-T	25G1	25G9	43B1	43D7
hTF	1	1	1	1	1	1	1	1	1	1	1	1	1	1
rTF	0	0	0	0	0	0	0	0	0	0	0	0	0	0
r141-194_h*	0	0	0	0	0	0	0	1	1	1	1	1	1	1
hTF_K68N	1	1	1	1	1	1	0	1	1	1	1	1	1	1
hTF_K149N	1	1	1	1	1	1	1	0	0	0	0	0	0	0
hTF_N171H_T197K	1	1	1	1	1	1	1	0	0	0	0	0	1	1

*rat amino acid segment replaced by human segment, resulting in 20 amino acid changes

Table 55: Epitope Bin assignment based on unbound chimeric constructs

Antibody	Constructs not bound by antibody	Epitope Bin
1F	rTF, h1-107_r, h1-77_r, h39-77_r, h78-107_r, h78-107_r.v2, h94-107_r	1
29E	rTF, h1-107_r, h1-77_r, h39-77_r, h78-107_r, h78-107_r.v2, h78-93_r	2
39A	rTF, h1-107_r, h1-77_r, h39-77_r	3
54E	rTF, h1-107_r, h1-77_r, h39-77_r	3
TF-011	rTF, h1-107_r, h1-77_r, h39-77_r, h78-107_r, h78-107_r.v2, h94-107_r	1
5G9	rTF, h1-107_r, h108-219_r, h159-219_r, h159-189_r, h159-174_r, h167-174_r, h190-219_r	4
M1593	rTF, h1-107_r, h1-77_r, h39-77_r, h94-107_r, h108-219_r, h108-158_r, h133-158_r	5
25A	rTF, h1-107_r, h108-219_r, h108-158_r, h133-158_r, h146-158_r, h159-219_r, h159-189_r, h159-174_r, h167-174_r	6
25A3	rTF, h1-107_r, h108-219_r, h108-158_r, h133-158_r, h146-158_r, h159-219_r, h159-189_r, h159-174_r, h167-174_r	6
25A5-T	rTF, h1-107_r, h108-219_r, h108-158_r, h133-158_r, h146-158_r, h159-219_r, h159-189_r, h159-174_r, h167-174_r	6
25G1	rTF, h1-107_r, h108-219_r, h108-158_r, h133-158_r, h146-158_r, h159-219_r, h159-189_r, h159-174_r, h167-174_r	6
25G9	rTF, h1-107_r, h108-219_r, h108-158_r, h133-158_r, h146-158_r, h159-219_r, h159-189_r, h159-174_r, h167-174_r	6
43B1	rTF, h1-107_r, h108-219_r, h108-158_r, h133-158_r, h146-158_r, h159-219_r, h159-189_r, h159-174_r, h167-174_r	7
43D7	rTF, h1-107_r, h108-219_r, h108-158_r, h133-158_r, h146-158_r, h159-219_r, h159-189_r, h159-174_r, h167-174_r	7
43D8	rTF, h1-107_r, h108-219_r, h108-158_r, h133-158_r, h146-158_r, h159-219_r, h159-189_r, h159-174_r, h167-174_r	7
43Ea	rTF, h1-107_r, h108-219_r, h108-158_r, h133-158_r, h146-158_r, h159-219_r, h159-189_r, h159-174_r, h167-174_r	7

[00737] While the invention has been particularly shown and described with reference to a preferred embodiment and various alternate embodiments, it will be understood by persons skilled in the relevant art that various changes in form and details can be made therein without departing from the spirit and scope of the invention.

[00738] All references, issued patents and patent applications cited within the body of the instant specification are hereby incorporated by reference in their entirety, for all purposes.

SEQUENCES

Table 13: Variable region sequences

Clone	VH Domains (SEQ ID NO)	VL Domains (SEQ ID NO)
1F	EVQLLESGGGLVQPGGSLRLSCAASGFT FSDYAMGWVRQAPGKGLEWVSTISGSG GLTYYADSVKGRFTISRDN SKNTLYLQM NSLRAEDTAVYYCAKAPYGYMDVWG KGT TVTVSS (SEQ ID NO:37)	DIQMTQSPSTLSASVGDRVITITCRASQIS SWLAWYQQKPGKAPKLLIYKASSLESG VPSRFSGSGSGTEFTLTISLQPD DFATYY CQQYKSYITFGGGTKVEIK (SEQ ID NO:38)
1G	EVQLLESGGGLVQPGGSLRLSCAASGFT FSSYAMAWVRQAPGKGLEWVSAISGSG GLTYYADSVKGRFTISRDN SKNTLYLQM NSLRAEDTAVYYCAKAPYGYMDVWG KGT TVTVSS (SEQ ID NO:75)	DIQMTQSPSTLSASVGDRVITITCRASQIS SWLAWYQQKPGKAPKLLIYKASSLESG VPSRFSGSGSGTEFTLTISLQPD DFATYY CQQYKSYITFGGGTKVEIK (SEQ ID NO:76)
25A	QVQLVQSGAEVKKPGASVKVSKASGY TFDVYGISWVRQAPGQGLEWMGWIAPI NGNTNYAQKLQGRVTMTTDTSTSTAYM ELRSLRSDDTAVYYCARDAGTYS PFGYG MDVWGQGTTTVTVSS (SEQ ID NO:113)	DIQMTQSPSTLSASVGDRVITITCRASQIS SWLAWYQQKPGKAPKLLIYKASSLESG VPSRFSGSGSGTEFTLTISLQPD DFATYY CQQFQSLPPFTFGGGTKVEIK (SEQ ID NO:114)
25A3	QVQLVQSGAEVKKPGASVKVSKASGY TFDVYGISWVRQAPGQGLEWMGWIAPI SGNTNYAQKLQGRVTMTTDTSTSTAYM ELRSLRSDDTAVYYCARDAGTYS PFGYG MDVWGQGTTTVTVSS (SEQ ID NO:151)	DIQMTQSPSTLSASVGDRVITITCQASQSI NNWLAWYQQKPGKAPKLLIYKAYNLES GVPSRFSGSGSGTEFTLTISLQPD DFATY YCQLFQSLPPFTFGGGTKVEIK (SEQ ID NO:152)
25A5	QVQLVQSGAEVKKPGASVKVSKASGY TFDVYGISWVRQAPGQGLEWMGWIAPI SGNTNYAQKLQGRVTMTTDTSTSTAYM ELRSLRSDDTAVYYCARDAGTYS PFGYG MDVWGQGTTTVTVSS (SEQ ID NO:189)	DIQMTQSPSTLSASVGDRVITITCRASESIS NWLAWYQQKPGKAPKLLIYKAYSLEYG VPSRFSGSGSGTEFTLTISLQPD DFATYY CQQFQKLPPFTFGGGTKVEIK (SEQ ID NO:190)
25A5-T	QVQLVQSGAEVKKPGASVKVSKASGY TFDAYGISWVRQAPGQGLEWMGWIAPI SGNTNYAQKLQGRVTMTTDTSTSTAYM ELRSLRSDDTAVYYCARDAGTYS PFGYG MDVWGQGTTTVTVSS (SEQ ID NO:836)	DIQMTQSPSTLSASVGDRVITITCRASESIS NWLAWYQQKPGKAPKLLIYKAYSLEYG VPSRFSGSGSGTEFTLTISLQPD DFATYY CQQFQKLPPFTFGGGTKVEIK (SEQ ID NO:837)
25G	QVQLVQSGAEVKKPGASVKVSKASGY TFRSYGISWVRQAPGQGLEWMGWVAPY NGNTNYAQKLQGRVTMTTDTSTSTAYM ELRSLRSDDTAVYYCARDAGTYS PYGY GMDVWGQGTTTVTVSS (SEQ ID NO:227)	DIQMTQSPSTLSASVGDRVITITCRASQIS SWLAWYQQKPGKAPKLLIYKASSLESG VPSRFSGSGSGTEFTLTISLQPD DFATYY CQQFQSLPPFTFGGGTKVEIK (SEQ ID NO:228)
25G1	QVQLVQSGAEVKKPGASVKVSKASGY TFRSYGISWVRQAPGQGLEWMGWVAPY SGNTNYAQKLQGRVTMTTDTSTSTAYM ELRSLRSDDTAVYYCARDAGTYS PYGY GMDVWGQGTTTVTVSS (SEQ ID NO:265)	DIQMTQSPSTLSASVGDRVITITCRASHSI DSWLAWYQQKPGKAPKLLIYKASYLES GVPSRFSGSGSGTEFTLTISLQPD DFATY YCQLFQSLPPFTFGGGTKVEIK (SEQ ID NO:266)
25G9	QVQLVQSGAEVKKPGASVKVSKASGY TFRSYGISWVRQAPGQGLEWMGWVAPY SGNTNYAQKLQGRVTMTTDTSTSTAYM ELRSLRSDDTAVYYCARDAGTYS PYGY GMDVWGQGTTTVTVSS (SEQ ID NO:303)	DIQMTQSPSTLSASVGDRVITITCQASQSI DSWLAWYQQKPGKAPKLLIYASAYLES GVPSRFSGSGSGTEFTLTISLQPD DFATY YCQRFQSLPPFTFGGGTKVEIK (SEQ ID NO:304)
29D	QVQLVESGGGVVQPGRLRLSCAASGFT FHSRGMHWVRQAPGKGLEWVAVITYD GINKYYADSV EGRFTISRDN SKNTLYLQ MNSLRAEDTAVYYCARDGVYGYDY WGQGT LVTVSS (SEQ ID NO:341)	DIVMTQSPDSLAVSLGERATINCKSSQSV LFSSNNKNYLAWYQQKPGQP PKLLIYW ASTRESGV PDRFSGSGSGTDFTLTISLQ AEDVAVYYCQQFHSYPLTFGGGT KVEIK (SEQ ID NO:342)
29E	QVQLVESGGGVVQPGRLRLSCAASGFT FRSYGMHWVRQAPGKGLEWVAVITYD GINKYYADSV EGRFTISRDN SKNTLYLQ	DIVMTQSPDSLAVSLGERATINCKSSQSV LFSSNNKNYLAWYQQKPGQP PKLLIYW ASTRESGV PDRFSGSGSGTDFTLTISLQ

Clone	VH Domains (SEQ ID NO)	VL Domains (SEQ ID NO)
	MNSLRAEDTAVYYCARDGVYGVYDYWGQGTTLVTVSS (SEQ ID NO:379)	AEDVAVYYCQQFHSYPLTFGGGKTKVEIK (SEQ ID NO:380)
39A	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSNAIGWVRQAPGQGLEWMGSIPIIGFANYAQKFQGRVTITADESTSTAYMELSLRSEDNAVYYCARDGSGYYGASSFGMDVWGQGTTLVTVSS (SEQ ID NO:417)	EIVMTQSPATLSVSPGERATLSCRASQSVSSNLAWYQQKPGQAPRLLIYGASTRATGIPARFSGSGSGTEFTLTISLQSEDFAVYYCEQYNNLPLTFGGGKTKVEIK (SEQ ID NO:418)
43B	QVQLQESGPGLVKPSQTLSTCTVSGGSISSGQYWSWIRQHPGKGLEWIGEIIYSGSTRYNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCARDAPYYGGGYYMMDVWGKGTTLVTVSS (SEQ ID NO:455)	EIVLTQSPGTLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQVGVVPYTFGGGKTKVEIK (SEQ ID NO:456)
43B1	QVQLQESGPGLVKPSQTLSTCTVSGGSISSGQYWSWIRQHPGKGLEWIGEIIYSGSTRYNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCARDAPYYGGGYYMMDVWGKGTTLVTVSS (SEQ ID NO:493)	EIVLTQSPGTLSPGERATLSCRASESVDSSYLAWYQQKPGQAPRLLIYGASTRQTGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQAGVVPYTFGGGKTKVEIK (SEQ ID NO:494)
43B7	QVQLQESGPGLVKPSQTLSTCTVSGGSISSGQYWSWIRQHPGKGLEWIGEIIYSGSTRYNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCARDAPYYGGGYYMMDVWGKGTTLVTVSS (SEQ ID NO:531)	EIVLTQSPGTLSPGERATLSCRASESVDSSYLAWYQQKPGQAPRLLIYGADSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQDGVVPYTFGGGKTKVEIK (SEQ ID NO:532)
43D	QVQLQQWGAGLLKPSETLSLTCAVYGGSLSGYYWSWIRQPPGKGLEWIGEIGASGSTRYNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCARDTPYYYEGGYYMMDVWGKGTTLVTVSS (SEQ ID NO:569)	EIVLTQSPGTLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQVGVVPYTFGGGKTKVEIK (SEQ ID NO:570)
43D7	QVQLQQWGAGLLKPSETLSLTCAVYGGSLSGYYWSWIRQPPGKGLEWIGEIGASGSTRYNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCARDTPYYYEGGYYMMDVWGKGTTLVTVSS (SEQ ID NO:607)	EIVLTQSPGTLSPGERATLSCRASDSVDSSYLAWYQQKPGQAPRLLIYGAFSRANGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQAGVVPYTFGGGKTKVEIK (SEQ ID NO:608)
43D8	QVQLQQWGAGLLKPSETLSLTCAVYGGSLSGYYWSWIRQPPGKGLEWIGEIGASGSTRYNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCARDTPYYYEGGYYMMDVWGKGTTLVTVSS (SEQ ID NO:645)	EIVLTQSPGTLSPGERATLSCRASQSVSSFLAWYQQKPGQAPRLLIYGAYSRTATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQAGVVPYTFGGGKTKVEIK (SEQ ID NO:646)
43E	QVQLQESGPGLVKPSQTLSTCTVSGGSISSGQYWSWIRQHPGKGLEWIGEIIYSGSTRYNPSLKSRTISVDTSKDQFSLKLSSVTAADTAVYYCARDTPYYDGGYYMMDVWGKGTTLVTVSS (SEQ ID NO:683)	EIVLTQSPGTLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQVGVVPYTFGGGKTKVEIK (SEQ ID NO:684)
43Ea	QVQLQESGPGLVKPSQTLSTCTVSGGSISSGQYWSWIRQHPGKGLEWIGEIIYSGSTRYNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCARDTPYYDGGYYMMDVWGKGTTLVTVSS (SEQ ID NO:721)	EIVLTQSPGTLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQVGVVPYTFGGGKTKVEIK (SEQ ID NO:722)
54E	QVQLVQSGAEVKKPGASVKVSCKASGYTFANYMHWRQAPGQGLEWMGINPSGGITVYAQKFQGRVTMTRDTSTSTVYMLESSLRSEDNAVYYCARGGSKVAALAFDIWGQGTMTVTVSS (SEQ ID NO:759)	DIQMTQSPSSLSASVGRVTITCQASQDISNSLNWYQQKPGKAPKLLIYDASNLETGVPSRFSGSRSGTDFTFTISLQPEDIATYYCQYNFHLPLTFGGGKTKVEIK (SEQ ID NO:760)

Table 14: Variable region sequence consensus

Group	VH Domain Consensus (SEQ ID NO)	VL Domain Consensus (SEQ ID NO)
1	EVQLLES GGLVQPGGSLRLSCAASGFT FSx[D/S]YAMx[A/G]WVRQAPGKGLEWV Sx[A/T]ISGSGGLTYADSVKGRFTISR NSKNTLYLQMNSLRAEDTAVYYCAKAP YGYMDVWVGKTTVTVSS (SEQ ID NO:761)	DIQMTQSPSTLSASVGDRVTITCRASQSI SSWLAWYQQKPGKAPKLLIYKASSLES GVPSRFSGSGSGTEFTLTISLQPDFFAT YYCQQYKSYITFGGGTKVEIK (SEQ ID NO:762)
25	QVQLVQSGAEVKKPGASVKVSCKASGY TFx[D/R]x[S/V/A]YGISWVRQAPGQGLE WMGWx[I/V]APYx[S/N]GNTNYAQKLQG RVTMTTDTSTSTAYMELRSLRSDDTAV YYCARDAGTYSPx[F/Y]GYGMDVWVGQ TTVTVSS (SEQ ID NO:763)	DIQMTQSPSTLSASVGDRVTITC _x [R/Q]A S _x [Q/E/H]S _{Lx} [S/D/N] _x [S/N]WLAWYQQKP GKAPKLLIY _x [K/S]A _x [S/Y] _x [S/Y/N]LE _x [S/ Y]GVPSRFSGSGSGTEFTLTISLQPDFFA TYYCQ _x [Q/L/R]FQ _x [S/K]LPPFTFGGGTK VEIK (SEQ ID NO:764)
29	QVQLVESGGGVVQPGSLRLSCAASGFT Fx[H/R]Sx[R/Y]GMHWVRQAPGKGLEWV AVITYDGINKYYADSVGRFTISRDNK NTLYLQMNSLRAEDTAVYYCARDGVY YGVYDYWGQGTTLVTVSS (SEQ ID NO:765)	DIVMTQSPDSLAVSLGERATINCKSSQSV LFSSNNKNYLAWYQQKPGQPPKLLIYW ASTRESGVPDRFSGSGSGTDFTLTISLQ AEDVAVYYCQQFHSYPLTFGGGKVEI K (SEQ ID NO:766)
39	QVQLVQSGAEVKKPGSSVKVSCKASGG TFSSNAIGWVRQAPGQGLEWMGSIPIIG FANYAQKFQGRVTITADESTSTAYMELS SLRSEDVAVYYCARDGSGYYGASSFGM DVWGQGTTLVTVSS (SEQ ID NO:767)	EIVMTQSPATLSVSPGERATLSCRASQSV SSNLAWYQQKPGQAPRLLIYGASTRAT GIPARFSGSGSGTEFTLTISLQSEDFAVY YCEQYNNLPLTFGGGKVEIK (SEQ ID NO:768)
43	QVQLQx[E/Q]x[S/W]Gx[P/A]GLx[V/L]KP Sx[Q/E]TLSLTCx[T/A]Vx[S/Y]GGs _x [I/L]S SGx[Q/Y]YWSWIRQx[H/P]PGKGLEWIGE Ix[Y/G]x[Y/A]SGSTRYNPSLKSRTVISVD TSKx[N/D]QFSLKLSSVTAADTAVYYCA RDx[T/A]PYYYx[E/G/D]GGYYYYMDVW GKGTTLVTVSS (SEQ ID NO:769)	EIVLTQSPGTLSPGERATLSCRASx[Q/E /D]SVx[S/D]SSx[Y/F]LAWYQQKPGQAPR LLIYGAs[S/D/F/Y] _x [S/T]Rx[A/Q] _x [T/N]GI PDRFSGSGSGTDFTLTISRLEPEDFAVYY CQQx[V/A/D]GVVPYTFGGGKVEIK (SEQ ID NO:770)
54	QVQLVQSGAEVKKPGASVKVSCKASGY TFANYMHWRQAPGQGLEWMGIINPS GGITVY AQKFQGRVTMTTRDTSTSTVYM ELSLRSEDVAVYYCARGGSKVAALAF DIWGQGTMTVTVSS (SEQ ID NO:771)	DIQMTQSPSSLSASVGDRVTITCQASQDI SNSLNWYQQKPGKAPKLLIYDASNLET GVPSRFSGSRSGTDFTFTISLQPEDATY YCQYNFHLPLTFGGGKVEIK (SEQ ID NO:772)

Table 15: Antibody 1F-CDR Sequences

	Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH</i> <i>CDR1</i>	DYAMG (SEQ ID NO:1)	GFTFSDY (SEQ ID NO:13)	GFTFSDYAMG (SEQ ID NO:19)	SDYAMG (SEQ ID NO:25)	GFTFSDYA (SEQ ID NO:31)
	<i>VH</i> <i>CDR2</i>	TISGSGGLTYYA DSVKG (SEQ ID NO:2)	GSGG (SEQ ID NO:14)	TISGSGGLTY (SEQ ID NO:20)	WVSTISGSGGLT Y (SEQ ID NO:26)	ISGSGGLT (SEQ ID NO:32)
	<i>VH</i> <i>CDR3</i>	APYGYMDV (SEQ ID NO:3)	PYGYMD (SEQ ID NO:15)	APYGYMDV (SEQ ID NO:21)	AKAPYGYMD (SEQ ID NO:27)	AKAPYGYMDV (SEQ ID NO:33)
VL CDR Seq.	<i>VL</i> <i>CDR1</i>	RASQSISSWLA (SEQ ID NO:4)	SQSISSW (SEQ ID NO:16)	RASQSISSWLA (SEQ ID NO:22)	SSWLAWY (SEQ ID NO:28)	QSISSW (SEQ ID NO:34)
	<i>VL</i> <i>CDR2</i>	KASSLES (SEQ ID NO:5)	KAS (SEQ ID NO:17)	KASSLES (SEQ ID NO:23)	LLIYKASSLE (SEQ ID NO:29)	KAS (SEQ ID NO:35)
	<i>VL</i> <i>CDR3</i>	QQYKSYIT (SEQ ID NO:6)	YKSYI (SEQ ID NO:18)	QQYKSYIT (SEQ ID NO:24)	QQYKSYI (SEQ ID NO:30)	QQYKSYIT (SEQ ID NO:36)
VH Sequence*:						
EVQLLESGGGLVQPGGSLRLSAA SGFTFSDYAMGWVVRQAPGKGLEWVSTISGSGGLTYVADSVKGRFTISRDN SKNTLYLQMNSLR AEDTA						
VL Sequence*:						
DIQMTQSPSTLSASVGDRTITTCRASQSISSWLA WYQQKPGKAPKLLIYKASSLES GVP SRFS GSGSGTEFTLTISSLQPD D FATTY CQQYKSYIT						
FGGGTKVEIK (SEQ ID NO:38)						

*Exemplary CDR sequences encompass amino acids as determined by Kabat plus Chothia

Table 16: Antibody 1G-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH</i>	GFTFSSYAMA (SEQ ID NO:39)	SYAMA (SEQ ID NO:45)	GFTFSSY (SEQ ID NO:51)	GFTFSSYAMA (SEQ ID NO:57)	SSYAMA (SEQ ID NO:63)	GFTFSSYA (SEQ ID NO:69)
	<i>CDR1</i>	AISGSGGLTYA DSVKG (SEQ ID NO:40)	AISGSGGLTYA DSVKG (SEQ ID NO:46)	GSGG (SEQ ID NO:52)	AISGSGGLTY (SEQ ID NO:58)	WVSAISGSGGLT Y (SEQ ID NO:64)	ISGSGGLT (SEQ ID NO:70)
	<i>VH</i>	APYGYMDV (SEQ ID NO:41)	APYGYMDV (SEQ ID NO:47)	PYGYMD (SEQ ID NO:53)	APYGYMDV (SEQ ID NO:59)	AKAPYGYMD (SEQ ID NO:65)	AKAPYGYMDV (SEQ ID NO:71)
	<i>CDR3</i>	RASQSISSWLA (SEQ ID NO:42)	RASQSISSWLA (SEQ ID NO:48)	SQSISSW (SEQ ID NO:54)	RASQSISSWLA (SEQ ID NO:60)	SSWLAWY (SEQ ID NO:66)	QSISW (SEQ ID NO:72)
VL CDR Seq.	<i>VL</i>	KASSLES (SEQ ID NO:43)	KASSLES (SEQ ID NO:49)	KAS (SEQ ID NO:55)	KASSLES (SEQ ID NO:61)	LLIYKASSLE (SEQ ID NO:67)	KAS (SEQ ID NO:73)
	<i>CDR2</i>	QQYKSYIT (SEQ ID NO:44)	QQYKSYIT (SEQ ID NO:50)	YKSYI (SEQ ID NO:56)	QQYKSYIT (SEQ ID NO:62)	QQYKSYI (SEQ ID NO:68)	QQYKSYIT (SEQ ID NO:74)
	<i>VL</i>	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMAWVRQAPGKGLEWVSAISGSGGLTYYADSVKGRFTISRDNKNTLYLQMNSLRAEDTA VYYCAKAPYGYMDVWGKGTITVSS (SEQ ID NO:75)					
	<i>CDR3</i>	DIQMTQSPSTLSASVGDRTITCRASQSISSWLAWYQQKPGKAPKLLIYKASSLESVPSRFSGSGSGTEFTLTISLQPDFFATYYCQQYKSYIT FGGGTKVEIK (SEQ ID NO:76)					

VH Sequence:

VL Sequence:

*Exemplary CDR sequences encompass amino acids as determined by Kabat plus Chothia

Table 17: Antibody 25A-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH CDR1</i>	GYTFDVYGIS (SEQ ID NO:77)	VYGIS (SEQ ID NO:83)	GYTFDVY (SEQ ID NO:89)	GYTFDVYGIS (SEQ ID NO:95)	DVYGIS (SEQ ID NO:101)	GYTFDVYG (SEQ ID NO:107)
	<i>VH CDR2</i>	WIAPYNGNTNY AQKLQG (SEQ ID NO:78)	WIAPYNGNTNY AQKLQG (SEQ ID NO:84)	PYNG (SEQ ID NO:90)	WIAPYNGNTN (SEQ ID NO:96)	WMGWIAPYNGN TN (SEQ ID NO:102)	IAPYNGNT (SEQ ID NO:108)
	<i>VH CDR3</i>	DAGTYSFPGYG MDV (SEQ ID NO:79)	DAGTYSFPGYG MDV (SEQ ID NO:85)	AGTYSFPGYGM D (SEQ ID NO:91)	DAGTYSFPGYG MDV (SEQ ID NO:97)	ARDAGTYSFPGY GMD (SEQ ID NO:103)	ARDAGTYSFPGT GMDV (SEQ ID NO:109)
	<i>VL CDR1</i>	RASQSISSWLA (SEQ ID NO:80)	RASQSISSWLA (SEQ ID NO:86)	SQSISSW (SEQ ID NO:92)	RASQSISSWLA (SEQ ID NO:98)	SSWLAWY (SEQ ID NO:104)	QSISSW (SEQ ID NO:110)
	<i>VL CDR2</i>	KASSLES (SEQ ID NO:81)	KASSLES (SEQ ID NO:87)	KAS (SEQ ID NO:93)	KASSLES (SEQ ID NO:99)	LLIYKASSLE (SEQ ID NO:105)	KAS (SEQ ID NO:111)
	<i>VL CDR3</i>	QQFQSLPPFT (SEQ ID NO:82)	QQFQSLPPFT (SEQ ID NO:88)	FQSLPPF (SEQ ID NO:94)	QQFQSLPPFT (SEQ ID NO:100)	QQFQSLPPF (SEQ ID NO:106)	QQFQSLPPFT (SEQ ID NO:112)
VH Sequence*: QVQLVQSGAEVKPGASVKVSKASGYTFDVYGISWVRQAPGQGLEWMGWIAPYNGNTNYAQLQGRVTMTTDTSTSTAYMELRSLRSDDD TAVYYCARDAGTYSFPGYGM MDV WGQGT TV TVSS (SEQ ID NO:113)							
VL Sequence*: DIQMTQSPSTLSASVGD RV TITCRASQSISSWLA WY QQKPGKAPKLLIYKASSLES GV PSRFRSGSGSGTEFTLTISSLQPD DF ATYYCQQFQSLPP FTFGGGTKVEIK (SEQ ID NO:114)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat plus Chothia

Table 18: Antibody 25A3-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH CDR1</i>	GYTFDVYGIS (SEQ ID NO:115)	VYGIS (SEQ ID NO:121)	GYTFDVY (SEQ ID NO:127)	GYTFDVYGIS (SEQ ID NO:133)	DVYGIS (SEQ ID NO:139)	GYTFDVYG (SEQ ID NO:145)
	<i>VH CDR2</i>	WIAPYSGNTNYA QKLQG (SEQ ID NO:116)	WIAPYSGNTNYA QKLQG (SEQ ID NO:122)	PYSG (SEQ ID NO:128)	WIAPYSGNTN (SEQ ID NO:134)	WMGWIAPYSGN TN (SEQ ID NO:140)	IAPYSGNT (SEQ ID NO:146)
	<i>VH CDR3</i>	DAGTYSFPGYG MDV (SEQ ID NO:117)	DAGTYSFPGYG MDV (SEQ ID NO:123)	AGTYSFPGYGM D (SEQ ID NO:129)	DAGTYSFPGYG MDV (SEQ ID NO:135)	ARDAGTYSFPGY GMD (SEQ ID NO:141)	ARDAGTYSFPGT GMDV (SEQ ID NO:147)
	<i>VL CDR1</i>	QASQSINNWLA (SEQ ID NO:118)	QASQSINNWLA (SEQ ID NO:124)	SQSINNW (SEQ ID NO:130)	QASQSINNWLA (SEQ ID NO:136)	NNWLAWY (SEQ ID NO:142)	QSINNW (SEQ ID NO:148)
	<i>VL CDR2</i>	KAYNLES (SEQ ID NO:119)	KAYNLES (SEQ ID NO:125)	KAY (SEQ ID NO:131)	KAYNLES (SEQ ID NO:137)	LLIYKAYNLE (SEQ ID NO:143)	KAY (SEQ ID NO:149)
	<i>VL CDR3</i>	QLFQSLPPFT (SEQ ID NO:120)	QLFQSLPPFT (SEQ ID NO:126)	FQSLPPF (SEQ ID NO:132)	QLFQSLPPFT (SEQ ID NO:138)	QLFQSLPPF (SEQ ID NO:144)	QLFQSLPPFT (SEQ ID NO:150)
VH Sequence*:							
QVQLVQSGAEVKPGASVKVSKASGYTFDVYGISWVRQAPGQGLEWMGWIAPYSGNTNYAQKQLQGRVTMTTDTSTSTAYMELRSLRSD							
TAVYYCARDAGTYSFPGYGMDVWGQGTITVTVSS (SEQ ID NO:151)							
VL Sequence*:							
DIQMTQSPSTLSASVGDRTITTCQASQSINNWLAWYQQKPGKAPKLLIYKAYNLESGVPSRFGSGSGTEFTLTISLQPDDEATYYCQLFQSLP							
PFTFGGGTKVEIK (SEQ ID NO:152)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat plus Chothia

Table 19a: Antibody 25A5-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH</i> <i>CDR1</i>	GYTFDVYGIS (SEQ ID NO:153)	VYGIS (SEQ ID NO:159)	GYTFDVY (SEQ ID NO:165)	GYTFDVYGIS (SEQ ID NO:171)	DVYGIS (SEQ ID NO:177)	GYTFDVYG (SEQ ID NO:183)
	<i>VH</i> <i>CDR2</i>	WIAPYSGNTNYA QKLQG (SEQ ID NO:154)	WIAPYSGNTNYA QKLQG (SEQ ID NO:160)	PYSG (SEQ ID NO:166)	WIAPYSGNTN (SEQ ID NO:172)	WMGWIAPYSGN TN (SEQ ID NO:178)	IAPYSGNT (SEQ ID NO:184)
	<i>VH</i> <i>CDR3</i>	DAGTYSFPGYG MDV (SEQ ID NO:155)	DAGTYSFPGYG MDV (SEQ ID NO:161)	AGTYSFPGYGM D (SEQ ID NO:167)	DAGTYSFPGYG MDV (SEQ ID NO:173)	ARDAGTYSFPGY GMD (SEQ ID NO:179)	ARDAGTYSFPGT GMDV (SEQ ID NO:185)
	<i>VL</i> <i>CDR1</i>	RASESISNWL (SEQ ID NO:156)	RASESISNWL (SEQ ID NO:162)	SEISINW (SEQ ID NO:168)	RASESISNWL (SEQ ID NO:174)	SNWLAWY (SEQ ID NO:180)	ESISNW (SEQ ID NO:186)
	<i>VL</i> <i>CDR2</i>	KAYSLEY (SEQ ID NO:157)	KAYSLEY (SEQ ID NO:163)	KAY (SEQ ID NO:169)	KAYSLEY (SEQ ID NO:175)	LLIYKAYSLEY (SEQ ID NO:181)	KAY (SEQ ID NO:187)
	<i>VL</i> <i>CDR3</i>	QQFQKLPPFT (SEQ ID NO:158)	QQFQKLPPFT (SEQ ID NO:164)	FQKLPPF (SEQ ID NO:170)	QQFQKLPPFT (SEQ ID NO:176)	QQFQKLPPF (SEQ ID NO:182)	QQFQKLPPFT (SEQ ID NO:188)
VH Sequence*: QVQLVQSGAEVKPGASVKVSKASGYTFDVYGISWVRQAPGQGLEWMGWIAPYSGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSD TAVYYCARDAGTYSFPGYGMVWGQGTITVTVSS (SEQ ID NO:189)							
VL Sequence*: DIQMTQSPSTLSASVGDRTITCRASESISNWLAWYQQKPGKAPKLLIYKAYSLEYGVPSRFSGSGTEFTLTISLQPDDEFATYYCQQFQKL PFTFGGGTKVEIK (SEQ ID NO:190)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat plus Chothia

Table 19b: Antibody 25A5-T-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	VH CDR1	GYTFDAYGIS (SEQ ID NO:884)	AYGIS (SEQ ID NO:890)	GYTFDAY (SEQ ID NO:896)	GYTFDAYGIS (SEQ ID NO:902)	DAYGIS (SEQ ID NO:908)	GYTFDAYG (SEQ ID NO:914)
	VH CDR2	WIAPYSGNTNYA QKLQG (SEQ ID NO:885)	WIAPYSGNTNYA QKLQG (SEQ ID NO:891)	PYSG (SEQ ID NO:897)	WIAPYSGNTN (SEQ ID NO:903)	WMGWIAPYSGN TN (SEQ ID NO:909)	IAPYSGNT (SEQ ID NO:915)
	VH CDR3	DAGTYSFPGYG MDV (SEQ ID NO:886)	DAGTYSFPGYG MDV (SEQ ID NO:892)	AGTYSFPGYGM D (SEQ ID NO:898)	DAGTYSFPGYG MDV (SEQ ID NO:904)	ARDAGTYSFPGY GMD (SEQ ID NO:910)	ARDAGTYSFPGT GMDV (SEQ ID NO:916)
VL CDR Seq.	VL CDR1	RASESISNWL (SEQ ID NO:887)	RASESISNWL (SEQ ID NO:893)	SEISINW (SEQ ID NO:899)	RASESISNWL (SEQ ID NO:905)	SNWLAWY (SEQ ID NO:911)	ESISNW (SEQ ID NO:917)
	VL CDR2	KAYSLEY (SEQ ID NO:888)	KAYSLEY (SEQ ID NO:894)	KAY (SEQ ID NO:900)	KAYSLEY (SEQ ID NO:906)	LLIYKAYSLEY (SEQ ID NO:912)	KAY (SEQ ID NO:918)
	VL CDR3	QQFQKLPPFT (SEQ ID NO:889)	QQFQKLPPFT (SEQ ID NO:895)	FQKLPPF (SEQ ID NO:901)	QQFQKLPPFT (SEQ ID NO:907)	QQFQKLPPF (SEQ ID NO:913)	QQFQKLPPFT (SEQ ID NO:919)
VH Sequence*: QVQLVQSGAEVKPGASVKVSKASGYTFDAYGISWVRQAPGQGLEWMGWIAPYSGNTNYAQKQLQGRVTMTTDTSTSTAYMELRSLRSD TAVYYCARDAGTYSFPGYGMVWGQGTITVSS (SEQ ID NO:836)							
VL Sequence*: DIQMTQSPSTLSASVGDRTITCRASESISNWLAWYQQKPGKAPKLLIYKAYSLEYGVPSRFSGSGGTEFTLTISLQPDDEFATYYCQQFQKL PFTFGGGTKVEIK (SEQ ID NO:837)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat plus Chothia

Table 20: Antibody 25G-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH</i> <i>CDR1</i>	GYTFRSYGIS (SEQ ID NO:191)	SYGIS (SEQ ID NO:197)	GYTFRSY (SEQ ID NO:203)	GYTFRSYGIS (SEQ ID NO:209)	RSYGIS (SEQ ID NO:215)	GYTFRSYG (SEQ ID NO:221)
	<i>VH</i> <i>CDR2</i>	WVAPYNGNTNY AQKLQG (SEQ ID NO:192)	WVAPYNGNTNY AQKLQG (SEQ ID NO:198)	PYNG (SEQ ID NO:204)	WVAPYNGNTN (SEQ ID NO:210)	WMGWVAPYNG NTN (SEQ ID NO:216)	VAPYNGNT (SEQ ID NO:222)
	<i>VH</i> <i>CDR3</i>	DAGTYSPTYGYG MDV (SEQ ID NO:193)	DAGTYSPTYGYG MDV (SEQ ID NO:199)	AGTYSPTYGYG D (SEQ ID NO:205)	DAGTYSPTYGYG MDV (SEQ ID NO:211)	ARDAGTYSPTYG YGMDV (SEQ ID NO:217)	ARDAGTYSPTYG YGMDV (SEQ ID NO:223)
	<i>VL</i> <i>CDR1</i>	RASQSISSWLA (SEQ ID NO:194)	RASQSISSWLA (SEQ ID NO:200)	SQSISSW (SEQ ID NO:206)	RASQSISSWLA (SEQ ID NO:212)	SSWLAWY (SEQ ID NO:218)	QSISSW (SEQ ID NO:224)
	<i>VL</i> <i>CDR2</i>	KASSLES (SEQ ID NO:195)	KASSLES (SEQ ID NO:201)	KAS (SEQ ID NO:207)	KASSLES (SEQ ID NO:213)	LLIYKASSLE (SEQ ID NO:219)	KAS (SEQ ID NO:225)
	<i>VL</i> <i>CDR3</i>	QQFQSLPPFT (SEQ ID NO:196)	QQFQSLPPFT (SEQ ID NO:202)	FQSLPPF (SEQ ID NO:208)	QQFQSLPPFT (SEQ ID NO:214)	QQFQSLPPF (SEQ ID NO:220)	QQFQSLPPFT (SEQ ID NO:226)
VH Sequence*: QVQLVQSGAEVKPGASVKVCKASGYTFRSYGISWVRQAPGQGLEWMGWVAPYNGNTNYAQKLQGRVTMTTDTSTAYMELRSLRSD DTAVYYCARDAGTYSPTYGYGMDVWGQGTITVTVSS (SEQ ID NO:227)							
VL Sequence*: DIQMTQSPSTLSASVGDRTITCRASQSISSWLAWYQQKPGKAPKLLIYKASSLESQVPSRFRSGSGSGTEFTLTISSLQPDDEATYYCQQFQSLPP FTFGGGTKVEIK (SEQ ID NO:228)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat plus Chothia

Table 21: Antibody 25G1-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH</i> <i>CDR1</i>	GYTFRSYGIS (SEQ ID NO:229)	SYGIS (SEQ ID NO:235)	GYTFRSY (SEQ ID NO:241)	GYTFRSYGIS (SEQ ID NO:247)	RSYGIS (SEQ ID NO:253)	GYTFRSYG (SEQ ID NO:259)
	<i>VH</i> <i>CDR2</i>	WVAPYSGNTNY AQKLQG (SEQ ID NO:230)	WVAPYSGNTNY AQKLQG (SEQ ID NO:236)	PYSG (SEQ ID NO:242)	WVAPYSGNT N (SEQ ID NO:248)	WMGWVAPYSG NTN (SEQ ID NO:254)	VAPYSGNT (SEQ ID NO:260)
	<i>VH</i> <i>CDR3</i>	DAGTYSYGYG MDV (SEQ ID NO:231)	DAGTYSYGYG MDV (SEQ ID NO:237)	AGTYSYGYGM D (SEQ ID NO:243)	DAGTYSYGYG MDV (SEQ ID NO:249)	ARDAGTYSYGYG YGMMDV (SEQ ID NO:255)	ARDAGTYSYGYG YGMMDV (SEQ ID NO:261)
	<i>VL</i> <i>CDR1</i>	RASHSIDSWLA (SEQ ID NO:232)	RASHSIDSWLA (SEQ ID NO:238)	SHSIDSW (SEQ ID NO:244)	RASHSIDSWLA (SEQ ID NO:250)	DSWLAWY (SEQ ID NO:256)	HSIDSW (SEQ ID NO:262)
	<i>VL</i> <i>CDR2</i>	KASYLES (SEQ ID NO:233)	KASYLES (SEQ ID NO:239)	KAS (SEQ ID NO:245)	KASYLES (SEQ ID NO:251)	LLIY KASYLE (SEQ ID NO:257)	KAS (SEQ ID NO:263)
	<i>VL</i> <i>CDR3</i>	QLFQSLPPFT (SEQ ID NO:234)	QLFQSLPPFT (SEQ ID NO:240)	FQSLPPF (SEQ ID NO:246)	QLFQSLPPFT (SEQ ID NO:252)	QLFQSLPPF (SEQ ID NO:258)	QLFQSLPPFT (SEQ ID NO:264)
VH Sequence*: QVQLVQSGAEVKPGASVKVCKASGYTFRSYGISWVRQAPGQGLEWMGWVAPYSGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDD TAVYYCARDAGTYSYGYGMDVWGQGTTVTVSS (SEQ ID NO:265)							
VL Sequence*: DIQMTQSPSTLSASVGDRTITCRASHSIDSWLAWYQQKPGKAPKLLIYKASYLESVPSRFSGSGGTEFTLTISLQPDDEATYYCQLFQSLPP FTFGGGTKVEIK (SEQ ID NO:266)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat plus Chothia

Table 22: Antibody 25G9-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH CDR1</i>	GYTFRSYGIS (SEQ ID NO:267)	SYGIS (SEQ ID NO:273)	GYTFRSY (SEQ ID NO:279)	GYTFRSYGIS (SEQ ID NO:285)	RSYGIS (SEQ ID NO:291)	GYTFRSYG (SEQ ID NO:297)
	<i>VH CDR2</i>	WVAPYSGNT NYAQKLQG (SEQ ID NO:268)	WVAPYSGNTNY AQKLQG (SEQ ID NO:274)	PYSG (SEQ ID NO:280)	WVAPYSGNTN (SEQ ID NO:286)	WMGWVAPYSG NTN (SEQ ID NO:292)	VAPYSGNT (SEQ ID NO:298)
	<i>VH CDR3</i>	DAGTYSPIGY GMDV (SEQ ID NO:269)	DAGTYSPIGYG MDV (SEQ ID NO:275)	AGTYSPIGYGM D (SEQ ID NO:281)	DAGTYSPIGYG MDV (SEQ ID NO:287)	ARDAGTYSPIGY YGMD (SEQ ID NO:293)	ARDAGTYSPIGY YGMDV (SEQ ID NO:299)
	<i>VL CDR1</i>	QASQSIDSWLA (SEQ ID NO:270)	QASQSIDSWLA (SEQ ID NO:276)	SQSIDSW (SEQ ID NO:282)	QASQSIDSWLA (SEQ ID NO:288)	DSWLAWY (SEQ ID NO:294)	QSIDSW (SEQ ID NO:300)
	<i>VL CDR2</i>	SASYLES (SEQ ID NO:271)	SASYLES (SEQ ID NO:277)	SAS (SEQ ID NO:283)	SASYLES (SEQ ID NO:289)	LLIYSASYLE (SEQ ID NO:295)	SAS (SEQ ID NO:301)
	<i>VL CDR3</i>	QRFQSLPPFT (SEQ ID NO:272)	QRFQSLPPFT (SEQ ID NO:278)	FQSLPPF (SEQ ID NO:284)	QRFQSLPPFT (SEQ ID NO:290)	QRFQSLPPF (SEQ ID NO:296)	QRFQSLPPFT (SEQ ID NO:302)
VH Sequence*: QVQLVQSGAEVKPGASVKVSKASGYTFRSYGISWVRQAPGQGLEWMGWVAPYSGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDD TAVYYCARDAGTYSPIGYGMDVWGQGTITVTVSS (SEQ ID NO:303)							
VL Sequence*: DIQMTQSPSTLSASVGDRTITTCQASQSIDSWLAWYQQKPGKAPKLLIYSASYLESVPFRFSGSGSGTEFTLTISLQPDDEATYYCQRFQSLPPF TFGGGTKEIK (SEQ ID NO:304)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat plus Chothia

Table 23: Antibody 29D-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	VH CDR1	GFTFHSRGMH (SEQ ID NO:305)	SRGMH (SEQ ID NO:311)	GFTFHSR (SEQ ID NO:317)	GFTFHSRGMH (SEQ ID NO:323)	HSRGMH (SEQ ID NO:329)	GFTFHSRG (SEQ ID NO:335)
	VH CDR2	VITYDGINKYYA DSVEG (SEQ ID NO:306)	VITYDGINKYYA DSVEG (SEQ ID NO:312)	YDGI (SEQ ID NO:318)	VITYDGINKY (SEQ ID NO:324)	WVAVITYDGINK Y (SEQ ID NO:330)	ITYDGINK (SEQ ID NO:336)
	VH CDR3	DGVYVGVDY (SEQ ID NO:307)	DGVYVGVDY (SEQ ID NO:313)	GVYGVYD (SEQ ID NO:319)	DGVYGVYDY (SEQ ID NO:325)	ARDGVYGVYD (SEQ ID NO:331)	ARDGVYGVYD Y (SEQ ID NO:337)
VL CDR Seq.	VL CDR1	KSSQSVLFSSNN KNYLA (SEQ ID NO:308)	KSSQSVLFSSNN KNYLA (SEQ ID NO:314)	SQSVLFSSNNK Y (SEQ ID NO:320)	KSSQSVLFSSNN KNYLA (SEQ ID NO:326)	LFSSNNKNYLA WY (SEQ ID NO:332)	QSVLFSSNNKNY (SEQ ID NO:338)
	VL CDR2	WASTRES (SEQ ID NO:309)	WASTRES (SEQ ID NO:315)	WAS (SEQ ID NO:321)	WASTRES (SEQ ID NO:327)	LLIYWASTRE (SEQ ID NO:333)	WAS (SEQ ID NO:339)
	VL CDR3	QQFHSYPLT (SEQ ID NO:310)	QQFHSYPLT (SEQ ID NO:316)	FHSYPL (SEQ ID NO:322)	QQFHSYPLT (SEQ ID NO:328)	QQFHSYPL (SEQ ID NO:334)	QQFHSYPLT (SEQ ID NO:340)
VH Sequence*: QVQLVESGGGVQPGRSRLRLSCAASGFTFHSRGMHVVRRQAPGKLEWVAVITYDGINKYYADSVVEGRFTISRDNKNTLYLQMNSLRAEDTA VYYCARDGVYGVYDYWGQGTLVTVSS (SEQ ID NO:341)							
VL Sequence*: DIVMTQSPDSLAVSLGERATINCKSSQSVLFSSNNKNYLAWYQQKPGQPPKLLIYWASTRESGVPDRFSGSGGTFTLTISLQAEDVAVYYCQ QFHSYPLTFGGGTKVEIK (SEQ ID NO:342)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat plus Chothia

Table 24: Antibody 29E-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH</i> <i>CDR1</i>	GFTFRSYGMH (SEQ ID NO:343)	SYGMH (SEQ ID NO:349)	GFTFRSY (SEQ ID NO:355)	GFTFRSYGMH (SEQ ID NO:361)	RSYGMH (SEQ ID NO:367)	GFTFRSYG (SEQ ID NO:373)
	<i>VH</i> <i>CDR2</i>	VITYDGINKYYA DSVEG (SEQ ID NO:344)	VITYDGINKYYA DSVEG (SEQ ID NO:350)	YDGI (SEQ ID NO:356)	VITYDGINKY (SEQ ID NO:362)	WVAVITYDGINK Y (SEQ ID NO:368)	ITYDGINK (SEQ ID NO:374)
	<i>VH</i> <i>CDR3</i>	DGVYVGVDY (SEQ ID NO:345)	DGVYVGVDY (SEQ ID NO:351)	GVYGVYD (SEQ ID NO:357)	DGVYVGVDY (SEQ ID NO:363)	ARDGVYGVYD (SEQ ID NO:369)	ARDGVYGVYD Y (SEQ ID NO:375)
VL CDR Seq.	<i>VL</i> <i>CDR1</i>	KSSQSVLFSSNN KNYLA (SEQ ID NO:346)	KSSQSVLFSSNN KNYLA (SEQ ID NO:352)	SQSVLFSSNN Y (SEQ ID NO:358)	KSSQSVLFSSNN KNYLA (SEQ ID NO:364)	LFSSNNKNYLA WY (SEQ ID NO:370)	QSVLFSSNNKNY (SEQ ID NO:376)
	<i>VL</i> <i>CDR2</i>	WASTRES (SEQ ID NO:347)	WASTRES (SEQ ID NO:353)	WAS (SEQ ID NO:359)	WASTRES (SEQ ID NO:365)	LLIYWASTRE (SEQ ID NO:371)	WAS (SEQ ID NO:377)
	<i>VL</i> <i>CDR3</i>	QQFHSYPLT (SEQ ID NO:348)	QQFHSYPLT (SEQ ID NO:354)	FHSYPL (SEQ ID NO:360)	QQFHSYPLT (SEQ ID NO:366)	QQFHSYPL (SEQ ID NO:372)	QQFHSYPLT (SEQ ID NO:378)
VH Sequence*: QVQLVESGGGVVQGRSLRLSCAASGFTFRSYGMHWVRQAPGKGLEWVAVITYDGINKYYADSVVEGRFTISRDNKNTLYLQMNSLRAEDTA VYYCARDGVYGVYDYGQGTIVTVSS (SEQ ID NO:379)							
VL Sequence*: DIVMTQSPDLSAVSLGERATINCKSSQSVLFSSNNKNYLAWYQQKPGQPPKLLIYWASTRESGVPDRFSGSGGTDFLTITSSSLQAEDVAVYYCQ QFHSYPLTFGGGKVEIK (SEQ ID NO:380)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat plus Chothia

Table 25: Antibody 39A-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	VH CDR1	GGTFSSNAIG (SEQ ID NO:381)	SNAIG (SEQ ID NO:387)	GGTFSSN (SEQ ID NO:393)	GGTFSSNAIG (SEQ ID NO:399)	SSNAIG (SEQ ID NO:405)	GGTFSSNA (SEQ ID NO:411)
	VH CDR2	SIPIIGFANYAQK FQG (SEQ ID NO:382)	SIPIIGFANYAQK FQG (SEQ ID NO:388)	PIIG (SEQ ID NO:394)	SIPIIGFAN (SEQ ID NO:400)	WMGSIPIIGFAN (SEQ ID NO:406)	IPIIGFA (SEQ ID NO:412)
	VH CDR3	DSGYYYGASSFG MDV (SEQ ID NO:383)	DSGYYYGASSFG MDV (SEQ ID NO:389)	SGYYYGASSFG MD (SEQ ID NO:395)	DSGYYYGASSFG MDV (SEQ ID NO:401)	ARDSGYYYGASS FGMD (SEQ ID NO:407)	ARDSGYYYGASS FGMDV (SEQ ID NO:413)
	VL CDR1	RASQSVSSNLA (SEQ ID NO:384)	RASQSVSSNLA (SEQ ID NO:390)	SQSVSSN (SEQ ID NO:396)	RASQSVSSNLA (SEQ ID NO:402)	SSNLAWY (SEQ ID NO:408)	QSVSSN (SEQ ID NO:414)
	VL CDR2	GASTRAT (SEQ ID NO:385)	GASTRAT (SEQ ID NO:391)	GAS (SEQ ID NO:397)	GASTRAT (SEQ ID NO:403)	LLIYGASTRA (SEQ ID NO:409)	GAS (SEQ ID NO:415)
	VL CDR3	EQYNNLPLT (SEQ ID NO:386)	EQYNNLPLT (SEQ ID NO:392)	YNNLPL (SEQ ID NO:398)	EQYNNLPLT (SEQ ID NO:404)	EQYNNLPL (SEQ ID NO:410)	EQYNNLPLT (SEQ ID NO:416)
VH Sequence*: QVQLVQSGAEVKPGSSVKVCKASGGTFSSNAIGWVRQAPGQGLEWMGSIPIIGFANYAQKFGQGRVTITADESTSTAYMELSSLRSEDATVY YCARDSGYYYGASSFGMDVWGQGTITVSS (SEQ ID NO:417)							
VL Sequence*: EIVMTQSPATLSVSPGERATLSCRASQSVSSNLAWYQQKPGQAPRLLIYGASTRATGIPARFSGSGSGTEFTLTISLQSEDFAVYYCEQYNNLPL TFGGGTKVEIK (SEQ ID NO:418)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat plus Chothia

Table 26: Antibody 43B-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH</i>	GGSSSGQYWS (SEQ ID NO:419)	SGQYWS (SEQ ID NO:425)	GGSSSGQ (SEQ ID NO:431)	GGSSSGQYWS (SEQ ID NO:437)	SSGQYWS (SEQ ID NO:443)	GGSSSGQY (SEQ ID NO:449)
	<i>CDR1</i>	EIYYSSTRYNPS LKS (SEQ ID NO:420)	EIYYSSTRYNPS LKS (SEQ ID NO:426)	YSG (SEQ ID NO:432)	EIYYSSTR (SEQ ID NO:438)	WIGEIYSGSTR (SEQ ID NO:444)	IYYSGST (SEQ ID NO:450)
	<i>VH</i>	DAPYYGGGY YYMDV (SEQ ID NO:421)	DAPYYGGGY YYMDV (SEQ ID NO:427)	APYYGGGY YMD (SEQ ID NO:433)	DAPYYGGGY YYMDV (SEQ ID NO:439)	ARDAPYYGGG YYYYMD (SEQ ID NO:445)	ARDAPYYGGG YYYYMDV (SEQ ID NO:451)
	<i>CDR3</i>	RASQSVSSSYLA (SEQ ID NO:422)	RASQSVSSSYLA (SEQ ID NO:428)	SQSVSSSY (SEQ ID NO:434)	RASQSVSSSYLA (SEQ ID NO:440)	SSSYLA (SEQ ID NO:446)	QSVSSSY (SEQ ID NO:452)
	<i>VL</i>	GASSRAT (SEQ ID NO:423)	GASSRAT (SEQ ID NO:429)	GAS (SEQ ID NO:435)	GASSRAT (SEQ ID NO:441)	LLIYGASSRA (SEQ ID NO:447)	GAS (SEQ ID NO:453)
	<i>CDR2</i>	QQVGVVPYT (SEQ ID NO:424)	QQVGVVPYT (SEQ ID NO:430)	VGVPY (SEQ ID NO:436)	QQVGVVPYT (SEQ ID NO:442)	QQVGVVPY (SEQ ID NO:448)	QQVGVVPYT (SEQ ID NO:454)
VL CDR Seq.	<i>VL</i>	QVQLQESGPGLVKPSQTLSTCTVSGSSSGQYWSWIRQHPGKGLEWIGEIYSGSTRYNPSLKS CARDAPYYGGGYYYMDVWGKGTITVSS (SEQ ID NO:455)					
	<i>CDR1</i>						
	<i>CDR2</i>						
	<i>CDR3</i>						
	<i>VL</i>						
	<i>CDR3</i>						

VH Sequence*:

QVQLQESGPGLVKPSQTLSTCTVSGSSSGQYWSWIRQHPGKGLEWIGEIYSGSTRYNPSLKS
CARDAPYYGGGYYYMDVWGKGTITVSS (SEQ ID NO:455)

VL Sequence*:

EIVLTQSPGTLSPGERATLSCRASQSVSSSYLA
TFGGGTKVEIK (SEQ ID NO:456)

*Exemplary CDR sequences encompass amino acids as determined by Kabat & Chothia

Table 27: Antibody 43B1-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH</i> <i>CDR1</i>	GGSSSGQYWS (SEQ ID NO:457)	SGQYWS (SEQ ID NO:463)	GGSSSGQ (SEQ ID NO:469)	GGSSSGQYWS (SEQ ID NO:475)	SSGQYWS (SEQ ID NO:481)	GGSSSGQY (SEQ ID NO:487)
	<i>VH</i> <i>CDR2</i>	EIYYSSTRYNPS LKS (SEQ ID NO:458)	EIYYSSTRYNPS LKS (SEQ ID NO:464)	YSG (SEQ ID NO:470)	EIYYSSTR (SEQ ID NO:476)	WIGEIYSGSTR (SEQ ID NO:482)	IYYSGST (SEQ ID NO:488)
	<i>VH</i> <i>CDR3</i>	DAPYYGGGY YYMDV (SEQ ID NO:459)	DAPYYGGGY YYMDV (SEQ ID NO:465)	APYYGGGY YMD (SEQ ID NO:471)	DAPYYGGGY YYMDV (SEQ ID NO:477)	ARDAPYYGGG YYYYMD (SEQ ID NO:483)	ARDAPYYGGG YYYYMDV (SEQ ID NO:489)
VL CDR Seq.	<i>VL</i> <i>CDR1</i>	RASEVDSSYLA (SEQ ID NO:460)	RASEVDSSYLA (SEQ ID NO:466)	SESVDSY (SEQ ID NO:472)	RASEVDSSYLA (SEQ ID NO:478)	DSSYLA (SEQ ID NO:484)	ESVDSSY (SEQ ID NO:490)
	<i>VL</i> <i>CDR2</i>	GASTRQT (SEQ ID NO:461)	GASTRQT (SEQ ID NO:467)	GAS (SEQ ID NO:473)	GASTRQT (SEQ ID NO:479)	LLIYGASTRQ (SEQ ID NO:485)	GAS (SEQ ID NO:491)
	<i>VL</i> <i>CDR3</i>	QQAGVVPYT (SEQ ID NO:462)	QQAGVVPYT (SEQ ID NO:468)	AGVVPY (SEQ ID NO:474)	QQAGVVPYT (SEQ ID NO:480)	QQAGVVPY (SEQ ID NO:486)	QQAGVVPYT (SEQ ID NO:492)
VH Sequence*: QVQLQESGPGLVKPSQTLSTCTVSGSSSGQYWSWIRQHPGKGLEWIGEIYSGSTRYNPSLKSRVTISVDTSKNQFSLKLSVTAADTAVYY CARDAPYYGGGYYYMDVWGKGTITVSS (SEQ ID NO:493)							
VL Sequence*: EIVLTQSPGTLSPGERATLSCRASEVDSSYLAWYQQKPGQAPRLLIYGASTRQTGIPDRFSGSGGTDFLTISRLEPEDFAVYYCQQAGVVP YTFGGGTKVEIK (SEQ ID NO:494)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat & Chothia

Table 28: Antibody 43B7-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH CDR1</i>	GGSSSGQYWS (SEQ ID NO:495)	SGQYWS (SEQ ID NO:501)	GGSSSGQ (SEQ ID NO:507)	GGSSSGQYWS (SEQ ID NO:513)	SSGQYWS (SEQ ID NO:519)	GGSSSGQY (SEQ ID NO:525)
	<i>VH CDR2</i>	EIYYSGSTYNPS LKS (SEQ ID NO:496)	EIYYSGSTYNPS LKS (SEQ ID NO:502)	YSG (SEQ ID NO:508)	EIYYSGST (SEQ ID NO:514)	WIGEIIYSGSTR (SEQ ID NO:520)	IYYSGST (SEQ ID NO:526)
	<i>VH CDR3</i>	DAPYYGGGYY YYMDV (SEQ ID NO:497)	DAPYYGGGYY YYMDV (SEQ ID NO:503)	APYYGGGYY YMD (SEQ ID NO:509)	DAPYYGGGYY YYMDV (SEQ ID NO:515)	ARDAPYYGGG YYYYMDV (SEQ ID NO:521)	ARDAPYYGGG YYYYMDV (SEQ ID NO:527)
	<i>VL CDR1</i>	RASEVDSSYLA (SEQ ID NO:498)	RASEVDSSYLA (SEQ ID NO:504)	SESVDSY (SEQ ID NO:510)	RASEVDSSYLA (SEQ ID NO:516)	DSSYLA (SEQ ID NO:522)	ESVDSSY (SEQ ID NO:528)
VL CDR Seq.	<i>VL CDR2</i>	GADSRAT (SEQ ID NO:499)	GADSRAT (SEQ ID NO:505)	GAD (SEQ ID NO:511)	GADSRAT (SEQ ID NO:517)	LLIYGADSRAT (SEQ ID NO:523)	GAD (SEQ ID NO:529)
	<i>VL CDR3</i>	QQDGVVPYT (SEQ ID NO:500)	QQDGVVPYT (SEQ ID NO:506)	DGVVPY (SEQ ID NO:512)	QQDGVVPYT (SEQ ID NO:518)	QQDGVVPY (SEQ ID NO:524)	QQDGVVPYT (SEQ ID NO:530)
VH Sequence*: QVQLQESGPGLVKPSQTLSTCTVSGSSSGQYWSWIRQHPGKGLEWIGEIIYSGSTRYNPSLKSRTVISVDTSKNQFSLKLSVTAADTAVYY CARDAPYYGGGYYVMDVWGKGTITVSS (SEQ ID NO:531)							
VL Sequence*: EIVLTQSPGTLSPGERATLSCRASEVDSSYLAWYQQKPGQAPRLLIYGADSRATGIPDRFSGSGGTDFLTISRLEPEDFAVYYCQQDGVVP YTFGGGTKVEIK (SEQ ID NO:532)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat & Chothia

Table 29: Antibody 43D-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH</i> <i>CDR1</i>	GGSLSGYYWS (SEQ ID NO:533)	GYYSWS (SEQ ID NO:539)	GGSLSGY (SEQ ID NO:545)	GGSLSGYYWS (SEQ ID NO:551)	SGYYWS (SEQ ID NO:557)	GGSLSGYY (SEQ ID NO:563)
	<i>VH</i> <i>CDR2</i>	EIGASGSTRYNPS LKS (SEQ ID NO:534)	EIGASGSTRYNPS LKS (SEQ ID NO:540)	ASG (SEQ ID NO:546)	EIGASGSTR (SEQ ID NO:552)	WIGEIGASGSTR (SEQ ID NO:558)	IGASGST (SEQ ID NO:564)
	<i>VH</i> <i>CDR3</i>	DTPYYEGGY YYMDV (SEQ ID NO:535)	DTPYYEGGY YYMDV (SEQ ID NO:541)	TPYYEGGY YMD (SEQ ID NO:547)	DTPYYEGGY YYMDV (SEQ ID NO:553)	ARDTPIYYEGG YYYYMD (SEQ ID NO:559)	ARDTPIYYEGG YYYYMDV (SEQ ID NO:565)
VL CDR Seq.	<i>VL</i> <i>CDR1</i>	RASQSVSSSYL A (SEQ ID NO:536)	RASQSVSSSYLA (SEQ ID NO:542)	SQSVSSSY (SEQ ID NO:548)	RASQSVSSSYLA (SEQ ID NO:554)	SSSYLA (SEQ ID NO:560)	QSVSSSY (SEQ ID NO:566)
	<i>VL</i> <i>CDR2</i>	GASSRAT (SEQ ID NO:537)	GASSRAT (SEQ ID NO:543)	GAS (SEQ ID NO:549)	GASSRAT (SEQ ID NO:555)	LLIYGASSRA (SEQ ID NO:561)	GAS (SEQ ID NO:567)
	<i>VL</i> <i>CDR3</i>	QQVGVVPYT (SEQ ID NO:538)	QQVGVVPYT (SEQ ID NO:544)	VGVPY (SEQ ID NO:550)	QQVGVVPYT (SEQ ID NO:556)	QQVGVVPY (SEQ ID NO:562)	QQVGVVPYT (SEQ ID NO:568)
VH Sequence*: QVQLQQWAGALLKPSSETLSLTCAVYGGSLSGYYWSWIRQPPGKGLEWIGEIGASGSTRYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYY CARDTPYYEGGYYYMDVWGKGTITVSS (SEQ ID NO:569)							
VL Sequence*: EIVLTQSPGTLSPGERATLSCRASQSVSSSYLAWSYQQKPGQAPRLLIYGASSRATGIPDRFSGSGGTDFLTISRLEPEDFAVYYCQQVGVVPY TFGGGTKVEIK (SEQ ID NO:570)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat & Chothia

Table 30: Antibody 43D7-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH CDR1</i>	GGSLSGYYWS (SEQ ID NO:571)	GYYS (SEQ ID NO:577)	GGSLSGY (SEQ ID NO:583)	GGSLSGYYWS (SEQ ID NO:589)	SGYYWS (SEQ ID NO:595)	GGSLSGYY (SEQ ID NO:601)
	<i>VH CDR2</i>	EIGASGSTRYNPS LKS (SEQ ID NO:572)	EIGASGSTRYNPS LKS (SEQ ID NO:578)	ASG (SEQ ID NO:584)	EIGASGSTR (SEQ ID NO:590)	WIGEIGASGSTR (SEQ ID NO:596)	IGASGST (SEQ ID NO:602)
	<i>VH CDR3</i>	DTPYYEGGY YYMDV (SEQ ID NO:573)	DTPYYEGGY YYMDV (SEQ ID NO:579)	TPYYEGGY YMD (SEQ ID NO:585)	DTPYYEGGY YYMDV (SEQ ID NO:591)	ARDTPYYEGG YYYYMD (SEQ ID NO:597)	ARDTPYYEGG YYYYMDV (SEQ ID NO:603)
	<i>VL CDR1</i>	RASDSDSYLA (SEQ ID NO:574)	RASDSDSYLA (SEQ ID NO:580)	SDSDSY (SEQ ID NO:586)	RASDSDSYLA (SEQ ID NO:592)	DSSYLAWY (SEQ ID NO:598)	DSVDSSY (SEQ ID NO:604)
VL CDR Seq.	<i>VL CDR2</i>	GAFSRAN (SEQ ID NO:575)	GAFSRAN (SEQ ID NO:581)	GAF (SEQ ID NO:587)	GAFSRAN (SEQ ID NO:593)	LLIYGAFSRA (SEQ ID NO:599)	GAF (SEQ ID NO:605)
	<i>VL CDR3</i>	QQAGVVPYT (SEQ ID NO:576)	QQAGVVPYT (SEQ ID NO:582)	AGVVPY (SEQ ID NO:588)	QQAGVVPYT (SEQ ID NO:594)	QQAGVVPY (SEQ ID NO:600)	QQAGVVPYT (SEQ ID NO:606)
VH Sequence*: QVQLQQWAGLLKPSETLSLTCAVYGGSLSGYYWSWIRQPPGKGLEWIGEIGASGSTRYNPSLKSRTISVDTSKNQFSLKLSVTAADTAVYY CARDTPYYEGGYYYMDVWGKGTITVSS (SEQ ID NO:607)							
VL Sequence*: EIVLTQSPGTLSPGERATLSCRASDSDSYLAWSYQQKPGQAPRLLIYGAFSRANGIPDRFSGSGGTDFLTISRLEPEDFAVYYCQQAGVVP YTFGGGTKVEIK (SEQ ID NO:608)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat & Chothia

Table 31: Antibody 43D8-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH</i> <i>CDR1</i>	GGSLSGYYWS (SEQ ID NO:609)	GYYSWS (SEQ ID NO:615)	GGSLSGY (SEQ ID NO:621)	GGSLSGYYWS (SEQ ID NO:627)	SGYYWS (SEQ ID NO:633)	GGSLSGYY (SEQ ID NO:639)
	<i>VH</i> <i>CDR2</i>	EIGASGSTRYNPS LKS (SEQ ID NO:610)	EIGASGSTRYNPS LKS (SEQ ID NO:616)	ASG (SEQ ID NO:622)	EIGASGSTR (SEQ ID NO:628)	WIGEIGASGSTR (SEQ ID NO:634)	IGASGST (SEQ ID NO:640)
	<i>VH</i> <i>CDR3</i>	DTPYYEGGY YYMDV (SEQ ID NO:611)	DTPYYEGGY YYMDV (SEQ ID NO:617)	TPYYEGGY YMD (SEQ ID NO:623)	DTPYYEGGY YYMDV (SEQ ID NO:629)	ARDTPYYEGG YYYYMD (SEQ ID NO:635)	ARDTPYYEGG YYYYMDV (SEQ ID NO:641)
	<i>VL</i> <i>CDR1</i>	RASQSVSSFLA (SEQ ID NO:612)	RASQSVSSFLA (SEQ ID NO:618)	SQSVSSSF (SEQ ID NO:624)	RASQSVSSFLA (SEQ ID NO:630)	SSSFLAWY (SEQ ID NO:636)	QSVSSSF (SEQ ID NO:642)
VL CDR Seq.	<i>VL</i> <i>CDR2</i>	GAYSRA (SEQ ID NO:613)	GAYSRA (SEQ ID NO:619)	GAY (SEQ ID NO:625)	GAYSRA (SEQ ID NO:631)	LLIYGAYSRA (SEQ ID NO:637)	GAY (SEQ ID NO:643)
	<i>VL</i> <i>CDR3</i>	QQAGVVPYT (SEQ ID NO:614)	QQAGVVPYT (SEQ ID NO:620)	AGVVPY (SEQ ID NO:626)	QQAGVVPYT (SEQ ID NO:632)	QQAGVVPY (SEQ ID NO:638)	QQAGVVPYT (SEQ ID NO:644)
VH Sequence*: QVQLQQWAGALLKPSETLSLTCAVYGGSLSGYYWSWIRQPPGKGLEWIGEIGASGSTRYNPSLKSRTVTSVDTSKNQFSLKLSVTAADTAVYY CARDTPYYEGGYYYMDVWGKGTITVSS (SEQ ID NO:645)							
VL Sequence*: EIVLTQSPGTLSPGERATLSCRASQSVSSFLAWYQQKPGQAPRLLIYGAYSRA TGIPDRFSGSGGTDFTLTISRLEPEDFAVYYCQQAGVVP YTFGGGTKVEIK (SEQ ID NO:646)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat & Chothia

Table 32: Antibody 43E-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH</i> <i>CDR1</i>	GGSSSGQYWS (SEQ ID NO:647)	SGQYWS (SEQ ID NO:653)	GGSSSGQ (SEQ ID NO:659)	GGSSSGQYWS (SEQ ID NO:665)	SSGQYWS (SEQ ID NO:671)	GGSSSGQY (SEQ ID NO:677)
	<i>VH</i> <i>CDR2</i>	EIYSGSTRYNPS LKS (SEQ ID NO:648)	EIYSGSTRYNPS LKS (SEQ ID NO:654)	YSG (SEQ ID NO:660)	EIYSGSTR (SEQ ID NO:666)	WIGEIYSGSTR (SEQ ID NO:672)	IYYSGST (SEQ ID NO:678)
	<i>VH</i> <i>CDR3</i>	DTPYYDGGYY YYMDV (SEQ ID NO:649)	DTPYYDGGYY YYMDV (SEQ ID NO:655)	TPYYDGGYY YMD (SEQ ID NO:661)	DTPYYDGGYY YYMDV (SEQ ID NO:667)	ARDTPYYDGG YYYYMD (SEQ ID NO:673)	ARDTPYYDGG YYYYMDV (SEQ ID NO:679)
	<i>VL</i> <i>CDR1</i>	RASQSVSSSYLA (SEQ ID NO:650)	RASQSVSSSYLA (SEQ ID NO:656)	SQSVSSSY (SEQ ID NO:662)	RASQSVSSSYLA (SEQ ID NO:668)	SSSYLA (SEQ ID NO:674)	QSVSSSY (SEQ ID NO:680)
VL CDR Seq.	<i>VL</i> <i>CDR2</i>	GASSRAT (SEQ ID NO:651)	GASSRAT (SEQ ID NO:657)	GAS (SEQ ID NO:663)	GASSRAT (SEQ ID NO:669)	LLIYGASSRA (SEQ ID NO:675)	GAS (SEQ ID NO:681)
	<i>VL</i> <i>CDR3</i>	QQVGVVPYT (SEQ ID NO:652)	QQVGVVPYT (SEQ ID NO:658)	VGVPY (SEQ ID NO:664)	QQVGVVPYT (SEQ ID NO:670)	QQVGVVPY (SEQ ID NO:676)	QQVGVVPYT (SEQ ID NO:682)
VH Sequence*: QVQLQESGPGLVKPSQTLSTCTVSGSSSGQYWSWIRQHPGKGLEWIGEIYSGSTRYNPSLKSRTVISVDTSKDQFSLKLSVTAADTAVYY CARDTPYYDGGYYYYMDVWGKGTITVTVSS (SEQ ID NO:683)							
VL Sequence*: EIVLTQSPGTLSPGERATLSCRASQSVSSSYLA YQQKPGQAPRLLIYGASSRATGIPDRFSGSGGTDFLTISRLEPEDFAVYYCQQVGVVPY TFGGGTKVEIK (SEQ ID NO:684)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat & Chothia

Table 33: Antibody 43Ea-CDR Sequences

		Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	VH CDR1	GGSSSGQYWS (SEQ ID NO:685)	SGQYWS (SEQ ID NO:691)	GGSSSGQ (SEQ ID NO:697)	GGSSSGQYWS (SEQ ID NO:703)	SSGQYWS (SEQ ID NO:709)	GGSSSGQY (SEQ ID NO:715)
	VH CDR2	EIYYSGSTYNPS LKS (SEQ ID NO:686)	EIYYSGSTYNPS LKS (SEQ ID NO:692)	YSG (SEQ ID NO:698)	EIYYSGST (SEQ ID NO:704)	WIGEIYSGSTR (SEQ ID NO:710)	IYYSGST (SEQ ID NO:716)
	VH CDR3	DTPYYDGGYY YYMDV (SEQ ID NO:687)	DTPYYDGGYY YYMDV (SEQ ID NO:693)	TPYYDGGYY YMD (SEQ ID NO:699)	DTPYYDGGYY YYMDV (SEQ ID NO:705)	ARDTPYYDGG YYYYMD (SEQ ID NO:711)	ARDTPYYDGG YYYYMDV (SEQ ID NO:717)
VL CDR Seq.	VL CDR1	RASQSVSSSYLA (SEQ ID NO:688)	RASQSVSSSYLA (SEQ ID NO:694)	SQSVSSSY (SEQ ID NO:700)	RASQSVSSSYLA (SEQ ID NO:706)	SSSYLA (SEQ ID NO:712)	QSVSSSY (SEQ ID NO:718)
	VL CDR2	GASSRAT (SEQ ID NO:689)	GASSRAT (SEQ ID NO:695)	GAS (SEQ ID NO:701)	GASSRAT (SEQ ID NO:707)	LLIYGASSRA (SEQ ID NO:713)	GAS (SEQ ID NO:719)
	VL CDR3	QQVGVVPYT (SEQ ID NO:690)	QQVGVVPYT (SEQ ID NO:696)	VGVPY (SEQ ID NO:702)	QQVGVVPYT (SEQ ID NO:708)	QQVGVVPY (SEQ ID NO:714)	QQVGVVPYT (SEQ ID NO:720)
VH Sequence*: QVQLQESGPGLVKPSQTLSTCTVSGGSSSGQYWSWIRQHPGKGLEWIGEIYSGSTRYNPSLKSRVTISVDTSKNQFSLKLSVTAADTAVYY CARDTPYYDGGYYYYMDVWGKGTITVTVSS (SEQ ID NO:721)							
VL Sequence*: EIVLTQSPGTLSPGERATLSCRASQSVSSSYLA TFGGGTKVEIK (SEQ ID NO:722)							

*Exemplary CDR sequences encompass amino acids as determined by Kabat & Chothia

Table 34: Antibody 54E-CDR Sequences

	Exemplary*	Kabat	Chothia	AbM	Contact	IMGT
VH CDR Seq.	<i>VH</i> <i>CDR1</i>	NYMMH (SEQ ID NO:723)	GYTFANY (SEQ ID NO:735)	GYTFANYMH (SEQ ID NO:741)	ANYMMH (SEQ ID NO:747)	GYTFANY (SEQ ID NO:753)
	<i>VH</i> <i>CDR2</i>	IINPSGGITVYAQ KFQG (SEQ ID NO:724)	PSGG (SEQ ID NO:736)	IINPSGGITV (SEQ ID NO:742)	WMGIINPSGGIT V (SEQ ID NO:748)	IINPSGGIT (SEQ ID NO:754)
	<i>VH</i> <i>CDR3</i>	GGSKVAALAFDI (SEQ ID NO:725)	GSKVAALAFD (SEQ ID NO:737)	GGSKVAALAFDI (SEQ ID NO:743)	ARGGSKVAALA FD (SEQ ID NO:749)	ARGGSKVAALA FDI (SEQ ID NO:755)
VL CDR Seq.	<i>VL</i> <i>CDR1</i>	QASQDISNSLN (SEQ ID NO:726)	SQDISNS (SEQ ID NO:738)	QASQDISNSLN (SEQ ID NO:744)	SNSLNWY (SEQ ID NO:750)	QDISNS (SEQ ID NO:756)
	<i>VL</i> <i>CDR2</i>	DASNLET (SEQ ID NO:727)	DAS (SEQ ID NO:739)	DASNLET (SEQ ID NO:745)	LLIYDASNLE (SEQ ID NO:751)	DAS (SEQ ID NO:757)
	<i>VL</i> <i>CDR3</i>	QQYNFHPLT (SEQ ID NO:728)	YNFHPL (SEQ ID NO:740)	QQYNFHPLT (SEQ ID NO:746)	QQYNFHPL (SEQ ID NO:752)	QQYNFHPLT (SEQ ID NO:758)
VH Sequence*: QVQLVQSGAEVKPGASVKVCKASGYTFANYMHVVRQAPGGQGLEWMGIINPSGGITVYAQKFQGRVTMTTRDTSTSTVYMESSLRSEDT AVYYCARGGSKVAALAFDIWGQGTMTVSS (SEQ ID NO:759)						
VL Sequence*: DIQMTQSPSSLASVGDRLVITTCQASQDISNSLNWYQQKPGKAPKLLIYDASNLETGVPSRFSGRSGTDFTFTISSLPEDIATYYCQQYNFHPLT FGGGTKVEIK (SEQ ID NO:760)						

*Exemplary CDR sequences encompass amino acids as determined by Kabat plus Chothia

Table 35: Consensus CDRs

Antibody Group	1	25	29	39	43	54
VH CDR Seq.*	VH CDR1	GFTFSx[D/S]YAM x[A/G] (SEQ ID NO: 773)	GYTFx[D/R]x[S/V] YGIS (SEQ ID NO: 779)	GGTFSSNAIG (SEQ ID NO: 791)	GGx[I/L]SSGx[Q/ Y]YWS (SEQ ID NO: 797)	GYTFANYMH (SEQ ID NO: 803)
	VH CDR2	x[A/T]ISGSGGLT YYADSVKG (SEQ ID NO: 774)	Wx[I/V]APYx[S/N]]GNTNYAQKLQG (SEQ ID NO: 780)	SIPIIGFANYAQK FQG (SEQ ID NO: 792)	Elx[Y/G]x[Y/A]JSG STRYNPSLKS (SEQ ID NO: 798)	IINPSGGITVYAAQ KFQG (SEQ ID NO: 804)
	VH CDR3	APYGYMDV (SEQ ID NO: 775)	DAGTYSPx[F/Y]G YGM DV (SEQ ID NO: 781)	DSGYYYGASSFG MDV (SEQ ID NO: 793)	Dx[T/A]PYYYx[E/ G/D]GGYYYYYMD V (SEQ ID NO: 799)	GGSKVAALAFDI (SEQ ID NO: 805)
	VL CDR1	RASQSISSWLA (SEQ ID NO: 776)	x[R/Q]ASx[Q/E/H] Slx[S/D/N]x[S/N] WLA (SEQ ID NO: 782)	KSSQSVLFSSNN KNYLA (SEQ ID NO: 788)	RASx[Q/E/D]SVx[S/D]SSx[Y/F]LA (SEQ ID NO: 800)	QASQDISNSLN (SEQ ID NO: 806)
	VL CDR2	KASSLES (SEQ ID NO: 777)	x[K/S]Ax[S/Y]x[S/ Y/N]LEx[S/Y] (SEQ ID NO: 783)	WASTRES (SEQ ID NO: 789)	GAX[S/D/F/Y]x[S/ T]Rx[A/Q]x[T/N] (SEQ ID NO: 801)	DASNLET (SEQ ID NO: 807)
	VL CDR3	QQYKSYIT (SEQ ID NO: 778)	Qx[Q/L/R]FQx[S/ K]LPPFT (SEQ ID NO: 784)	QQFHSYPLT (SEQ ID NO: 790)	QQx[V/A/D]GVVP YT (SEQ ID NO: 802)	QQYNFHPLT (SEQ ID NO: 808)

*Exemplary CDR sequences encompass amino acids as determined by Kabat plus Chothia

Table 36: Human, Cynomolgus Monkey, and Mouse TF Sequences

Species	Human (<i>Homo sapiens</i>)	Cynomolgus Monkey (<i>Macaca fascicularis</i>)	Mouse (<i>Mus musculus</i>)
Full-length sequence [signal sequence underlined]	METPAWPRVPRPETAV <u>ARTLLLGWVFAQVAG</u> <u>ASGTTNTVAAYNLTW</u> KSTNFKTILEWEPKPV NQYTVQISTKSGDW KSKCFYTTDTECDLTD EIVKDVKQTYLARVFS YPAGNVESTGSAGEPL YENSPEFTPYLETNLG QPTIQSFEQVGTKVNV TVEDERTLVRRNNTFL SLRDVFGKDLIYTLYY WKSSSSGKKTAKTNTN EFLIDVDKGENYCFSV QAVIPSRVTNRKSTDSP VECMGQEKGEFREIFYI IGAVFVVIILVILAILSL HKCRKAGVGQSWKEN SPLNVS (SEQ ID NO:809)	METPAWPRVPRPETAV <u>ARTLLLGWVFAQVAG</u> <u>ASGTTNTVAAYNLTW</u> KSTNFKTILEWEPKPIN QYVTVQISTKSGDWKS KCFYTADTECDLTDEI VKDVKQTYLARVFSYP AGHVESTGSTEEPPEY NSPEFTPYLETNLGQPT IQSFEQVGTKVNVTVQ DEWTLVRRNDTFLSLR DVFGKDLIYTLYYWKS SSSGKKTAKTNTNEFLI DVDKGENYCFSVQAVI PSRRTANRKSTDSPVE CMGHEKGESREIFYIIG AVVVFVVIILVILAILSH KCKKARVGRSWKENS PLNVA (SEQ ID NO:813)	MAILVRPRLAALAPT <u>FLGCLLLQVTAGAGIP</u> EKAFNLTWISTDFKTIL EWQPKPTNYTYTVQIS DRSRNWKNKCFSTTDT ECDLTDEIVKDVTWAY EAKVLSVPRRNSVHGD GDQLVIHGEEPFTNAP KFLPYRDTNLGQPVIQ QFEQDGRKLNVVVKD SLTLVRKNGTFLTLRQ VFGKDLGYIITYRKGS TGKKTNTNTNEFSIDV EEGVS YCFFVQAMIFS RKTNQSPGSPCTE QWKSFLGETLIIVGAV VLLATIFIILLSISLCKR RKNRAGQKGKNTPSR LA (SEQ ID NO:817)
Extracellular domain (ECD)	SGTTNTVAAYNLTWK STNFKTILEWEPKPVN QYVTVQISTKSGDWKS KCFYTTDTECDLTDEI VKDVKQTYLARVFSYP AGNVESTGSAGEPLYE NSPEFTPYLETNLGQPT IQSFEQVGTKVNVTVQ DERTLVRRNNTFLSLR DVFGKDLIYTLYYWKS SSSGKKTAKTNTNEFLI DVDKGENYCFSVQAVI PSRTVNRKSTDSPVEC MGQEKGEFRE (SEQ ID NO:810)	SGTTNTVAAYNLTWK STNFKTILEWEPKPINQ VYTVQISTKSGDWKSK CFYTADTECDLTDEIV KDVKQTYLARVFSYPA GHVESTGSTEEPPEYENS PEFTPYLETNLGQPTIQ SFEQVGTKVNVTVQDE WTLVRRNDTFLSLRDV FGKDLIYTLYYWKSSS SGKKTAKTNTNEFLID VDKGENYCFSVQAVIP SRRTANRKSTDSPVEC MGHEKGESRE (SEQ ID NO:814)	AGIPEKAFNLTWISTDF KTILEWQPKPTNYTYT VQISDRSRNWKNKCF TTDTECDLTDEIVKDV TWAYEAKVLSVPRRN SVHGDGDQLVIHGEEP PFTNAPKFLPYRDTNL GQPVIQQFEQDGRKLN VVVKDSLTLVRKNGTF LTLRQVFGKDLGYIITY RKGSSTGKKTNTNTN EFSIDVEEGVS YCFFVQ AMIFSRKTNQNSPGSST VCTEQWKSFLGE (SEQ ID NO:818)
Sequence of TF ECD-His (TF-His) protein	SGTTNTVAAYNLTWK STNFKTILEWEPKPVN QYVTVQISTKSGDWKS KCFYTTDTECDLTDEI VKDVKQTYLARVFSYP AGNVESTGSAGEPLYE NSPEFTPYLETNLGQPT IQSFEQVGTKVNVTVQ DERTLVRRNNTFLSLR DVFGKDLIYTLYYWKS SSSGKKTAKTNTNEFLI DVDKGENYCFSVQAVI PSRTVNRKSTDSPVEC MGQEKGEFRETGHHH HHH (SEQ ID NO:811)	SGTTNTVAAYNLTWK STNFKTILEWEPKPINQ VYTVQISTKSGDWKSK CFYTADTECDLTDEIV KDVKQTYLARVFSYPA GHVESTGSTEEPPEYENS PEFTPYLETNLGQPTIQ SFEQVGTKVNVTVQDE WTLVRRNDTFLSLRDV FGKDLIYTLYYWKSSS SGKKTAKTNTNEFLID VDKGENYCFSVQAVIP SRRTANRKSTDSPVEC MGHEKGESRETGHHH HHH (SEQ ID NO:815)	AGIPEKAFNLTWISTDF KTILEWQPKPTNYTYT VQISDRSRNWKNKCF TTDTECDLTDEIVKDV TWAYEAKVLSVPRRN SVHGDGDQLVIHGEEP PFTNAPKFLPYRDTNL GQPVIQQFEQDGRKLN VVVKDSLTLVRKNGTF LTLRQVFGKDLGYIITY RKGSSTGKKTNTNTN EFSIDVEEGVS YCFFVQ AMIFSRKTNQNSPGSST VCTEQWKSFLGETGH HHHHH (SEQ ID NO:819)

Species	Human (<i>Homo sapiens</i>)	Cynomolgus Monkey (<i>Macaca fascicularis</i>)	Mouse (<i>Mus musculus</i>)
Sequence of TF ECD-Fc (TF-Fc) fusion protein	SGTTNTVAAYNLTWK STNFKTILEWEPKPVN QVYTVQISTKSGDWKS KCFYTTDTECDLTDEI VKDVKQTYLARVFSYP AGNVESTGSAGEPLYE NSPEFTPYLETNLGQPT IQSFEQVGTKVNVTV DERTLVRNNNTFLSLR DVFGKDLIYTLYYWKS SSSGKKTAKTNTNEFLI VDKGENYCFSVQAVI PSRTVNRKSTDSPVEC MGQEKGEFRETGENL YFQGDKTHTCPPCPAP ELLGGPSVFLFPPKPKD TLMISRTPEVTCVVVD VSHEDPEVKFNWYVD GVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNK ALPAPIEKTISKAKGQP REPQVYTLPPSREEMT KNQVSLTCLVKGFYPS DIAVEWESNGQPENNY KTTTPVLDSGDSFFLYS KLTVDKSRWQQGNVF SCSVMHEALHNHYTQ KSLSLSPGK (SEQ ID NO:812)	SGTTNTVAAYNLTWK STNFKTILEWEPKPINQ VYTVQISTKSGDWKSK CFYTADTECDLTDEIV KDVKQTYLARVFSYPA GHVESTGSTEEPYPYENS PEFTPYLETNLGQPTIQ SFEQVGTKVNVTVQDE WTLVRRNDTFLSLRDV FGKDLIYTLYYWKSSS SGKKTAKTNTNEFLID VDKGENYCFSVQAVIP SRRTANRKSTDSPVEC MGHEKGESRETGENL YFQGDKTHTCPPCPAP ELLGGPSVFLFPPKPKD TLMISRTPEVTCVVVD VSHEDPEVKFNWYVD GVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNK ALPAPIEKTISKAKGQP REPQVYTLPPSREEMT KNQVSLTCLVKGFYPS DIAVEWESNGQPENNY KTTTPVLDSGDSFFLYS KLTVDKSRWQQGNVF SCSVMHEALHNHYTQ KSLSLSPGK (SEQ ID NO:816)	AGIPEKAFNLTWISTDF KTILEWQPKPTNYTYT VQISDRSRNWKNKCF TTDTECDLTDEIVKDV TWAYEAKVLSVPRRN SVHGDGDQLVIHGEEP PFTNAPKFLPYRDTNL GQPVIQQFEQDGRKLN VVVKDSLTLVRKNGTF LTLRQVFGKDLGYIITY RKGSSSTGKKTNITNTN EFSIDVEEGVSYCFFVQ AMIFSRKTQNSPGSSST VCTEQWKSFLGETGEN LYFQGDKTHTCPPCPA PELLGGPSVFLFPPKPK DTLMISRTPEVTCVVV DVSHEDPEVKFNWYV DGVEVHNAKTKPREE QYNSTYRVVSVLTVLH QDWLNGKEYKCKVSN KALPAPIEKTISKAKGQ PREPQVYTLPPSREEM TKNQVSLTCLVKGFYP SDIAVEWESNGQPENN YKTTTPVLDSGDSFFL YSKLTVDKSRWQQGN VFSCSVMHEALHNHY TQKSLSLSPGK (SEQ ID NO:820)

Table 39: Sequences of Anti-TF Antibodies

Antibody	VH domain	VL domain
10H10 (M1593)	EVQLVQSGAEVKKPGESLRISCKGS GYTFAPYWIEWVRQMPGKGLEWM GDILPGTGFTTYSFQGHVTISADK SISTAYLQWSSLKASDTAMYYCARS GYYGNSGFAYWGQGTLLTVSS (SEQ ID NO:821)	DIVMTQTPLSLPVTPGEPASISCKSSQ SLLSSGNQKNYLTWYLQKPGQSPQL LIYWASTRESGVPRFSGSGSGTDFT LKISRVEAEDVGVYYCQNDYTYPLT FGQGTKLEIK (SEQ ID NO:822)
TF-011	EVQLLESGLLVQPGGSLRLSCAAS GFTFSNYAMSWVRQAPGKGLEWVS SISGSGDYTTYTDSVKGRFTISRDN KNTLYLQMNSLRAEDTAVYYCARS PWGYYLDSWGQGTLLTVSS (SEQ ID NO:828)	DIQMTQSPPSLSASAGDRVITITCRAS QGISSLAWYQQKPEKAPKSLIYAA SSLQSGVPSRFSGSGSGTDFTLTIS QPEDFATYYCQQYNSYPYTFGQGTK LEIK (SEQ ID NO:829)
5G9 (humanized TF8-5G9, CNTO 860)	QVQLVESGGGVVQPGRSLRLSCKAS GFNIKDYMHWRQAPGKGLEWIG LIDPENGNTIYDPKFQGRFTISADNS KNTLFLQMDSLRPEDTAVYYCARD NSYYFDYWGGQGTPTVSS (SEQ ID NO:830)	DIQMTQSPSSLSASVGDRTITCKAS QDIRKYLNWYQQKPGKAPKLLIYY ATSLADGVPSRFSGSGSGTDYFTTIS SLQPEDIATYYCLQHGESPYTFGQGT KLEIT (SEQ ID NO:831)

Table 41: Pig TF sequences

Species	Pig (<i>Sus scrofa</i>)
Full-length sequence [signal sequence underlined]	<u>MATPTGPPVSCPKAAVARALLLGWVLVQVAGATGTTD</u> VIVAYN LTWKSTNFKTILEWEPKPINYVYTVQISPRLGDWKNKCFHTTDTE CDVTDEIMRNVKETYYVARVLSYPADTVLTAQEPFTNSPPFTPYL DTNLGQPVIQSFEQVGTKLNVTVEAARTLVRVNGTFLRLRDVFG KDLNYTLYYWRASSTGKKKATTNTNEFLIDVDKGENYCFSVQA VIPSRRVNQKSPESRIECTSQEKAVSRELFLIVGAVVFAVIVFVLV LSVSLYKCRKERAGPSGKENAPLNVA (SEQ ID NO:824)
Extracellular domain (ECD)	TGTTDVIVAYNLTWKSTNFKTILEWEPKPINYVYTVQISPRLGDW KNKCFHTTDTECDVTDEIMRNVKETYYVARVLSYPADTVLTAQEP PFTNSPPFTPYLDTNLGQPVIQSFEQVGTKLNVTVEAARTLVRVN GTFLRLRDVFGKDLNYTLYYWRASSTGKKKATTNTNEFLIDVDK GENYCFSVQAVIPSRRVNQKSPESRIECTSQEKAVSRE (SEQ ID NO:825)
Sequence of TF ECD-His (TF-His) protein	TGTTDVIVAYNLTWKSTNFKTILEWEPKPINYVYTVQISPRLGDW KNKCFHTTDTECDVTDEIMRNVKETYYVARVLSYPADTVLTAQEP PFTNSPPFTPYLDTNLGQPVIQSFEQVGTKLNVTVEAARTLVRVN GTFLRLRDVFGKDLNYTLYYWRASSTGKKKATTNTNEFLIDVDK GENYCFSVQAVIPSRRVNQKSPESRIECTSQEKAVSRETGHHHHH H (SEQ ID NO:826)
Sequence of TF ECD-Fc (TF-Fc) fusion protein	TGTTDVIVAYNLTWKSTNFKTILEWEPKPINYVYTVQISPRLGDW KNKCFHTTDTECDVTDEIMRNVKETYYVARVLSYPADTVLTAQEP PFTNSPPFTPYLDTNLGQPVIQSFEQVGTKLNVTVEAARTLVRVN GTFLRLRDVFGKDLNYTLYYWRASSTGKKKATTNTNEFLIDVDK GENYCFSVQAVIPSRRVNQKSPESRIECTSQEKAVSRETGENLYF QGDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVTV DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTL PPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTP VLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQK SLSLSPGK (SEQ ID NO:827)

Table 49: Rabbit TF sequences

Species	Rabbit (<i>Oryctolagus cuniculus</i>)
Full-length sequence [signal sequence underlined]	<u>MAPPTRLQVPRPGTAVPYTVLLGWLLAQVARAADTTGR</u> AYNLTWKSTNFKTILEWEPKSIDHVYTVQISTRLENWKS CFLTAETECDLTDEVVKDVGQTYMARVLSYPARNGNTT GFPEPPFRNSPEFTPYLDTNLGQPTIQSFEQVGTKLNVTV QDARTLVRRTGTFLSLRAVFGKDLNYTLYYWRASSTGK KTATTNTNEFLIDVDKGENYCFSVQAVIPSRRKRKQSPES LTECTSREQGRAREMFFIIGAVVVVALLIIVLSVTVYKCRK ARAGPSGKESSPLNIA (SEQ ID NO:832)

Species	Rabbit (<i>Oryctolagus cuniculus</i>)
Extracellular domain (ECD)	ADTTGRAYNLTWKSTNFKTILEWEPKSIDHVTYTVQISTRL ENWWSKCFLEAETECDLTDEVVKDVGQTYMARVLSYPA RNGNTTGFPPEPPFRNSPEFTPYLDTNLGQPTIQSFEQVGT KLNVTYVQDARTLVRRNGTFLSLRAVFGKDLNLTLYYWR ASSTGKKTATTNTNEFLIDVDKGENYCFSVQAVIPSRKRK QRSPELTECTSREQGRAREM (SEQ ID NO:833)
Sequence of TF ECD-His (TF-His) protein	ADTTGRAYNLTWKSTNFKTILEWEPKSIDHVTYTVQISTRL ENWWSKCFLEAETECDLTDEVVKDVGQTYMARVLSYPA RNGNTTGFPPEPPFRNSPEFTPYLDTNLGQPTIQSFEQVGT KLNVTYVQDARTLVRRNGTFLSLRAVFGKDLNLTLYYWR ASSTGKKTATTNTNEFLIDVDKGENYCFSVQAVIPSRKRK QRSPELTECTSREQGRAREMTGHHHHHH (SEQ ID NO:834)
Sequence of TF ECD-Fc (TF-Fc) fusion protein	ADTTGRAYNLTWKSTNFKTILEWEPKSIDHVTYTVQISTRL ENWWSKCFLEAETECDLTDEVVKDVGQTYMARVLSYPA RNGNTTGFPPEPPFRNSPEFTPYLDTNLGQPTIQSFEQVGT KLNVTYVQDARTLVRRNGTFLSLRAVFGKDLNLTLYYWR ASSTGKKTATTNTNEFLIDVDKGENYCFSVQAVIPSRKRK QRSPELTECTSREQGRAREMENLYFQGDKTHTCPPCPAP ELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTKAKGQPREPQVY TLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPN NYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSQSV HEALHNHYTQKSLSLSPGK (SEQ ID NO:835)

Table 56: Rat TF ECD and chimeric construct ECD sequences

Rat/Chimeric construct	Extracellular domain (ECD) sequence
rTF (rat TF)	AGTPPGKAFNLTWISTDFKTILEWQPKPTNYTYTVQISDRSRNWKY KCTGTTDTECDLTDEIVKDVNWTYEARVLSVPWRNSTHGKETLFG THGEEPPFTNARKFLPYRDTKIGQPVIQKYEQGGTKLVTVKDSFT LVRKNGTFLTLRQVFGNDLGILTYRKDSSTGRKTNTHHTNEFLID VEKGVSYCFFAQAVIFSRKTNHKSPEITKCTEQWKSVLGE (SEQ ID NO:838)
h1-107_r	AGTPPGKAFNLTWISTDFKTILEWQPKPTNYTYTVQISDRSRNWKY KCTGTTDTECDLTDEIVKDVNWTYEARVLSVPWRNSTHGKETLFG THGEEPPFTNARKFLPYRDTKLGQPTIQSFEQVGTGVNVTVEDERT LVRRNNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLID VDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:839)
h1-77_r	AGTPPGKAFNLTWISTDFKTILEWQPKPTNYTYTVQISDRSRNWKY KCTGTTDTECDLTDEIVKDVNWTYEARVLSYPAGNVESTGSAGEP LYENSPEFTPYLETNLGQPTIQSFEQVGTGVNVTVEDERTLVRRNN TFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGEN YCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:840)
h1-38_r	AGTPPGKAFNLTWISTDFKTILEWQPKPTNYTYTVQISTKSGDWKS KCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAGEPL YENSPEFTPYLETNLGQPTIQSFEQVGTGVNVTVEDERTLVRRNNT FLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKGENY CFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:841)

Rat/Chimeric construct	Extracellular domain (ECD) sequence
h39-77_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISDRSRNW KYKCTGTTDTECDLTDEIVKDVNWTYEARVLSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRR NNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:842)
h78-107_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSVPWRNSTHGTHGE EPPFTNARKFLPYRDTKLGQPTIQSFEQVGTKVNVTVEDERTLVRR NNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:843)
h78-107_r.v2	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSVPWRNSTHGKETL FGTHGEEPPFTNARKFLPYRDTKLGQPTIQSFEQVGTKVNVTVEDE RTLVRNNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFL IDVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:844)
h78-93_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSVPWRNSTHGKETL FGTHGEEPPYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDER TLVRNNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLI DVDKGENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:845)
h94-107_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLFTNARKFLPYRDTKLGQPTIQSFEQVGTKVNVTVEDERTLVRR NNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:846)
h108-219_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNIGQPVIQKYEQGGTKLKVTVKDSFTLVRK NGTFLTLRQVFGNDLG YILTYRKDSSTGRKTNTTHTNEFLIDVEKG VSYCFFAQAVIFSRTNHNKSPESITKCTEQWKSVLGE (SEQ ID NO:847)
h108-158_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNIGQPVIQKYEQGGTKLKVTVKDSFTLVRK NGTFLTLRQVFGNDLG YILTYRKSSSSGKKTAKTNTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:848)
h108-132_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNIGQPVIQKYEQGGTKLKVTVKDSFTLVRK NNTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:849)
h133-158_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRR NGTFLTLRQVFGNDLG YILTYRKSSSSGKKTAKTNTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:850)
h133-145_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG

Rat/Chimeric construct	Extracellular domain (ECD) sequence
	EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRK NGTFLTLRQVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:851)
h133-139_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRK NGTFLSLRDVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:852)
h140-145_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRK NNTFLTLRQVFGKDLIYTLYYWKSSSSGKKTAKTNTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:853)
h146-158_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRK NNTFLSLRDVFGNDLGYILTYRKSSSSGKKTAKTNTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:854)
h146-151_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRK NNTFLSLRDVFGNDLGYILTYRKSSSSGKKTAKTNTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:855)
h152-158_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRK NNTFLSLRDVFGKDLGYILTYRKSSSSGKKTAKTNTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:856)
h159-219_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRK NNTFLSLRDVFGKDLIYTLYYWKDSSTGRKTNTHTNEFLIDVEKG VSYCFFAQAVIFSRTVNRKSTSPVECMGQEKGEFRE (SEQ ID NO:857)
h159-189_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRK NNTFLSLRDVFGKDLIYTLYYWKDSSTGRKTNTHTNEFLIDVEKG VSYCFFAQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:858)
h159-174_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRK NNTFLSLRDVFGKDLIYTLYYWKDSSTGRKTNTHTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:859)
h159-166_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRK NNTFLSLRDVFGKDLIYTLYYWKDSSTGRKTAKTNTNEFLIDVDK

Rat/Chimeric construct	Extracellular domain (ECD) sequence
	GENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:860)
h167-174_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRR NNTFLSLRDVFGKDLIYTLYYWKSSSSGKKNTTHTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:861)
h175-189_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRR NNTFLSLRDVFGKDLIYTLYYWKSSSSGKKAKTNTNEFLIDVEKG VSYCFFAQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:862)
h190-219_r	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRR NNTFLSLRDVFGKDLIYTLYYWKSSSSGKKAKTNTNEFLIDVDKG ENYCFSVQAVIFSRTNHNKSPESITKCTEQWKSVLGE (SEQ ID NO:863)
hTF_K68N	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVNQTLYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRR NNTFLSLRDVFGKDLIYTLYYWKSSSSGKKAKTNTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:865)
hTF_K149N	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRR NNTFLSLRDVFGNDLIYTLYYWKSSSSGKKAKTNTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:866)
hTF_N171H_T197K	SGTTNTVAAYNLTWKSTNFKTILEWEPKPVNQVYTVQISTKSGDW KSKCFYTTDTECDLTDEIVKDVKQTYLARVFSYPAGNVESTGSAG EPLYENSPEFTPYLETNLGQPTIQSFEQVGTKVNVTVEDERTLVRR NNTFLSLRDVFGKDLIYTLYYWKSSSSGKKAKTNTNEFLIDVDKG ENYCFSVQAVIPSRTVNRKSTDSPVECMGQEKGEFRE (SEQ ID NO:867)
r141-194_h	AGTPPGKAFNLTWISTDFKTILEWQPKPTNYTYTVQISDRSRNWKY KCTGTTDTECDLTDEIVKDVNWTYEARVLSVPWRNSTHGKETLFG THGEEPPFTNARKFLPYRDTKIGQPVIQKYEQGGTKLKVTVKDSFT LVRRNNTFLSLRDVFGKDLIYTLYYWKSSSSGKKAKTNTNEFLID VDKGENYCFSVQAVIFSRTNHNKSPESITKCTEQWKSVLGE (SEQ ID NO:864)

Table 57: Variable region sequence consensus

Group	VH Domain Consensus (SEQ ID NO)	VL Domain Consensus (SEQ ID NO)
Lineage 25A	QVQLVQSGAEVKKPGASVKVSCAS GYTFDx[V/A]YGISWVRQAPGQGLEW MGWIAPYx[N/S]GNTNYAQKLQGRVT MTTDTSTSTAYMELRSLRSDDTAVVY CARDAGTYSFPGYGMVWGQGTTVT VSS (SEQ ID NO:868)	DIQMTQSPSTLSASVGDRVITTCx[R/Q] ASx[Q/E]SIx[S/N]x[S/N]WLAWYQKPK GKAPKLLIYKAx[S/Y]x[S/N]LEx[S/Y]G VPSRFSGSGSGTEFTLTISLQPDFFAT YYCQx[Q/L]FQx[S/K]LPPFTFGGGTKV EIK (SEQ ID NO:869)

Group	VH Domain Consensus (SEQ ID NO)	VL Domain Consensus (SEQ ID NO)
Lineage 25G	QVQLVQSGAEVKKPGASVKVSCAS GYTFRSYGISWVRQAPGQGLEWMGW VAPY _x [N/S]GNTNYAQKLQGRVTMTT DTSTSTAYMELRSLRSDDTAVYYCAR DAGTYSPTYGYGMDVWVGQGTTVTVSS (SEQ ID NO:870)	DIQMTQSPSTLSASVGDRVITTC _x [R/Q] AS _x [Q/H]SI _x [S/D]SWLAWYQQKPGKA PKLLIY _x [K/S]AS _x [S/Y]LESGVPSRFSG SGSGTEFTLTISLQPDFFATYYCQ _x [Q/ L/R]FQSLPPFTFGGGTKVEIK (SEQ ID NO:871)

Table 58: Consensus CDRs

Antibody Group	Lineage 25A	Lineage 25G
VH CDR Seq.*	<i>VH CDR1</i> GYTFD _x [V/A]YGIS (SEQ ID NO:872)	GYTFRSYGIS (SEQ ID NO:878)
	<i>VH CDR2</i> WIAPY _x [N/S]GNTNYAQKLQG (SEQ ID NO:873)	WVAPY _x [N/S]GNTNYAQKLQG (SEQ ID NO:879)
	<i>VH CDR3</i> DAGTYSPTFGYGM DV (SEQ ID NO:874)	DAGTYSPTYGYGM DV (SEQ ID NO:880)
VL CDR Seq.*	<i>VL CDR1</i> x[R/Q]AS _x [Q/E]SI _x [S/N]x[S/N]W LA (SEQ ID NO:875)	x[R/Q]AS _x [Q/H]SI _x [S/D]SWLA (SEQ ID NO:881)
	<i>VL CDR2</i> KA _x [S/Y]x[S/N]LE _x [S/Y] (SEQ ID NO:876)	x[K/S]AS _x [S/Y]LES (SEQ ID NO:882)
	<i>VL CDR3</i> Q _x [Q/L]FQ _x [S/K]LPPFT (SEQ ID NO:877)	Q _x [Q/L/R]FQSLPPFT (SEQ ID NO:883)

*Exemplary CDR sequences encompass amino acids as determined by Kabat plus Chothia

Table 59: Antibody sequences for TF antibodies

variable regions in bold; cysteines involved in drug conjugation underlined. The clones in Table 13 have the same heavy chain constant regions. The clones in Table 13 have the same light chain constant regions.

Clone	HEAVY CHAIN	LIGHT CHAIN
25A	QVQLVQSGAEVKKPGASVKVSCA SGYTFD VYGISWVRQAPGQGLEWM GW IAPYSGNTNYAQKLQGRVTMTT DTSTSTAYMELRSLRSDDTAVYYCA RD AGTYSPTFGYGM DVWVGQGTTVT VSSASTKGPSVFPLAPSSKSTSGGTA LGCLVKDYFPEPVTVSWNSGALTSGV HTFPAVLQSSGLYSLSSVTVPSSSLG TQTYICNVNHKPSNTKVDKRVEPKSC <u>DKTHTC</u> <u>PPC</u> PAPELLGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYCKV SNKALPAPIEKTISKAKGQPREPQVYT LPPSREEMTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTTPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPG (SEQ ID NO: 944)	DIQMTQSPSTLSASVGDRVITTCRAS QSISSW LAWYQQKPGKAPKLLIYKA SS LESGVPSRFSGSGSGTEFTLTISL QPDD FATYYCQQFQSLPPFTFGGGT KVEIKRTVAAPS VFIFFPSDEQLKSGTA SVVCLLNFPYPREAKVQWKVDNALQS GNSQESVTEQDSKDSTYSLSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSF <u>NRGEC</u> (SEQ ID NO: 947)
25A3	QVQLVQSGAEVKKPGASVKVSCA SGYTFD VYGISWVRQAPGQGLEWM GW IAPYSGNTNYAQKLQGRVTMTT	DIQMTQSPSTLSASVGDRVITTCQAS QSI NNWLAWYQQKPGKAPKLLIYKA YN LESGVPSRFSGSGSGTEFTLTISL

Clone	HEAVY CHAIN	LIGHT CHAIN
	DTSTSTAYMELRSLRSDDTAVYYCA RDAGTYSPPFGYGM DVWQG GT TVT VSSASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVT TVSWNSGALTSGV HTFPAVLQSSGLYSLSSV TVPS SSLG TQTYICNVNHKPSNTK VDKRVEPKSC DKTHTCPPCPAPELLGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYT LPPSREEMTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPG (SEQ ID NO: 944)	QPDDFATYYCQLFQSLPPFTFGGGT KVEIKRTVAAPSVFIFPPSDEQLKSGTA SVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSF NRGEC (SEQ ID NO: 948)
25A5	QVQLVQSGAEVKKPGASVKVSCKA SGYTFDVGISWVRQAPGQGLEWM GWIAPYSGNTNYAQKLQGRVTMTT DTSTSTAYMELRSLRSDDTAVYYCA RDAGTYSPPFGYGM DVWQG GT TVT VSSASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVT TVSWNSGALTSGV HTFPAVLQSSGLYSLSSV TVPS SSLG TQTYICNVNHKPSNTK VDKRVEPKSC DKTHTCPPCPAPELLGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYT LPPSREEMTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPG (SEQ ID NO: 944)	DIQMTQSPSTLSASVGDRVTITCRAS ESISNWLAWYQQKPGKAPKLLIYKA YSLEYGVPSRFSGSGSGTEFTLTISL QPDDFATYYCQQFQKLPPFTFGGGT KVEIKRTVAAPSVFIFPPSDEQLKSGTA SVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSF NRGEC (SEQ ID NO: 921)
25A5-T	QVQLVQSGAEVKKPGASVKVSCKA SGYTFDAYGISWVRQAPGQGLEWM GWIAPYSGNTNYAQKLQGRVTMTT DTSTSTAYMELRSLRSDDTAVYYCA RDAGTYSPPFGYGM DVWQG GT TVT VSSASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVT TVSWNSGALTSGV HTFPAVLQSSGLYSLSSV TVPS SSLG TQTYICNVNHKPSNTK VDKRVEPKSC DKTHTCPPCPAPELLGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYT LPPSREEMTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVM	DIQMTQSPSTLSASVGDRVTITCRAS ESISNWLAWYQQKPGKAPKLLIYKA YSLEYGVPSRFSGSGSGTEFTLTISL QPDDFATYYCQQFQKLPPFTFGGGT KVEIKRTVAAPSVFIFPPSDEQLKSGTA SVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSF NRGEC (SEQ ID NO: 921)

Clone	HEAVY CHAIN	LIGHT CHAIN
	HEALHNHYTQKSLSLSPG (SEQ ID NO:920)	
25G	<p>QVQLVQSGAEVKKPGASVKVSCKA SGYTFRSYGISWVRQAPGQGLEWM GWVAPYNGNTNYAQKLQGRVTMT TDTSTSTAYMELRSLRSDDTAVYYC ARDAGTYSPIGYGMDVWGQGT TVSSASTKGPSVFPLAPSSKSTSGGTA ALGCLVKDYFPEPVTVSWNSGALTSG VHTFPAVLQSSGLYSLSSVTVPSSSL GTQTYICNVNHKPSNTKVDKRVEPKS CDKTHTCPPCPAPELLGGPSVFLFPPK PKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKC KVSNAKALPAIEKTISKAKGQPREPQV YTLPPSREEMTKNQVSLTCLVKGFYP SDIAVEWESNGQPENNYKTPPVLD DGSFFLYSKLTVDKSRWQQGNVFC VMHEALHNHYTQKSLSLSPG (SEQ ID NO: 945)</p>	<p>DIQMTQSPSTLSASVGDRVTITCRAS QSISSWLAWYQQKPGKAPKLLIYKA SSLESGVPSRFSGSGSGTEFTLTIS LPDFAFYCQQFQSLPPFTFGGGT KVEIKRTVAAPSVEFPPSDEQLKSGT SVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKSTYSLSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSF NRGEC (SEQ ID NO: 947)</p>
25G1	<p>QVQLVQSGAEVKKPGASVKVSCKA SGYTFRSYGISWVRQAPGQGLEWM GWVAPYSGNTNYAQKLQGRVTMT TDTSTSTAYMELRSLRSDDTAVYYC ARDAGTYSPIGYGMDVWGQGT TVSSASTKGPSVFPLAPSSKSTSGGTA ALGCLVKDYFPEPVTVSWNSGALTSG VHTFPAVLQSSGLYSLSSVTVPSSSL GTQTYICNVNHKPSNTKVDKRVEPKS CDKTHTCPPCPAPELLGGPSVFLFPPK PKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKC KVSNAKALPAIEKTISKAKGQPREPQV YTLPPSREEMTKNQVSLTCLVKGFYP SDIAVEWESNGQPENNYKTPPVLD DGSFFLYSKLTVDKSRWQQGNVFC VMHEALHNHYTQKSLSLSPG (SEQ ID NO: 922)</p>	<p>DIQMTQSPSTLSASVGDRVTITCRAS HSIDSWLAWYQQKPGKAPKLLIYKA SYLESGVPSRFSGSGSGTEFTLTIS LPDFAFYCQLFQSLPPFTFGGGT KVEIKRTVAAPSVEFPPSDEQLKSGT SVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKSTYSLSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSF NRGEC (SEQ ID NO: 949)</p>
25G9	<p>QVQLVQSGAEVKKPGASVKVSCKA SGYTFRSYGISWVRQAPGQGLEWM GWVAPYSGNTNYAQKLQGRVTMT TDTSTSTAYMELRSLRSDDTAVYYC ARDAGTYSPIGYGMDVWGQGT TVSSASTKGPSVFPLAPSSKSTSGGTA ALGCLVKDYFPEPVTVSWNSGALTSG VHTFPAVLQSSGLYSLSSVTVPSSSL GTQTYICNVNHKPSNTKVDKRVEPKS CDKTHTCPPCPAPELLGGPSVFLFPPK PKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKC</p>	<p>DIQMTQSPSTLSASVGDRVTITCQAS QSIDSWLAWYQQKPGKAPKLLIYSA SYLESGVPSRFSGSGSGTEFTLTIS LPDFAFYCQRFQSLPPFTFGGGT KVEIKRTVAAPSVEFPPSDEQLKSGT SVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKSTYSLSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSF NRGEC (SEQ ID NO:923)</p>

Clone	HEAVY CHAIN	LIGHT CHAIN
	KVSNKALPAIEKTISKAKGQPREPQV YTLPPSREEMTKNQVSLTCLVKGFYP SDIAVEWESNGQPENNYKTTPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCS VMHEALHNHYTQKSLSLSPG (SEQ ID NO:922)	
Lineage e 25A consen sus	QVQLVQSGAEVKKPGASVKVSCKA SGYTFD_x[V/A]YGISWVRQAPGQGL EWMGWIAPY_x[N/S]GNTNYAQKLQ GRVTMTTDTSTSTAYMELRSLRSDD TAVYYCARDAGTYSPPFGYGMDVW GQGTTVTVSSASTKGPSVFPLAPSSK STSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVV TVPSSSLGTQTYICNVNHKPSNTKVD KRVEPKSCDKTHTCPPCPAPELLGGPS VFLFPPKPKDTLMISRTPEVTCVVDV SHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNG KEYKCKVSNKALPAIEKTISKAKGQP REPQVYTLPPSREEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTP PVLDS DGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 946)	DIQMTQSPSTLSASVGDRVTITC_x[R/ Q]AS_x[Q/E]SI_x[S/N]_x[S/N]WLAWYQQ KPGKAPKLLIYKA_x[S/Y]_x[S/N]LE_x[S/ Y]GVPSRFSGSGSGTEFTLTISLQPD DFATYYCQ_x[Q/L]FQ_x[S/K]LPPFTFG GGTKVEIKRTVAAPS FIFPPSDEQLK SGTASVVCLLNNFYPREAKVQWKVDN ALQSGNSQESVTEQDSKDSTYLSSTL TLISKADYEKHKVYACEVTHQGLSSPV TKSFNRGEC (SEQ ID NO: 950)
43D7	QVQLQQWGAGLLKPSETLSLTCAV YGGSLSGYYWSWIRQPPGKGLEWI GEIGASGSTRYNPSLKSRTISVDTS KNQFSLKLSSVTAADTAVYYCARDT PYYYEGGYYYMDVWGKGTTVTV SSASTKGPSVFPLAPSSKSTSGGTAAL GCLVKDYFPEPVTVSWNSGALTSGVH TFPAVLQSSGLYSLSSVTVPSSSLGT QTYICNVNHKPSNTKVDKRVEPKSCD KTHTCPPCPAPELLGGPSVFLFPPKPK DTLMISRTPEVTCVVDVSHEDPEVK FNWYVDGVEVHNAKTKPREEQYNST YRVVSVLTVLHQDWLNGKEYKCKVS NKALPAIEKTISKAKGQPREPQVYTL PPSREEMTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTPVLDS DGSF FLYSKLTVDKSRWQQGNVFSCSVMH EALHNHYTQKSLSLSPG (SEQ ID NO:924)	EIVLTQSPGTLSLSPGERATLSCRASD SVDSSYLAWYQQKPGQAPRLLIYGA FSRANGIPDRFSGSGSGTDFTLTISRL EPEDFAVYYCQAGAVVPYTFGGGK VEIKRTVAAPS FIFPPSDEQLKSGTA VVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDSTYLSSTLTLISK ADYEKHKVYACEVTHQGLSSPVTKSF NRGEC (SEQ ID NO:925)
43D8	QVQLQQWGAGLLKPSETLSLTCAV YGGSLSGYYWSWIRQPPGKGLEWI GEIGASGSTRYNPSLKSRTISVDTS KNQFSLKLSSVTAADTAVYYCARDT PYYYEGGYYYMDVWGKGTTVTV SSASTKGPSVFPLAPSSKSTSGGTAAL GCLVKDYFPEPVTVSWNSGALTSGVH TFPAVLQSSGLYSLSSVTVPSSSLGT QTYICNVNHKPSNTKVDKRVEPKSCD	EIVLTQSPGTLSLSPGERATLSCRAS QSVSSSFLAWYQQKPGQAPRLLIYG AYS RATGIPDRFSGSGSGTDFTLTISR LEPEDFAVYYCQAGVVPYTFGGGT KVEIKRTVAAPS FIFPPSDEQLKSGTA SVVCLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDSTYLSSTLTLISK ADYEKHKVYACEVTHQGLSSPVTKSF NRGEC (SEQ ID NO:927)

Clone	HEAVY CHAIN	LIGHT CHAIN
	KTHTCPPCPAPELLGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVK FNWYVDGVEVHNAKTKPREEQYNST YRVVSVLTVLHQDWLNGKEYKCKVS NKALPAPIEKTISKAKGQPREPQVYTL PPSREEMTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLDSDGSF FLYSKLTVDKSRWQQGNVFSCSVMH EALHNHYTQKSLSLSPG (SEQ ID NO:926)	

CLAIMS

1. A method of treating an ocular disease in a subject in need thereof comprising administering to the subject an isolated antibody, wherein the antibody binds to the extracellular domain of human Tissue Factor (TF), wherein the antibody binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FVIIa.
2. The method of claim 1, wherein the antibody is administered as a dose via intraocular injection and wherein if the dose administered to the subject is greater than 6 mg of antibody/eye, the dose is administered in two or more injections.
3. The method of claim 1, wherein the antibody is administered to the subject via a plurality of doses comprising at least a first dose and at least a second dose and wherein the time between the first and second doses is at least 28 days.
4. The method of claim 1, wherein the antibody is administered as a dose via intraocular injection, wherein if the dose administered to the subject is greater than 6 mg of antibody/eye, the dose is administered in two or more injections, and wherein the antibody is administered to the subject via a plurality of doses comprising at least a first dose and at least a second dose and wherein the time between the first and second doses is at least 28 days.
5. The method of claim 1, wherein the antibody is administered via intravitreal injection.
6. The method of claim 1, wherein the antibody (i) specifically binds to rabbit TF or (ii) is cross-reactive with rabbit TF.
7. The method of claim 1, wherein the antibody (i) specifically binds pig TF or (ii) is cross-reactive with pig TF.
8. The method of any one of claims 1-7, wherein the antibody does not inhibit human thrombin generation as determined by thrombin generation assay (TGA).
9. The method of any one of claims 1-7, wherein the antibody does not inhibit or inhibits human thrombin generation to a lesser extent, as determined by thrombin generation assay (TGA), compared to a comparator therapy.
10. The method of claim 9, wherein the comparator therapy is an anti-TF protein, an anti-TF antibody, an anti-TF immunoconjugate or an anti-TF drug.
11. The method of claim 9 or 10, wherein the comparator therapy is ICON-1.

12. The method of claim 9 or 10, wherein the comparator therapy is an antibody comprising a V_H sequence of SEQ ID NO:821 and a V_L sequence of SEQ ID NO:822.

13. The method of claim 12, wherein binding between the isolated antibody and a variant TF extracellular domain comprising a mutation at amino acid residue 149 of the sequence shown in SEQ ID NO:810 is less than 50% of the binding between the isolated antibody and the extracellular domain of TF of the sequence shown in SEQ ID NO:810, as determined by the median fluorescence intensity value of the isolated antibody relative to an isotype control in a live cell staining assay.

14. The method of any one of claims 1 to 13, wherein the antibody comprises all three heavy chain Complementary Determining Regions (CDRs) and all three light chain CDRs from an antibody group in Table 35, wherein the all three heavy chain CDRs and the all three light chain CDRs are from the same antibody group.

15. The method of any one of the claims 1 to 13, wherein the antibody comprises all three heavy chain Complementary Determining Regions (CDRs) and all three light chain CDRs from an antibody in any one of Tables 15-34, wherein the all three heavy chain CDRs and the all three light chain CDRs are from the same antibody.

16. The method of claim 15, wherein the isolated human antibody comprises all three heavy chain CDRs and all three light chain CDRs from: the antibody designated 25A, the antibody designated 25A5, the antibody designated 25A5-T, the antibody designated 25G, the antibody designated 25G1, the antibody designated 25G9, the antibody designated 43B, the antibody designated 43B1, the antibody designated 43B7, the antibody designated 43D, the antibody designated 43D7, the antibody designated 43D8, the antibody designated 43E, or the antibody designated 43Ea.

17. The method of claim 16, wherein the isolated human antibody comprises all three heavy chain CDRs and all three light chain CDRs from: the antibody designated 25A, the antibody designated 25A5, the antibody designated 25A5-T, the antibody designated 25G, the antibody designated 25G1, or the antibody designated 25G9.

18. The method of claim 16, wherein the isolated human antibody comprises all three heavy chain CDRs and all three light chain CDRs from: the antibody designated 43B, the antibody designated 43B1, the antibody designated 43B7, the antibody designated 43D, the antibody designated 43D7, the antibody designated 43D8, the antibody designated 43E, or the antibody designated 43Ea.

19. The method of any one of claims 1 to 13, wherein the antibody comprises a VH Domain sequence and VL domain sequence from Table 14, wherein the VH and VL domain sequences are from the same group in Table 14.

20. The method of any one of claims 1 to 13, wherein the antibody comprises a VH Domain sequence and VL domain sequence from Table 13, wherein the VH and VL domain sequences are from the same clone in Table 13.

21. The method of any one of claims 1 to 14, wherein the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:779; a VH-CDR2 comprising the sequence set forth in SEQ ID NO:780; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:781; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:782; a VL-CDR2 comprising the sequence set forth in SEQ ID NO:783; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:784.

22. The method of any one of claims 1 to 14, wherein the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:872; a VH-CDR2 comprising the sequence set forth in SEQ ID NO:873; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:874; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:875; a VL-CDR2 comprising the sequence set forth in SEQ ID NO:876; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:877.

23. The method of claim 22, wherein the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:884; a VH-CDR2 comprising the sequence set forth in SEQ ID NO:885; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:886; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:887; a VL-CDR2 comprising the sequence set forth in SEQ ID NO:888; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:889.

24. The method of any one of claims 1 to 14 or claim 22, wherein the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:868 and a VL sequence comprising the sequence set forth in SEQ ID NO:869.

25. The method of claim 24, wherein the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:189 and a VL sequence comprising the sequence set forth in SEQ ID NO:190.

- 26.** The method of claim 24, wherein the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:836 and a VL sequence comprising the sequence set forth in SEQ ID NO:837.
- 27.** The method of claim 23 or 26, wherein the antibody comprises: a heavy chain comprising the sequence set forth in SEQ ID NO:920 and a light chain comprising the sequence set forth in SEQ ID NO:921.
- 28.** The method of any one of claims 1 to 14, wherein the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:878; a VH-CDR2 comprising the sequence set forth in SEQ ID NO:879; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:880; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:881; a VL-CDR2 comprising the sequence set forth in SEQ ID NO:882; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:883.
- 29.** The method of claim 28, wherein the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:267; a VH-CDR2 comprising the sequence set forth in SEQ ID NO:268; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:269; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:270; a VL-CDR2 comprising the sequence set forth in SEQ ID NO:271; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:272.
- 30.** The method of any one of claims 1 to 14 or claim 28, wherein the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:870 and a VL sequence comprising the sequence set forth in SEQ ID NO:871.
- 31.** The method of claim 30, wherein the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:303 and a VL sequence comprising the sequence set forth in SEQ ID NO:304.
- 32.** The method of claim 29 or 31, wherein the antibody comprises: a heavy chain comprising the sequence set forth in SEQ ID NO:922 and a light chain comprising the sequence set forth in SEQ ID NO:923.
- 33.** The method of any one of claims 1 to 14, wherein the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:797; a VH-CDR2 comprising the sequence set forth in SEQ ID NO:798; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:799; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:800; a VL-CDR2

comprising the sequence set forth in SEQ ID NO:801; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:802.

34. The method of claim 33, wherein the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:571; a VH-CDR2 comprising the sequence set forth in SEQ ID NO:572; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:573; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:574; a VL-CDR2 comprising the sequence set forth in SEQ ID NO:575; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:576.

35. The method of claim 33, wherein the antibody comprises: a VH-CDR1 comprising the sequence set forth in SEQ ID NO:609; a VH-CDR2 comprising the sequence set forth in SEQ ID NO:610; a VH-CDR3 comprising the sequence set forth in SEQ ID NO:611; a VL-CDR1 comprising the sequence set forth in SEQ ID NO:612; a VL-CDR2 comprising the sequence set forth in SEQ ID NO:613; and a VL-CDR3 comprising the sequence set forth in SEQ ID NO:614.

36. The method of any one of claims 1 to 14 or claim 33, wherein the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:769 and a VL sequence comprising the sequence set forth in SEQ ID NO:770.

37. The method of claim 36, wherein the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:569 and a VL sequence comprising the sequence set forth in SEQ ID NO:570.

38. The method of claim 36, wherein the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:607 and a VL sequence comprising the sequence set forth in SEQ ID NO:608.

39. The method of claim 36, wherein the antibody comprises: a VH sequence comprising the sequence set forth in SEQ ID NO:645 and a VL sequence comprising the sequence set forth in SEQ ID NO:646.

40. The method of claim 34 or 38, wherein the antibody comprises: a heavy chain comprising the sequence set forth in SEQ ID NO:924 and a light chain comprising the sequence set forth in SEQ ID NO:925.

41. The method of claim 35 or 39, wherein the antibody comprises: a heavy chain comprising the sequence set forth in SEQ ID NO:926 and a light chain comprising the sequence set forth in SEQ ID NO:927.

42. The method of any one of the preceding claims, wherein the antibody competes for binding to human TF with the antibody designated 25A, the antibody designated 25A5, the antibody designated 25A5-T, the antibody designated 25G, the antibody designated 25G1, the antibody designated 25G9, the antibody designated 43B, the antibody designated 43B1, the antibody designated 43B7, the antibody designated 43D, the antibody designated 43D7, the antibody designated 43D8, the antibody designated 43E, or the antibody designated 43Ea.

43. The method of claim 42, wherein the antibody competes for binding to human TF with the antibody designated 25A, the antibody designated 25A5, the antibody designated 25A5-T, the antibody designated 25G, the antibody designated 25G1, or the antibody designated 25G9.

44. The method of claim 42, wherein the antibody competes for binding to human TF with the antibody designated 43B, the antibody designated 43B1, the antibody designated 43B7, the antibody designated 43D, the antibody designated 43D7, the antibody designated 43D8, the antibody designated 43E, or the antibody designated 43Ea.

45. The method of any of the preceding claims, wherein the antibody binds to the same human TF epitope bound by the antibody designated 25A, the antibody designated 25A5, the antibody designated 25A5-T, the antibody designated 25G, the antibody designated 25G1, the antibody designated 25G9, the antibody designated 43B, the antibody designated 43B1, the antibody designated 43B7, the antibody designated 43D, the antibody designated 43D7, the antibody designated 43D8, the antibody designated 43E, or the antibody designated 43Ea.

46. The method of claim 45, wherein the antibody binds to the same human TF epitope bound by the antibody designated 25A, the antibody designated 25A5, the antibody designated 25A5-T, the antibody designated 25G, the antibody designated 25G1, or the antibody designated 25G9.

47. The method of claim 45, wherein the antibody binds to the same human TF epitope bound by the antibody designated 43B, the antibody designated 43B1, the antibody designated 43B7, the antibody designated 43D, the antibody designated 43D7, the antibody designated 43D8, the antibody designated 43E, or the antibody designated 43Ea.

48. The method of any one of the preceding claims, wherein the antibody does not inhibit human thrombin generation as determined by thrombin generation assay (TGA), does not reduce the thrombin peak on a thrombin generation curve (Peak IIa) compared to an isotype control, does not increase the time from the assay start to the thrombin peak on a thrombin generation curve (ttPeak) compared to an isotype control, does not decrease the endogenous thrombin potential (ETP) as determined by the area under a thrombin generation curve compared to an isotype control, allows human thrombin generation as determined by thrombin generation assay (TGA), maintains the thrombin peak on a thrombin generation curve (Peak IIa) compared to an isotype control, maintains the time from the assay start to the thrombin peak on a thrombin generation curve (ttPeak) compared to an isotype control, preserves the endogenous thrombin potential (ETP) as determined by the area under a thrombin generation curve compared to an isotype control, binds human TF at a human TF binding site that is distinct from a human TF binding site bound by human FX, does not interfere with the ability of TF:FVIIa to convert FX into FXa, and does not compete for binding to human TF with FVIIa.

49. The method of any one of the preceding claims, wherein the antibody (i) specifically binds to cynomolgus monkey TF or (ii) is cross-reactive with cynomolgus monkey TF.

50. The method of any one of the preceding claims, wherein the antibody (i) specifically binds to mouse TF or (ii) is cross-reactive with mouse TF.

51. The method of any one of the preceding claims, wherein the three heavy chain CDRs and the three light chain CDRs are determined using exemplary, Kabat, Chothia, AbM, Contact, or IMGT numbering.

52. The method of any one of the preceding claims, wherein the affinity of the antibody to human TF is greater compared to a comparator therapy.

53. The method of claim 52, wherein the comparator therapy is an anti-TF protein, an anti-TF antibody, an anti-TF immunoconjugate or an anti-TF drug.

54. The method of any one of claims 52 or 53, wherein the comparator therapy is ICON-1.

55. The method of any one of the preceding claims, wherein the antibody comprises an Fc region.

56. The method of claim 55, wherein the Fc region of the antibody comprises one or more mutations that result in an increased immune response.

57. The method of claim 55 or 56, wherein the antibody interacts with an Fc γ Receptor (Fc γ R) expressed by an immune cell via the Fc domain, and wherein the antibody increases an immune response in the subject upon specific binding between the antibody and one or more TF-expressing cells, thereby treating the ocular disease.

58. The method of claim 56 or 57, wherein the immune response is enhanced Fc effector function.

59. The method of any one of claims 56 to 58, wherein the immune response increases compared to a comparator therapy.

60. The method of claim 59, wherein the immune response is any one of: (i) increased T cell activity, (ii) increased activation of $\alpha\beta$ and/or $\gamma\delta$ T cells, (iii) increased cytotoxic T cell activity, (iv) increased NK and/or NKT cell activity, (v) alleviation of $\alpha\beta$ and/or $\gamma\delta$ T-cell suppression, (vi) increased pro-inflammatory cytokine secretion, (vii) increased IL-2 secretion, (viii) increased interferon- γ production, (ix) increased Th1 response, (x) decreased Th2 response, (xi) decreased or eliminated cell number and/or activity of at least one of regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), immature myeloid cells (iMCs), mesenchymal stromal cells, or TIE2-expressing monocytes, (xii) reduced regulatory cell activity, and/or the reduced activity of one or more of myeloid derived suppressor cells (MDSCs), iMCs, mesenchymal stromal cells, TIE2- expressing monocytes, (xiii) decreased or eliminated M2 macrophages, (xiv) reduced M2 macrophage pro-tumorigenic activity, (xv) decreased or eliminated N2 neutrophils, (xvi) reduced N2 neutrophils pro-tumorigenic activity, (xvii) reduced inhibition of T cell activation, (xviii) reduced inhibition of CTL activation, (xix) reduced inhibition of NK and/or NKT cell activation, (xx) reversed $\alpha\beta$ and/or $\gamma\delta$ T cell exhaustion, (xxi) increased $\alpha\beta$ and/or $\gamma\delta$ T cell response, (xxii) increased activity of cytotoxic cells, (xxiii) stimulated antigen-specific memory responses, (xxiv) elicitation of apoptosis or lysis of diseased cells, (xxv) stimulation of cytotoxic or cytostatic effect on diseased cells, (xxvi) induced direct killing of diseased cells, (xxvii) increased Th17 activity, (xxviii) modulated myeloid cell polarization, (xxix) modulated myeloid cell shifting toward a pro-inflammatory response, (xxx) shifted myeloid cells from M2 toward M1 phenotype, (xxxi) modulation of myeloid cells in the ocular tissues, (xxxii) increased anti-inflammatory mediator secretion, (xxxiii) enhanced myeloid and lymphoid infiltration into the diseased or damaged site, and (xxxiv) induction of complement dependent cytotoxicity and/or antibody dependent cell-mediated cytotoxicity.

- 61.** The method of claim 59, wherein the immune response is any one of: (i) antibody-dependent cellular cytotoxicity (ADCC), (ii) antibody-dependent cellular phagocytosis (ADCP), and (iii) C1q binding to activate complement dependent cytotoxicity (CDC).
- 62.** The method of any one of claims 56 to 61, wherein the immune response is antibody-dependent cellular cytotoxicity (ADCC).
- 63.** The method of any one of claims 56 to 61, wherein the immune response is antibody-dependent cellular phagocytosis (ADCP).
- 64.** The method of any one of claims 56 to 61, wherein the immune response is C1q binding to activate complement dependent cytotoxicity (CDC).
- 65.** The method of any one of claims 59 to 64, wherein the comparator therapy is an anti-TF protein, an anti-TF antibody, an anti-TF immunoconjugate or an anti-TF drug.
- 66.** The method of any one of claims 59 to 65, wherein the comparator therapy is ICON-1.
- 67.** The method of any one of the preceding claims, comprising administering the antibody of any one of the preceding claims to the subject by intravitreal injection, suprachoroidal injection or subretinal injection.
- 68.** The method of claim 67, comprising administering the antibody of any one of the preceding claims to the subject by intravitreal injection.
- 69.** The method of any one of the preceding claims wherein the ocular disease is neovascularization, age-related macular degeneration (AMD), retinal vein occlusion, retinopathy of prematurity or diabetic retinopathy.
- 70.** The method of claim 69, wherein the diabetic retinopathy is proliferative diabetic retinopathy or non-proliferative diabetic retinopathy.
- 71.** The method of claim 69, wherein the AMD is neovascular AMD.
- 72.** The method of claim 69, wherein the neovascularization is macular neovascularization.
- 73.** The method of claim 69, wherein the AMD is wet AMD.
- 74.** The method of claim 69, wherein the AMD is dry AMD.
- 75.** The method of any of the preceding claims, wherein upon administration to a subject, the antibody decreases the total leakiness area on the retina relative to baseline levels.

- 76.** The method of any of the preceding claims, wherein upon administration to a subject, the antibody decreases the total leakiness area on the retina relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug.
- 77.** The method of any one of claims 1 to 74, wherein upon administration to a subject, the antibody does not alter the total leakiness area on the retina relative to baseline levels.
- 78.** The method of any one of claims 75 to 77, wherein the total leakiness area is determined using fluorescein angiography or OCT-angiography.
- 79.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody reduces choroidal neovascularization (CNV) area relative to baseline levels.
- 80.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody reduces choroidal neovascularization (CNV) area relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug.
- 81.** The method of claim 79 or 80, wherein the CNV area is reduced by at least about 10%, at least about 20%, at least about 30%, at least about 40% or at least about 50%.
- 82.** The method of any one of claims 79 to 81, wherein the CNV area is determined using fluorescein angiography or OCT-angiography.
- 83.** The method of any of the preceding claims, wherein upon administration to a subject, the antibody does not result in ocular inflammation.
- 84.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in reduced ocular inflammation relative to baseline levels.
- 85.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in reduced ocular inflammation relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug.
- 86.** The method of any one of claims 83 to 85, wherein the ocular inflammation is as determined using direct or indirect ophthalmoscopy.
- 87.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in reduced retinal thickness relative to baseline levels.
- 88.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in reduced retinal thickness relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug .

- 89.** The method of any one of the preceding claims, wherein the retinal thickness is reduced by at least about 50 μm , at least about 100 μm , at least about 150 μm , at least about 175 μm , at least about 200 μm , at least about 225 μm or at least about 250 μm .
- 90.** The method of any one of claims 1 to 88, wherein the retinal thickness is reduced by at least about 10%, at least about 20%, at least about 30%, at least about 40% or at least about 50%.
- 91.** The method of any one of the preceding claims, wherein the reduced retinal thickness is reduced central retinal subfield thickness (CST), reduced center point thickness (CPT), or reduced central foveal thickness (CFT).
- 92.** The method of claim 87 or 88, wherein retinal thickness is central retina thickness (CRT).
- 93.** The method of claim 92, wherein following administration to the subject, the CRT value increases to a value within the range of 200-270 μm .
- 94.** The method of any one of claims 87 to 93, wherein the retinal thickness is measured by spectral domain optical coherence tomography (SD OCT).
- 95.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in reduced mean foveal thickness relative to baseline levels.
- 96.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in reduced mean foveal thickness relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug .
- 97.** The method of claim 95 or 96, wherein the mean foveal thickness is measured by SD OCT.
- 98.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in reduced macular edema relative to baseline levels.
- 99.** The method of claim 98, wherein the macular edema is determined by fluorescein angiography, or optical coherence tomography (OCT).
- 100.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in reduced leakage into the macula relative to baseline levels.
- 101.** The method of claim 100, wherein the leakage into the macula is determined by fluorescein angiography, or optical coherence tomography (OCT).

- 102.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in reduced or absent biomarkers of inflammation relative to baseline levels.
- 103.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in reduced or absent biomarkers of inflammation relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug .
- 104.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in reduced or absent biomarkers of inflammation, as compared to a comparator therapy.
- 105.** The method of claim 104, wherein the comparator therapy is an anti-TF protein, an anti-TF antibody, an anti-TF immunoconjugate or an anti-TF drug.
- 106.** The method of claim 104, wherein the comparator therapy is ICON-1.
- 107.** The method of any one of claims 102 to 106, wherein the biomarkers of inflammation comprise one or more inflammatory cytokines.
- 108.** The method of claim 107, wherein the inflammatory cytokines comprise at least one of: IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF).
- 109.** The method of any one of claims 102 to 108, wherein the biomarkers of inflammation are measured by ELISA.
- 110.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in reduced or equal anti-drug antibody (ADA) levels relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug .
- 111.** The method of claim 110, wherein the ADA levels are systemic ADA levels.
- 112.** The method of claim 110, wherein the ADA levels are within vitreous humor and/or aqueous humor.
- 113.** The method of any one of claims 110 to 112, wherein the ADA levels are measured by ECL bridging assay.
- 114.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody does not significantly alter intraocular pressure (IOP) relative levels to baseline levels.
- 115.** The method of claim 114, wherein the IOP is measured by tonometry.

- 116.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in reduced mononuclear cell infiltration relative to baseline levels.
- 117.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in reduced mononuclear cell infiltration relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug .
- 118.** The method of claim 116 or 117, wherein the mononuclear cell infiltration comprises M2 macrophages.
- 119.** The method of any one of claims 116 to 118, wherein the mononuclear cell infiltration is infiltration of mononuclear cells into one or more of: retina, choroid, vitreous body, iris, ciliary body, limbus, and conjunctiva.
- 120.** The method of any one of claims 116 to 119, wherein the mononuclear cell infiltration is measured by immunohistochemical (IHC) analysis.
- 121.** The method of any one of the preceding claims, wherein upon administration of the antibody to a subject, the exposure to the antibody in target tissues is comparable to that from a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug.
- 122.** The method of any one of the preceding claims, wherein upon administration of the antibody to a subject, the concentration of the antibody in the vitreous humor is significantly higher than the concentration in the serum for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days following the administration.
- 123.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in less severe adverse ocular changes relative to baseline levels, wherein the adverse ocular changes comprise the presence of one or more of: anterior chamber cells, flare and fibrin, keratic precipitates/corneal endothelial cells, cellular deposits on the anterior lens capsule, incomplete pupil dilation, cells on corneal endothelium and lens capsule, vitreous haze, vitreous cells, retinal sheathing, and chorio-retinal hemorrhages.
- 124.** The method of any one of the preceding claims, wherein upon administration to a subject, the antibody results in less severe adverse ocular changes relative to a different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug , wherein the adverse ocular changes comprise the presence of one or more of: anterior chamber cells, flare and fibrin, keratic precipitates/corneal endothelial cells, cellular deposits on the anterior lens

capsule, incomplete pupil dilation, cells on corneal endothelium and lens capsule, vitreous haze, vitreous cells, retinal sheathing, and chorio-retinal hemorrhages.

125. The method of any one of the preceding claims, wherein the different anti-TF antibody, anti-TF drug, anti-VEGF antibody or anti-VEGF drug comprises: aflibercept, ranibizumab, brolucizumab, pegaptanib, bevacizumab, abicipar pegol, RG7716, KSI-301 or ICON-1.

126. The method of any one of the preceding claims, wherein the antibody binds human TF with a K_D less than 10^{-7} M.

127. The method of any one of the preceding claims, wherein the antibody binds human TF with a K_D between 10^{-7} M and 10^{-11} M.

128. The method of any one of the preceding claims, wherein K_D for mouse TF as measured by ForteBio is 360 nM or less.

129. The method of any one of the preceding claims, wherein K_D for mouse TF as measured by ForteBio is between 6.12 nM and 360 nM.

130. The method of any one of the preceding claims, wherein K_D for mouse TF as measured by ForteBio is 21 nM or less.

131. The method of any one of the preceding claims, wherein K_D for mouse TF as measured by ForteBio is between about 2.4 nM and 21 nM.

132. The method of any one of the preceding claims, wherein the subject is a mammal.

133. The method of any one of the preceding claims, wherein the subject is a human.

134. The method of any one of the preceding claims, wherein the administration is intraocular.

135. The method of any one of the preceding claims, wherein the antibody is administered by intravitreal injection.

136. The method of any one of the preceding claims, wherein the antibody is administered once a month or once every two months.

137. The method of any one of claims 1-133, wherein the antibody is administered biweekly.

138. The method of any one of claims 1-133, wherein the antibody is administered weekly.

139. The method of any one of the preceding claims, wherein the antibody is administered at 0.6 mg of antibody/eye.

140. The method of any one of claims 1-138, wherein the antibody is administered at 0.6, 2, 3.6, 4, or 6 mg of antibody/eye.

141. The method of claim 140, wherein the antibody is administered in a single injection of the antibody/eye.

142. The method of any one of claims 1-138, wherein the antibody is administered at 11.9 or 12 mg of antibody/eye.

143. The method of claim 142, wherein the antibody is administered in a single injection of the antibody/eye.

144. The method of any one of claims 1-138, wherein the antibody is administered at less than 0.6, 2, 3.6, 4, 6, 11.9 or 12 mg of antibody/eye.

Study Design Pig Laser-CNV Model

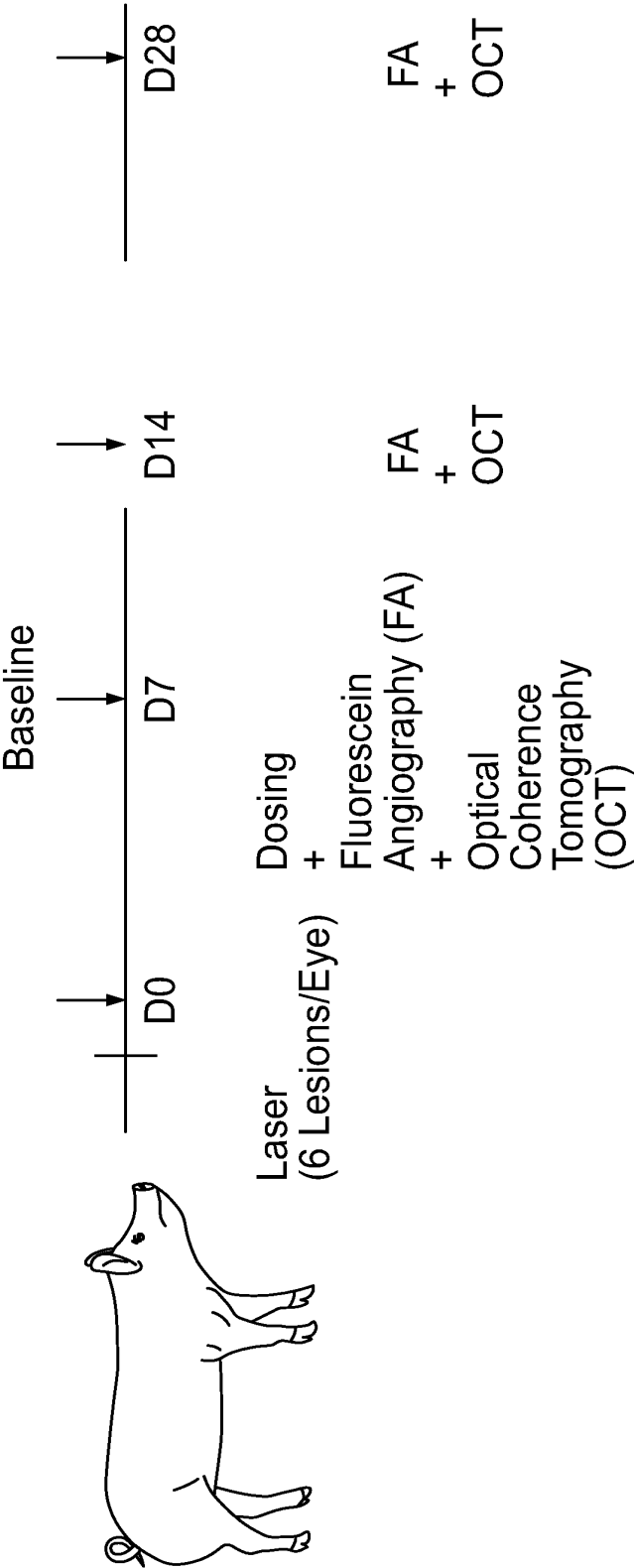


FIG. 1

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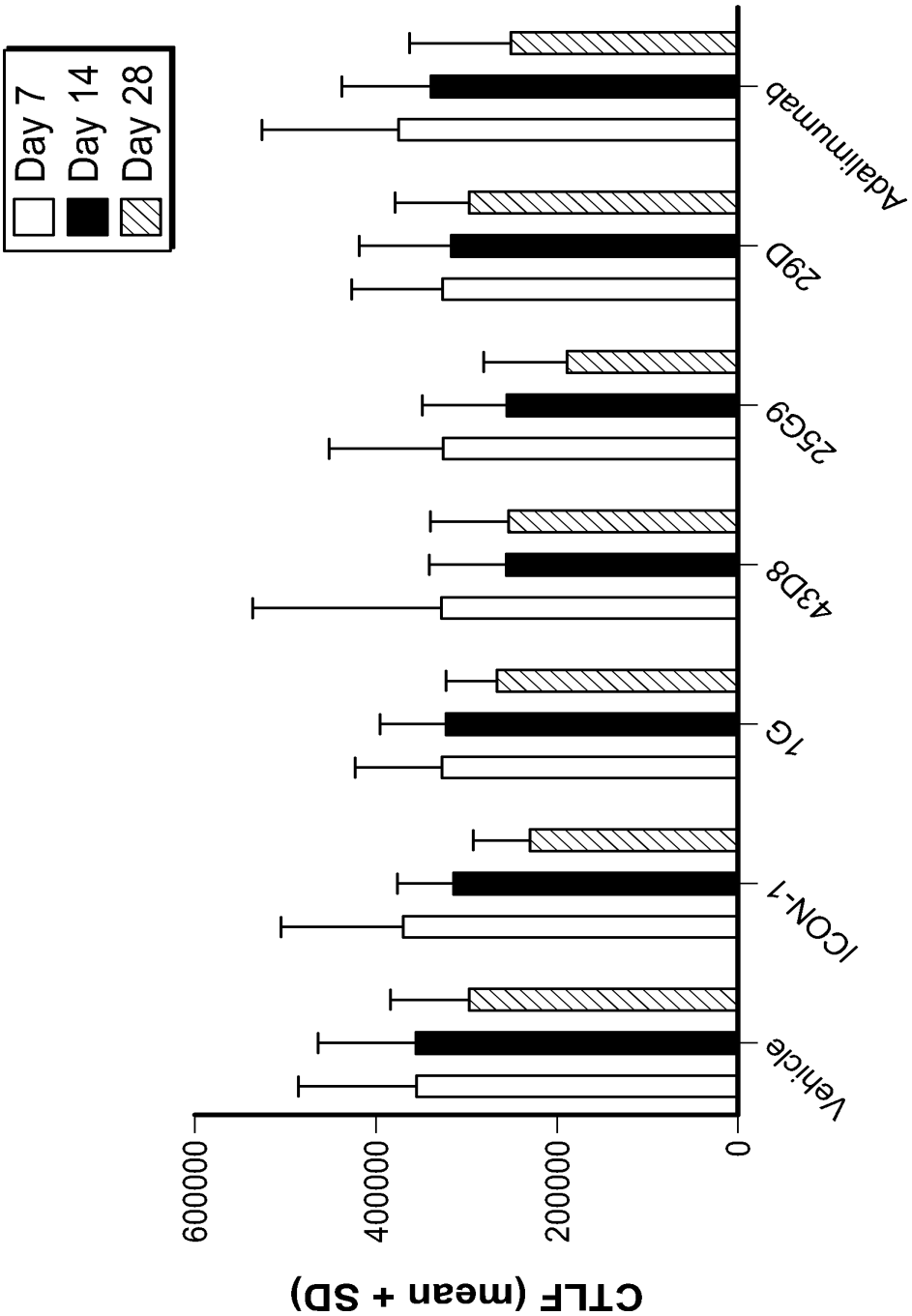


FIG. 2A

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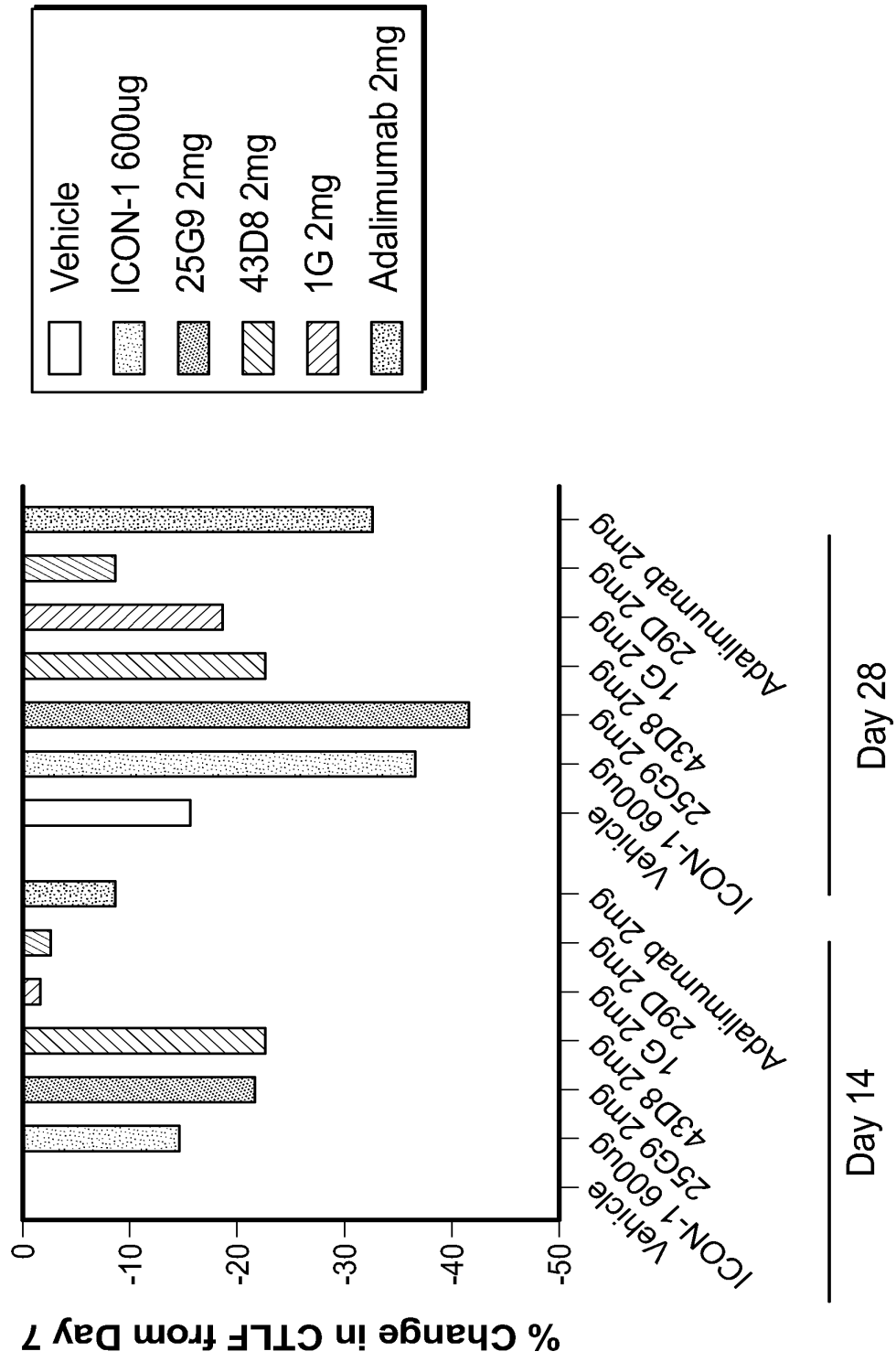


FIG. 2B

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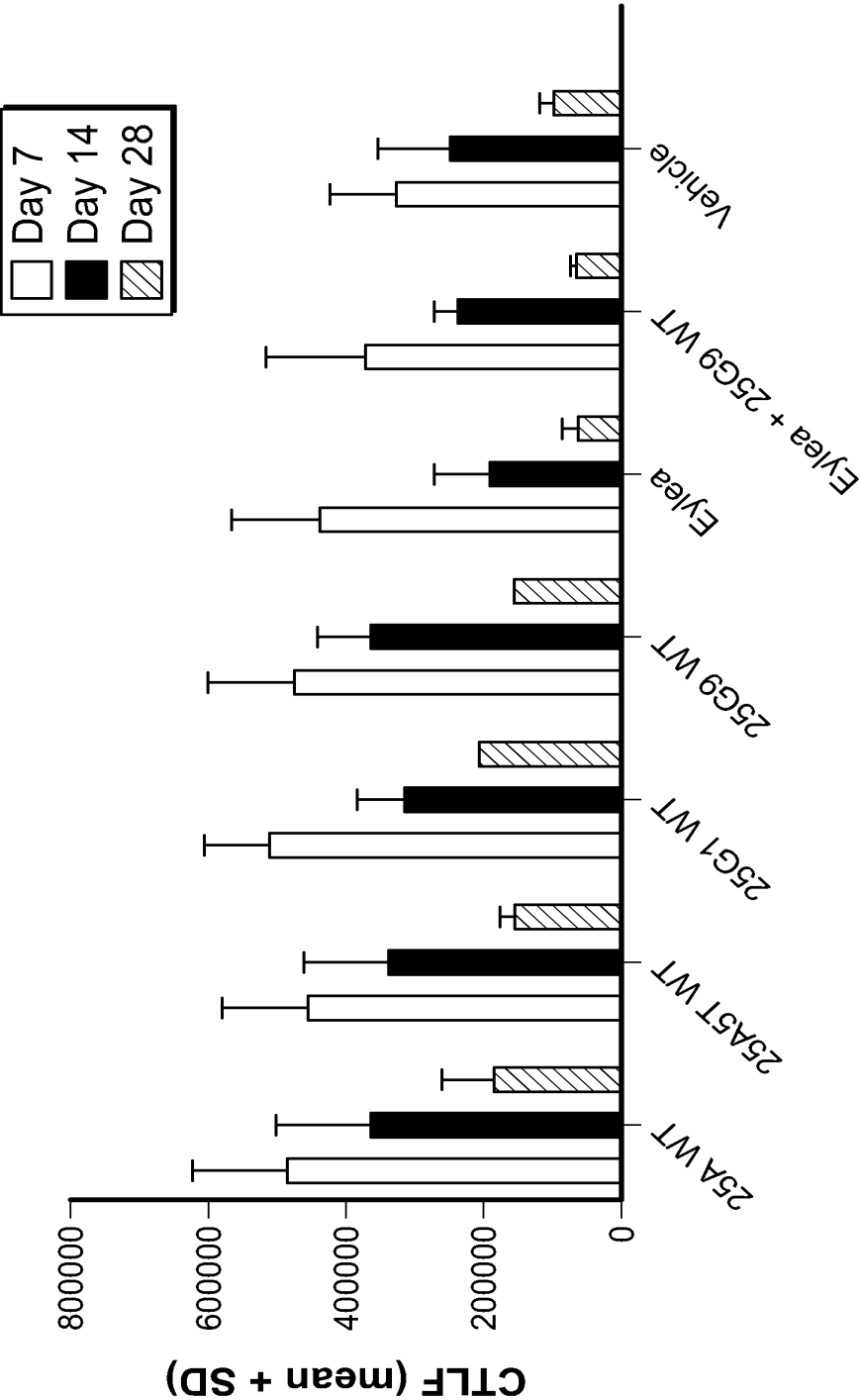


FIG. 3

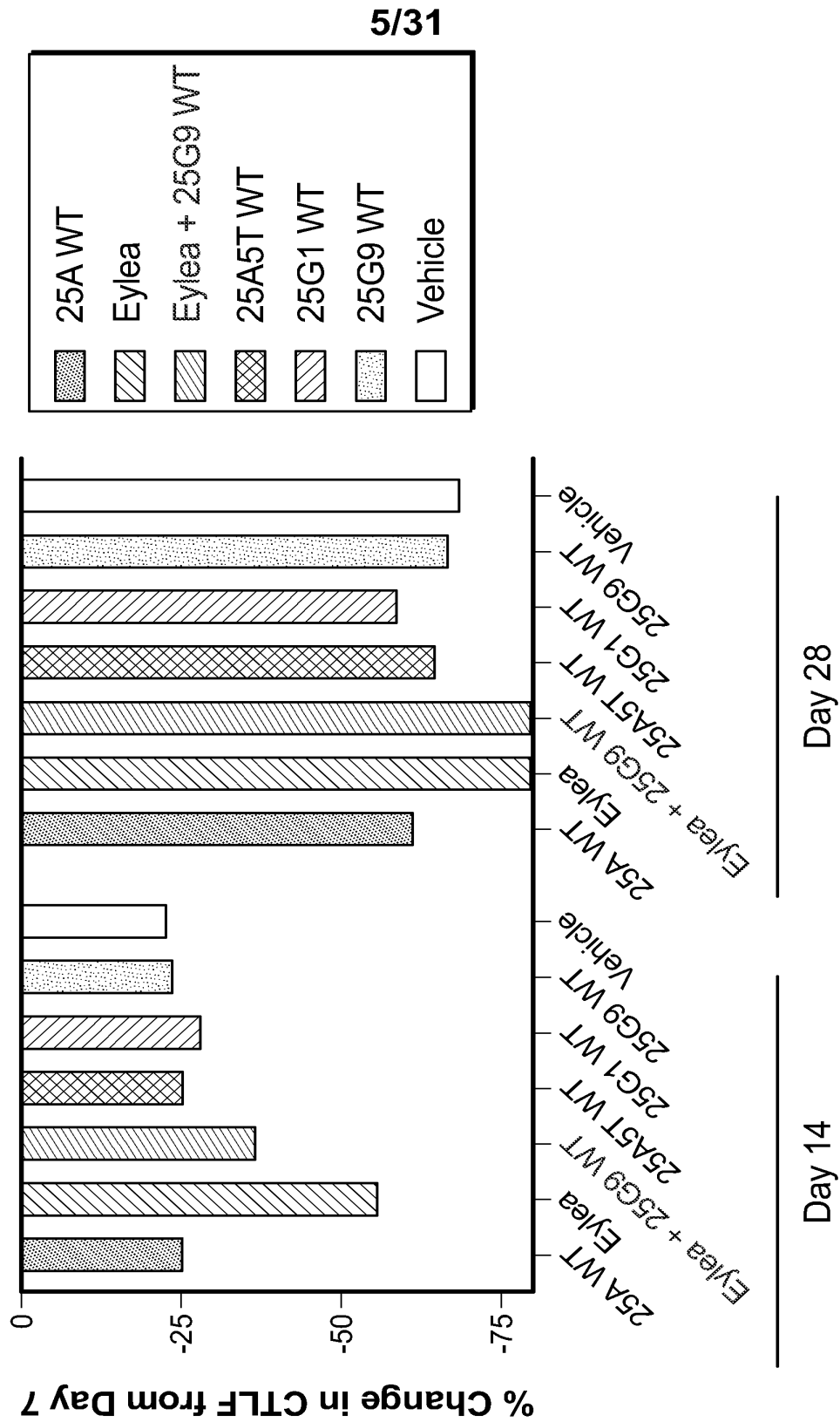


FIG. 4

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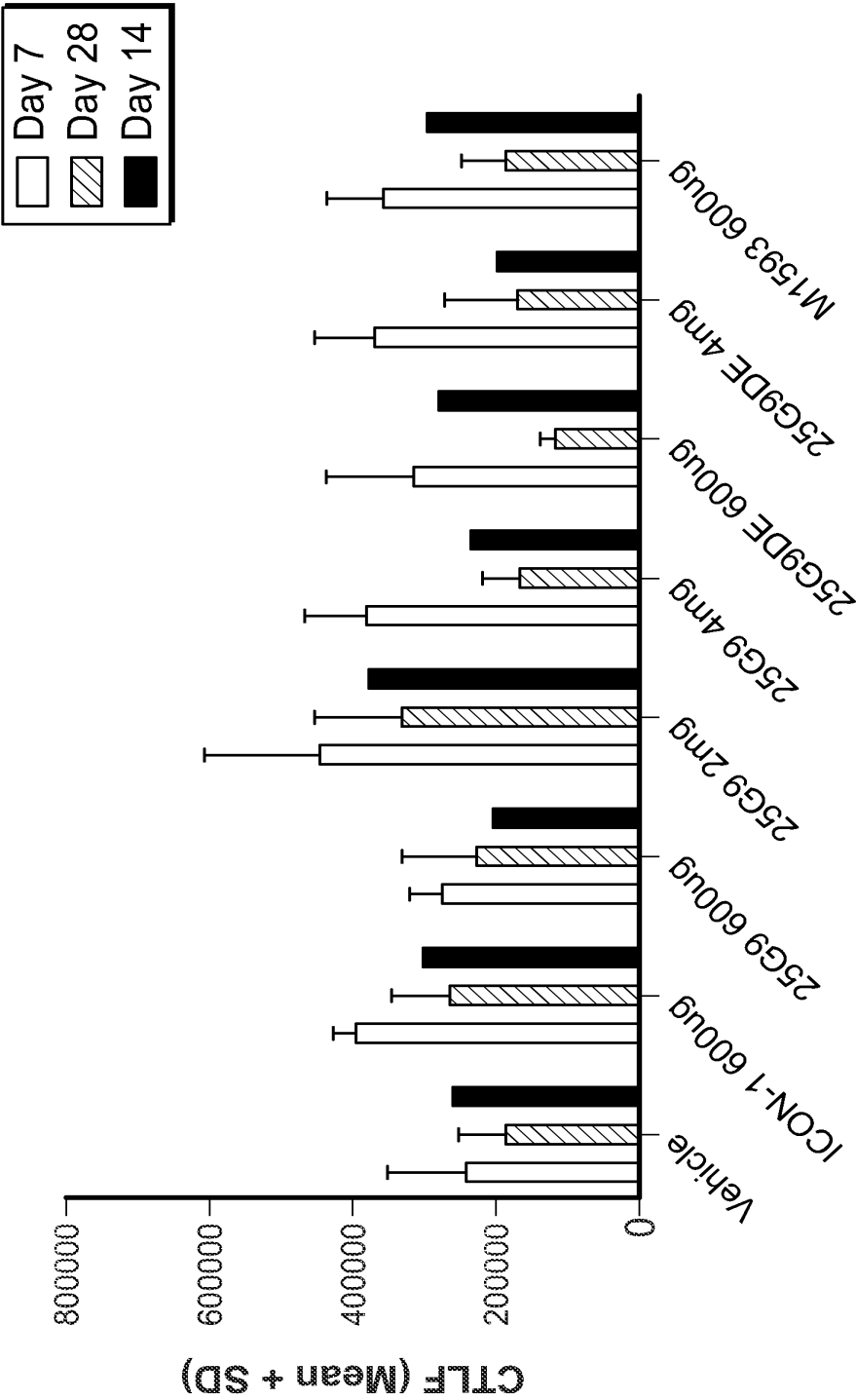


FIG. 5

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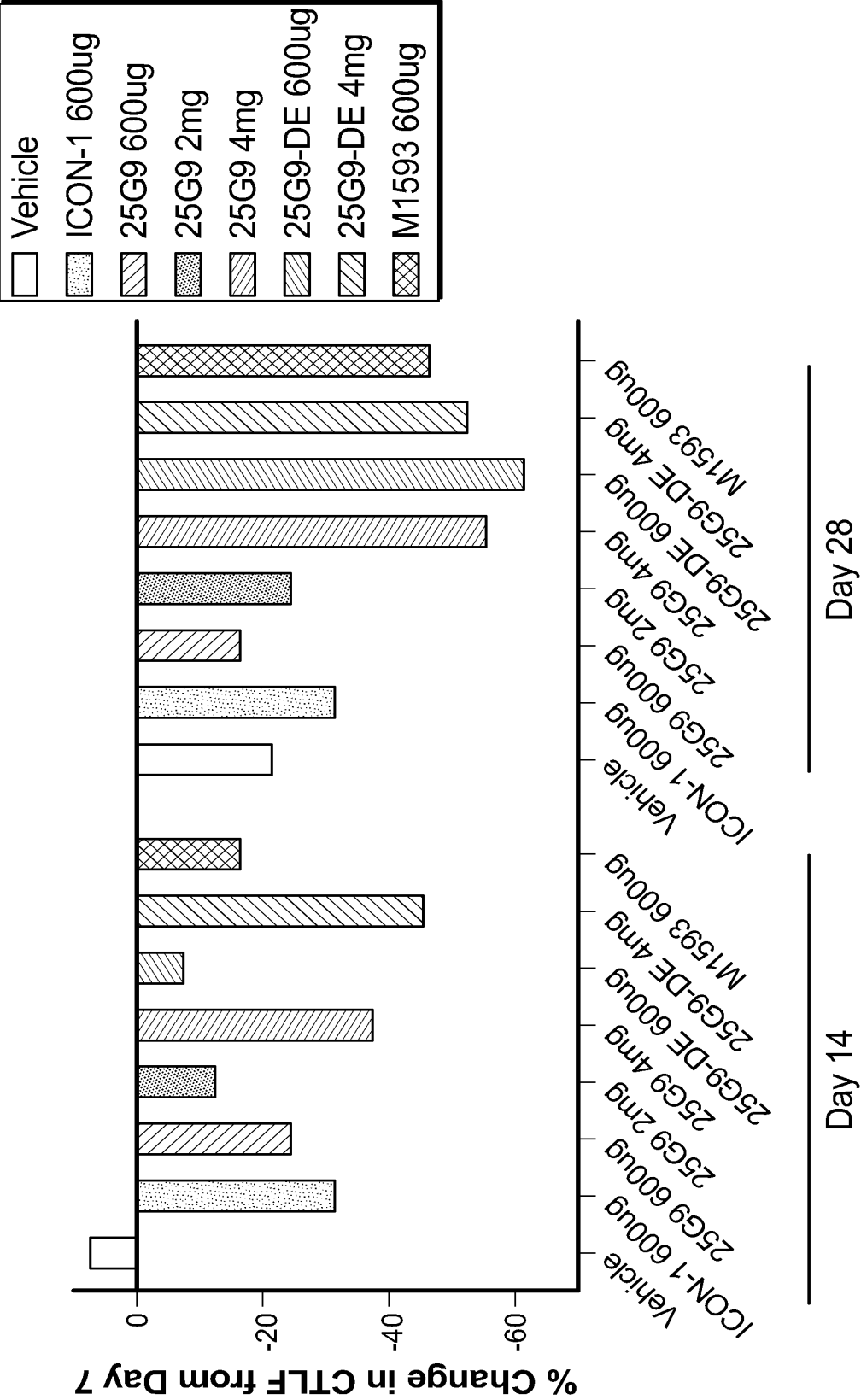


FIG. 6

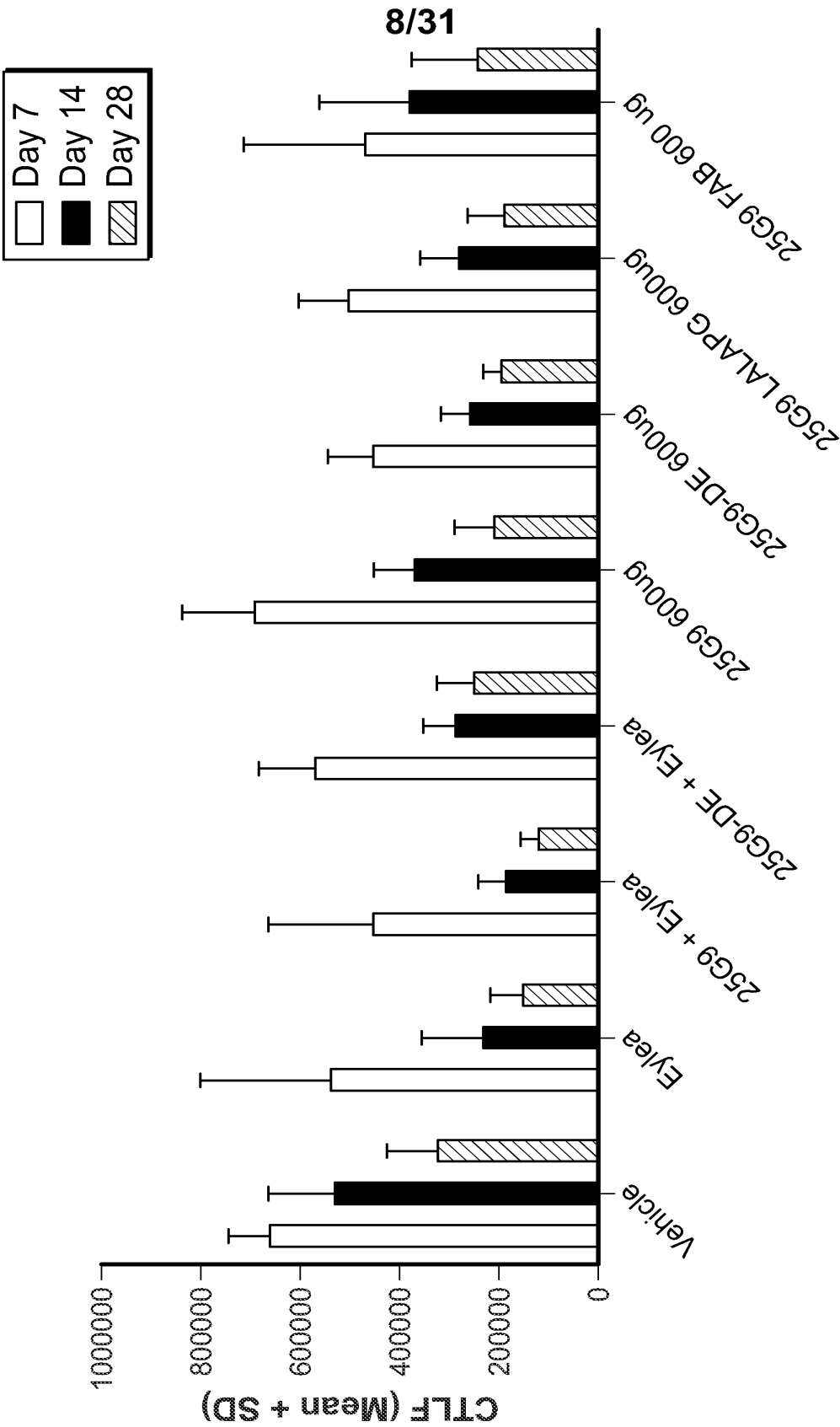


FIG. 7

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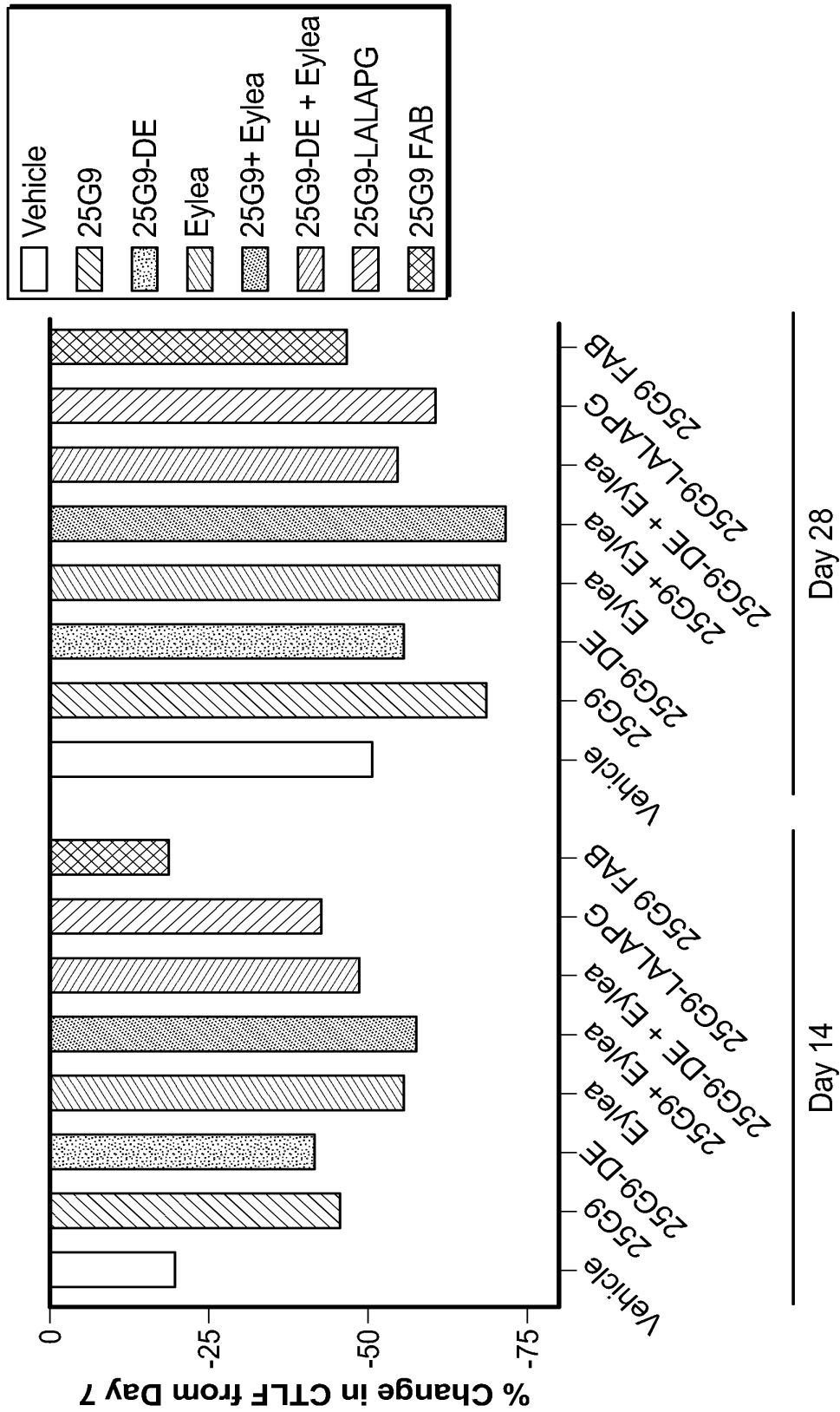


FIG. 8

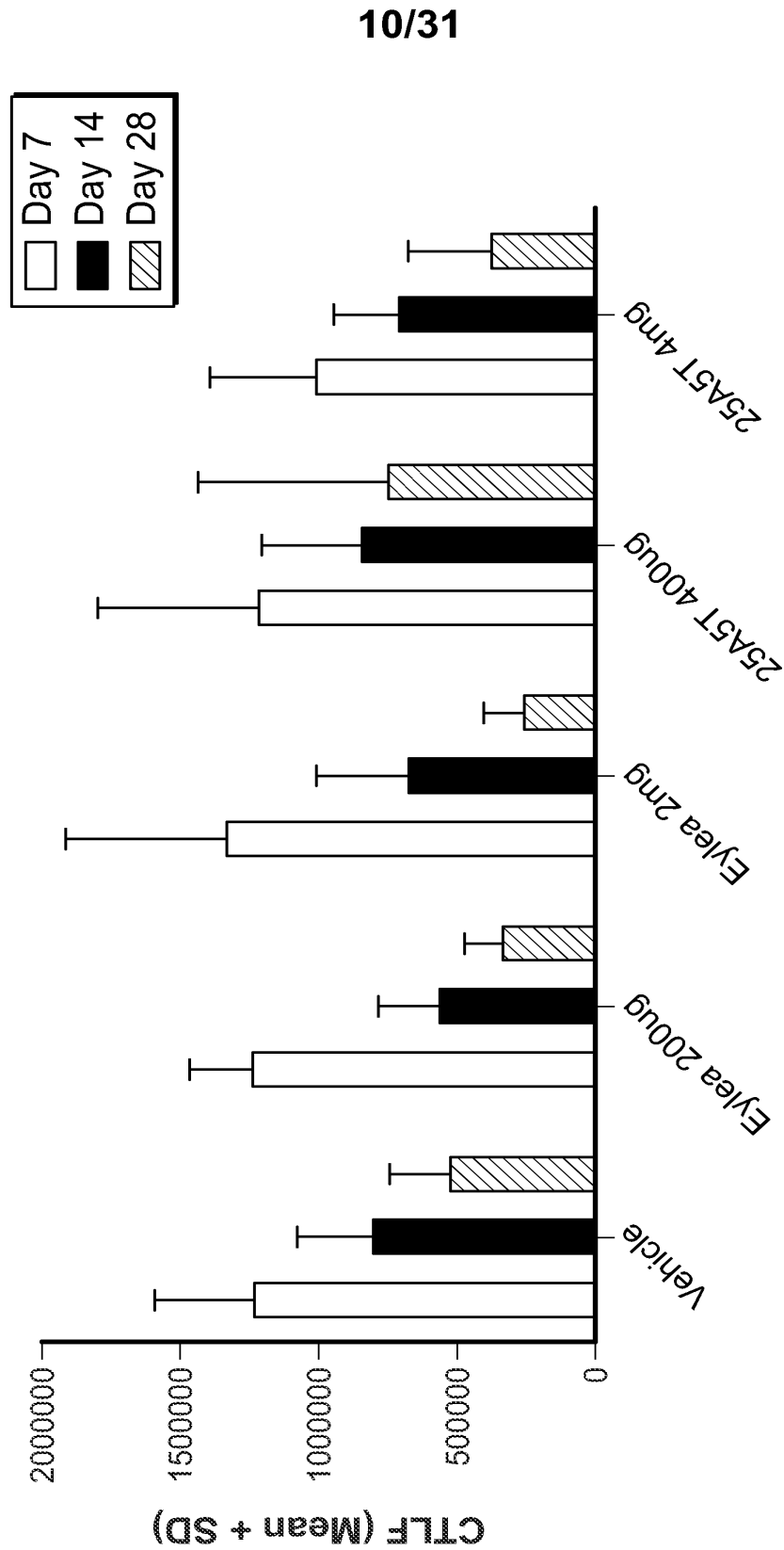


FIG. 9

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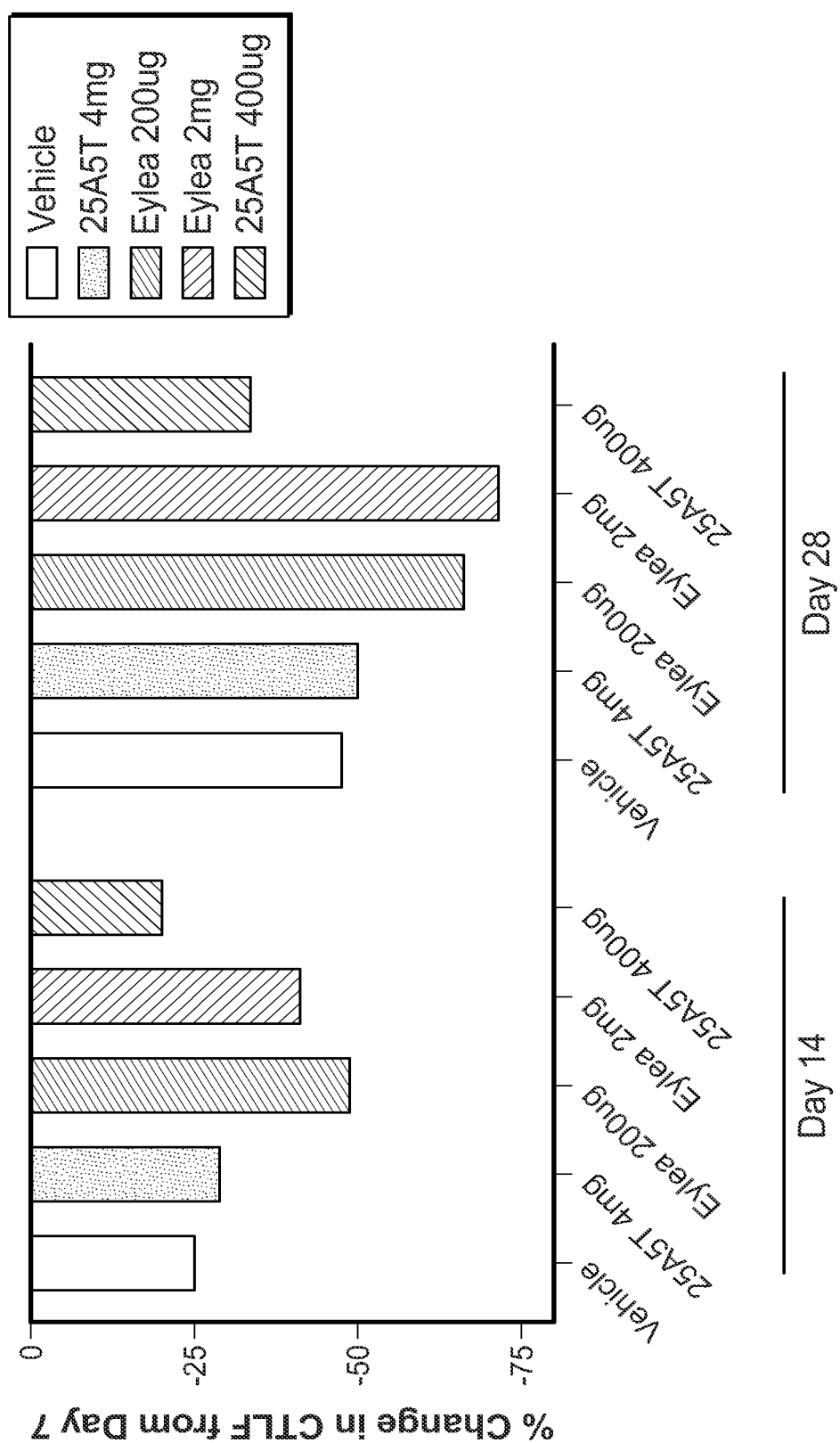


FIG. 10

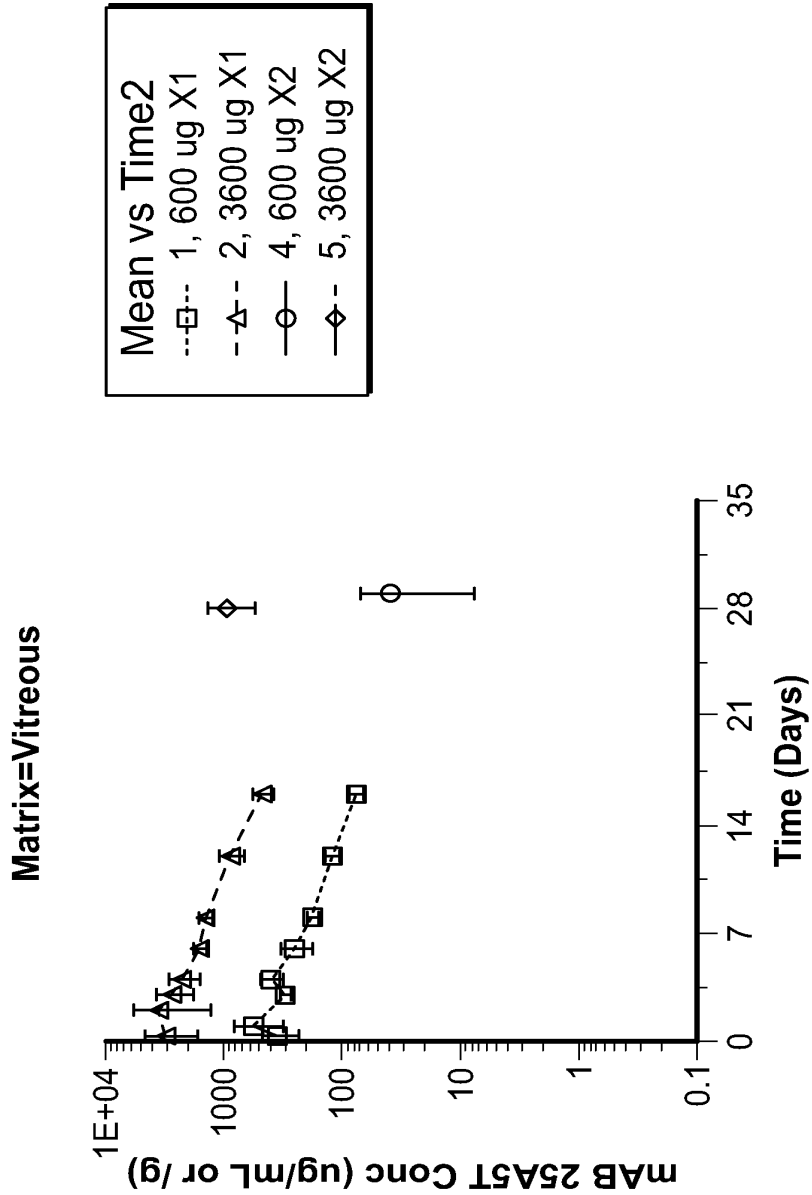


FIG. 11

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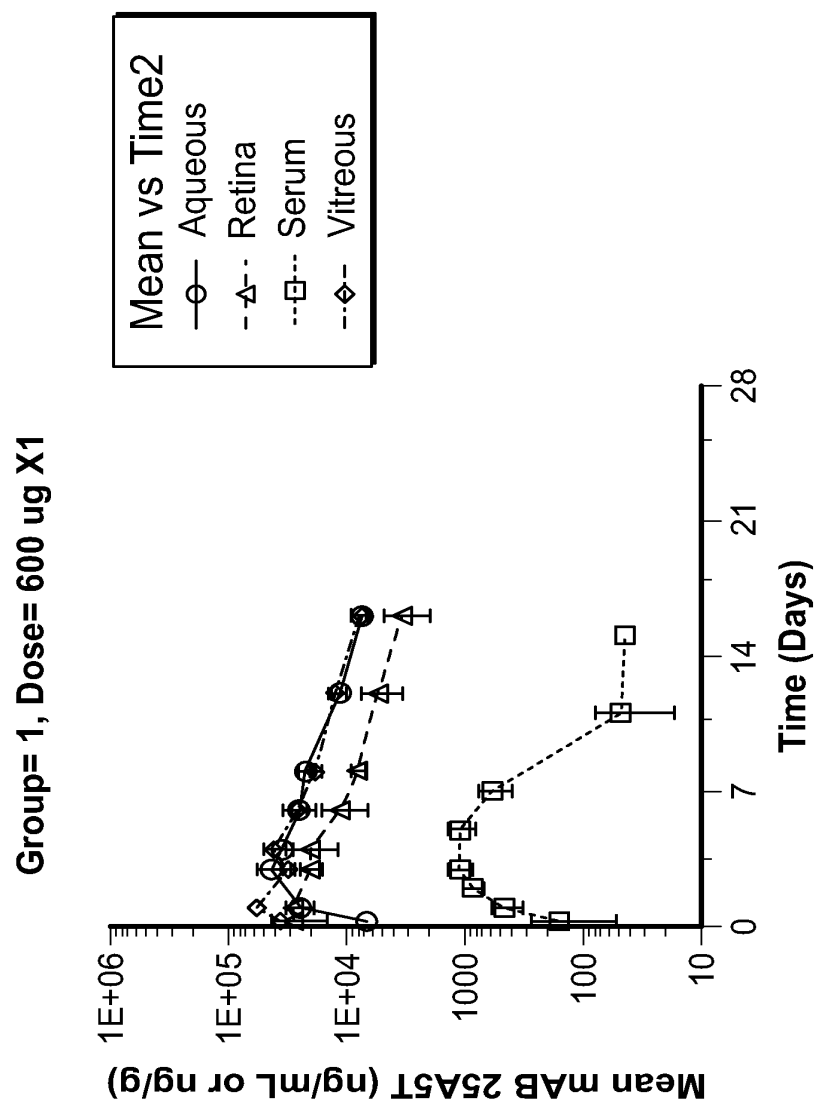


FIG. 12A

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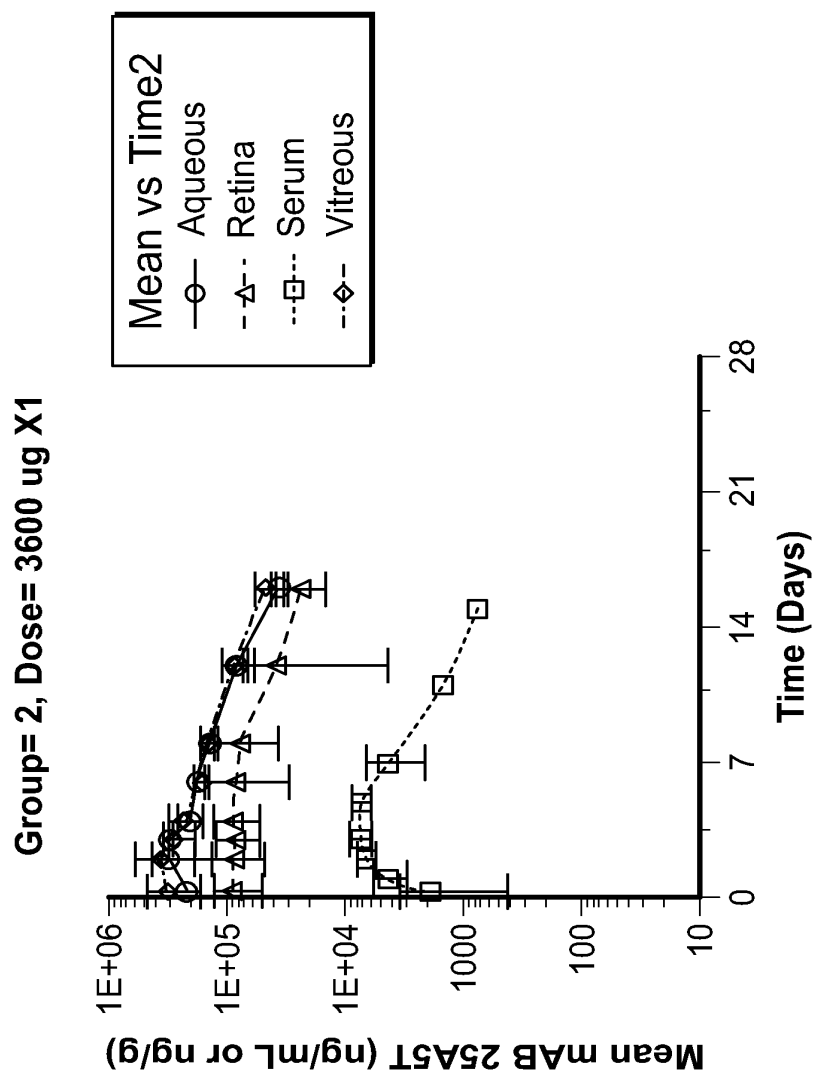


FIG. 12B

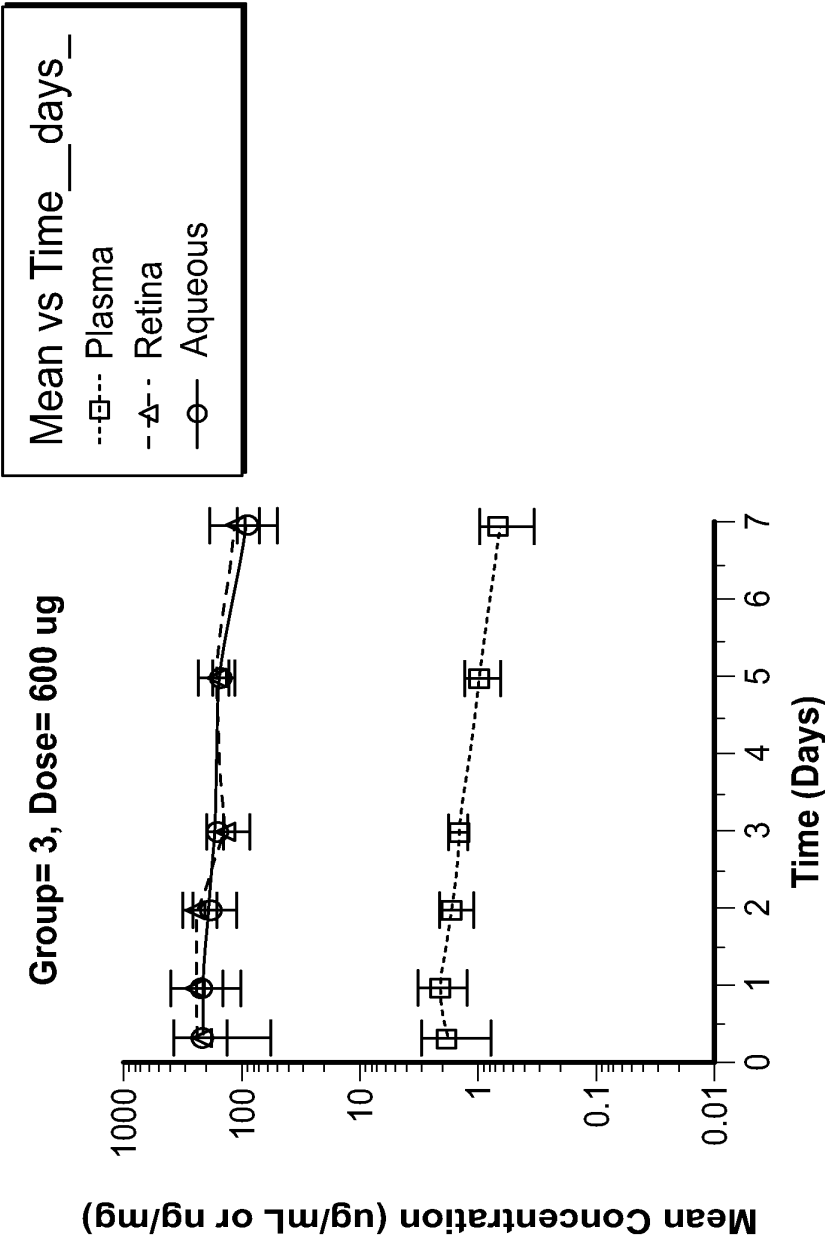


FIG. 13

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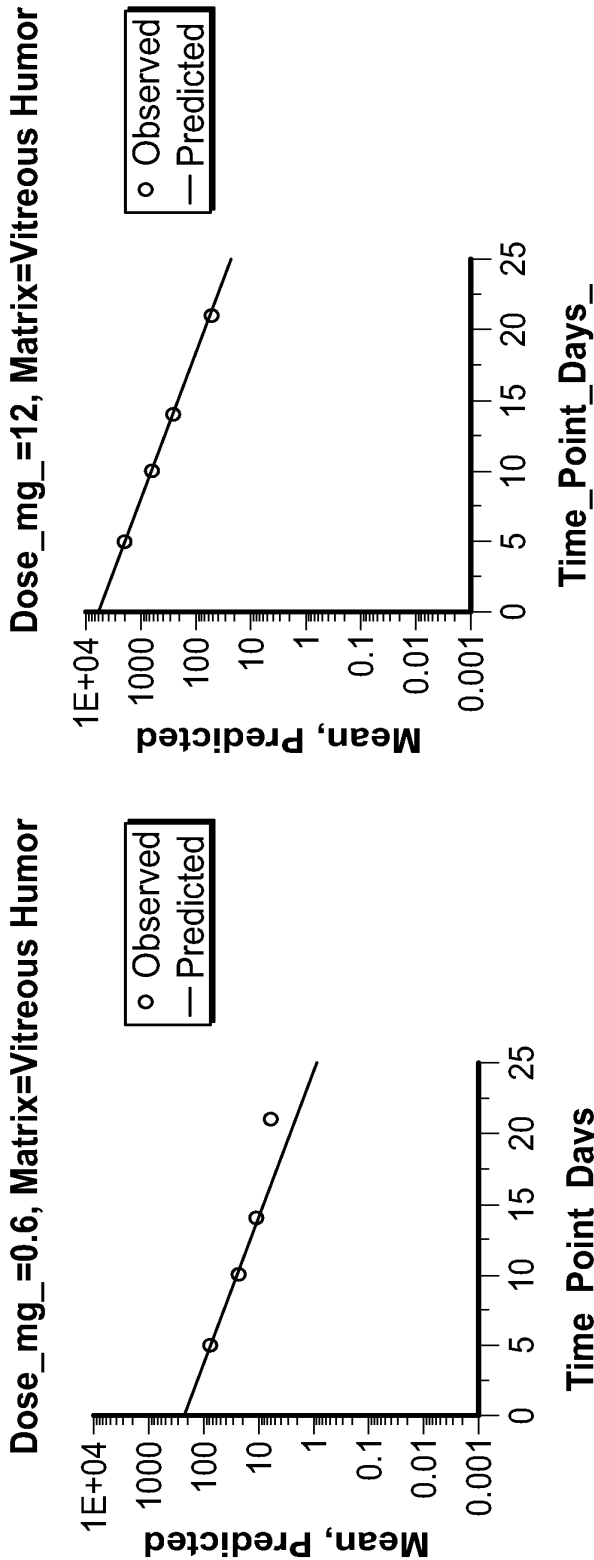


FIG. 14

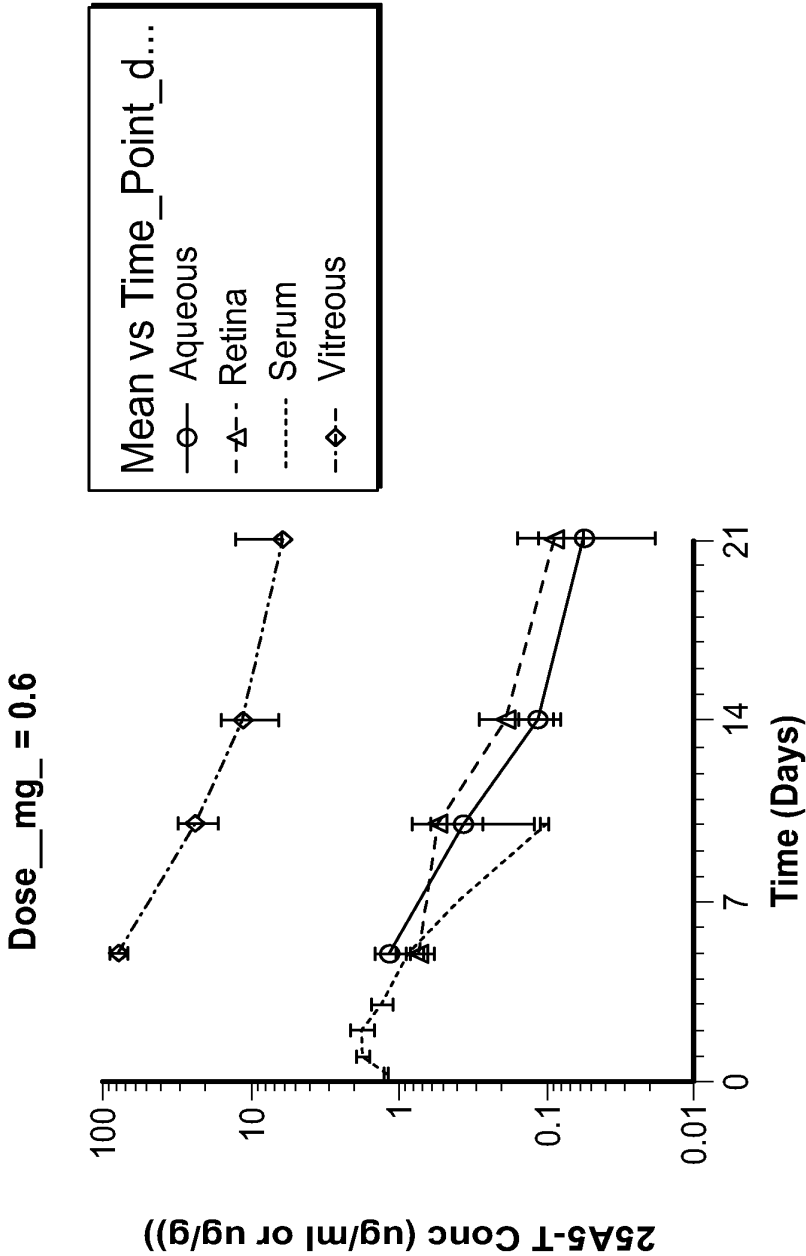


FIG. 15A

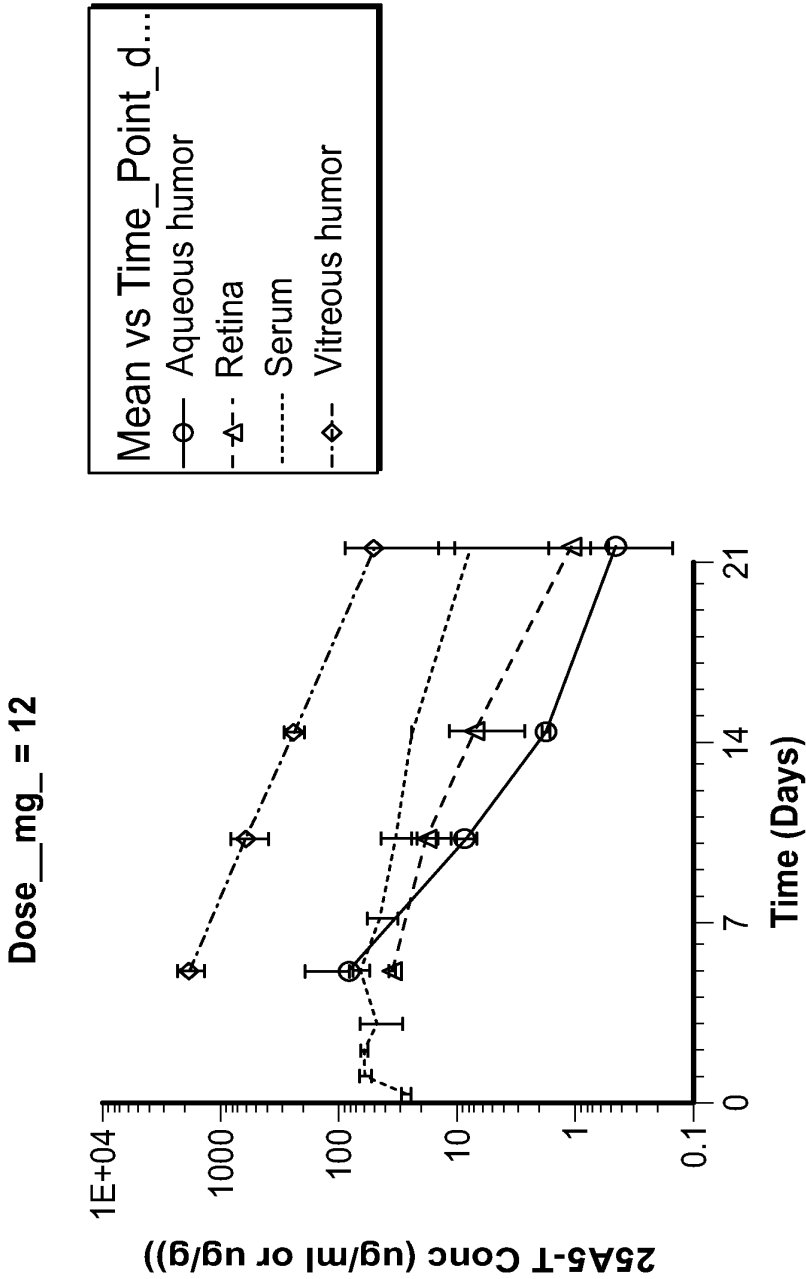


FIG. 15B

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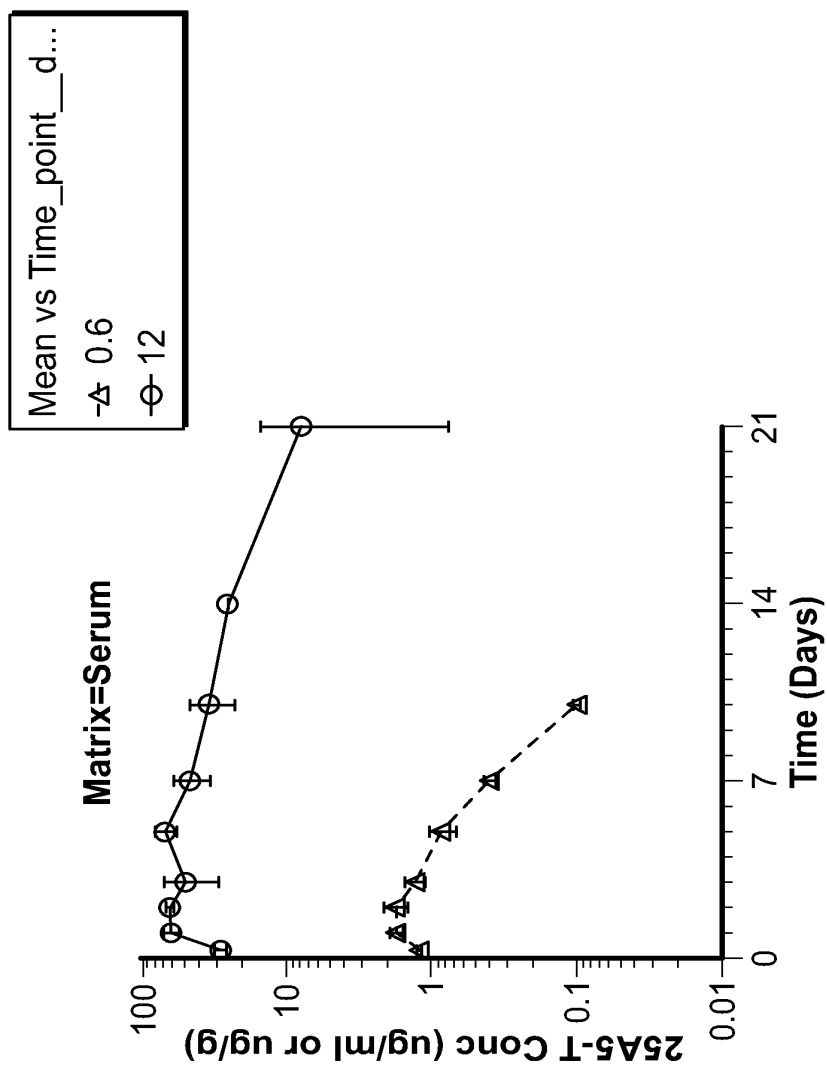


FIG. 16

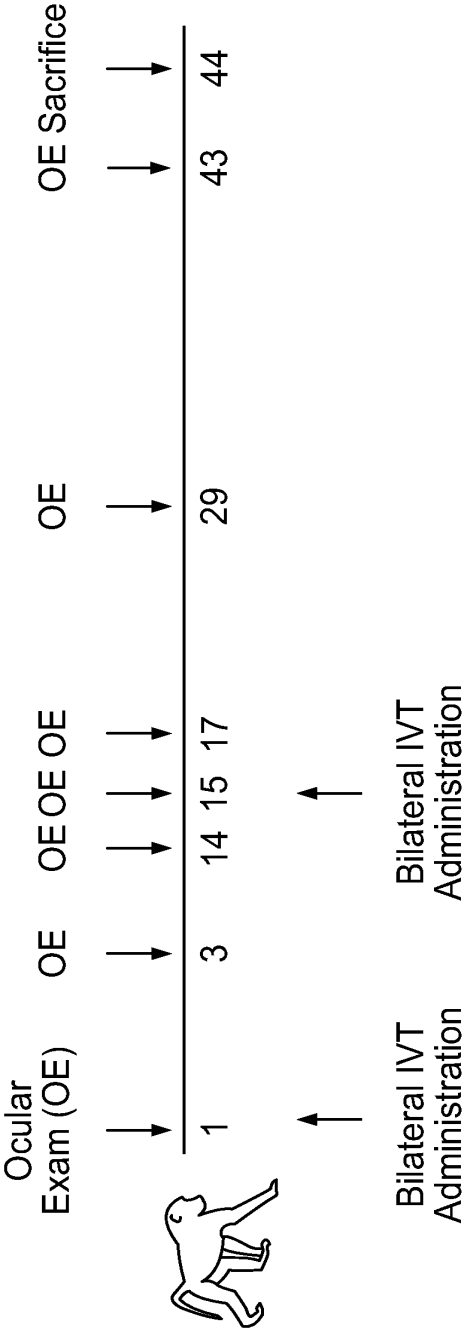


FIG. 17

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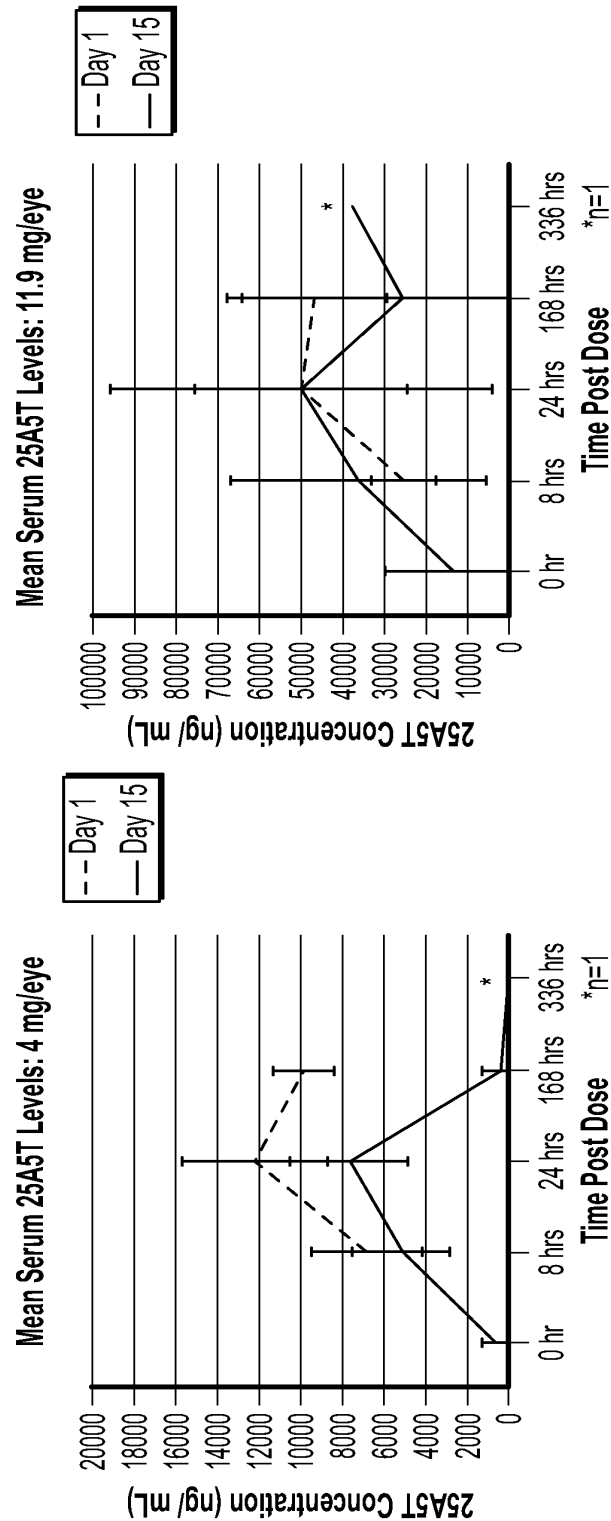


FIG. 18

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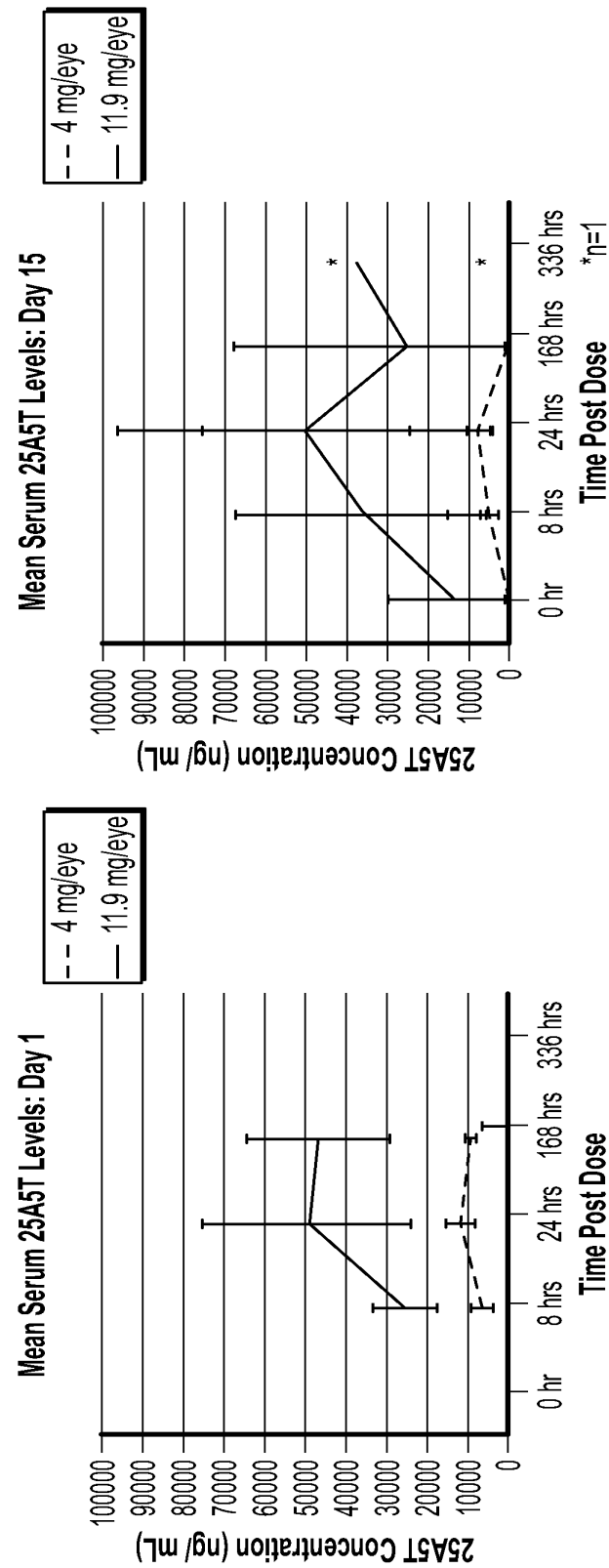


FIG. 19

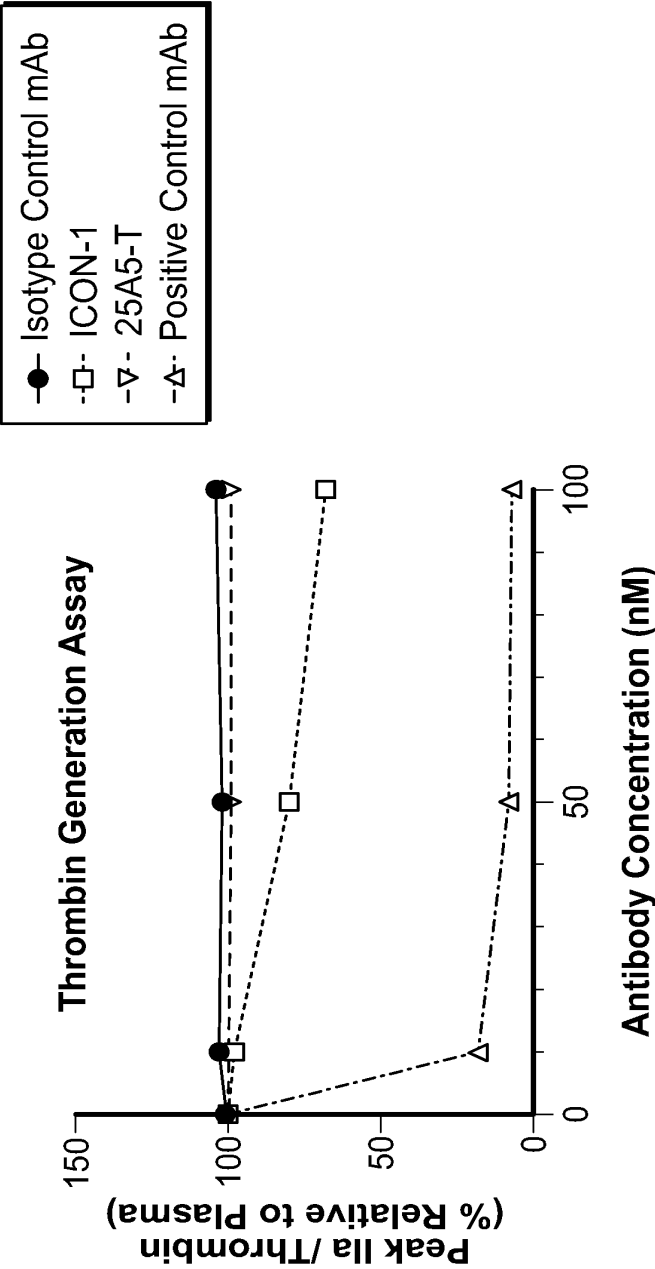


FIG. 20

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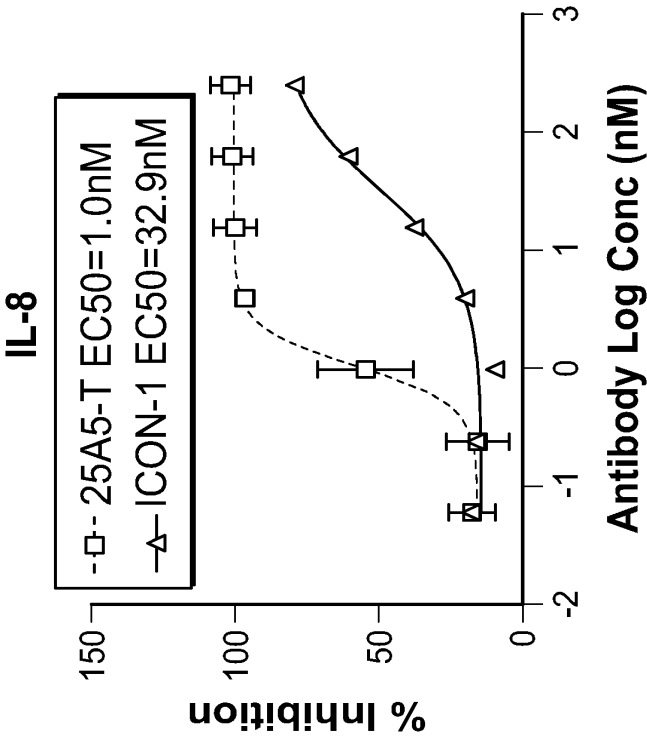


FIG. 21B

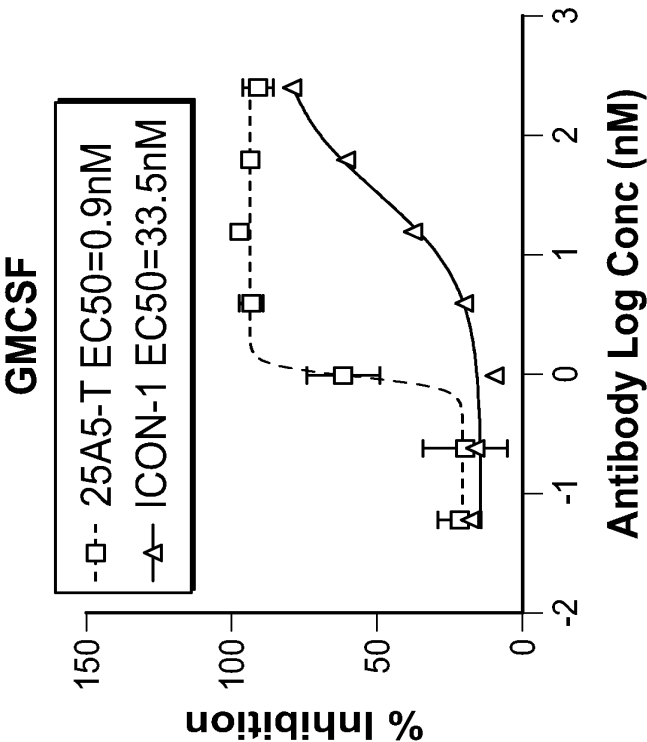


FIG. 21A

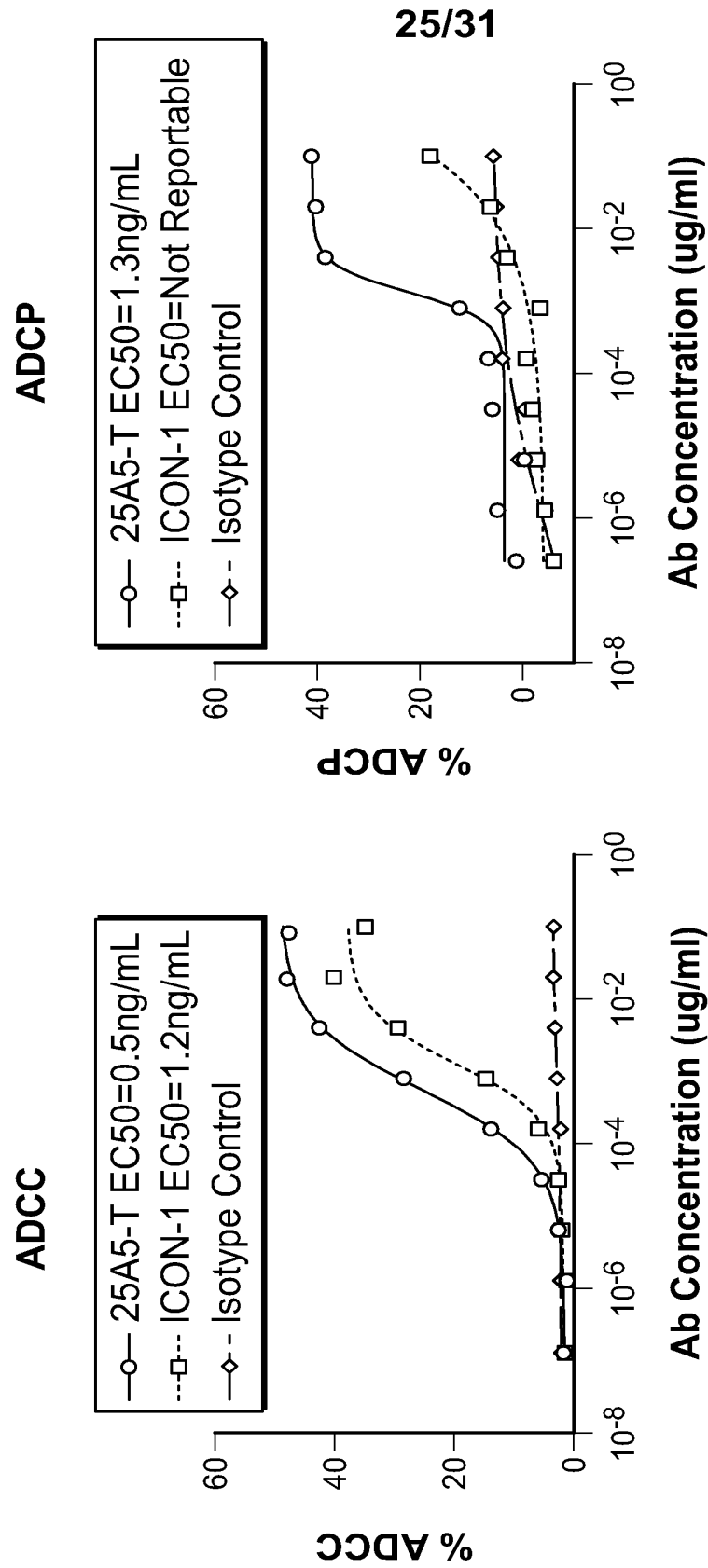


FIG. 22A

FIG. 22B

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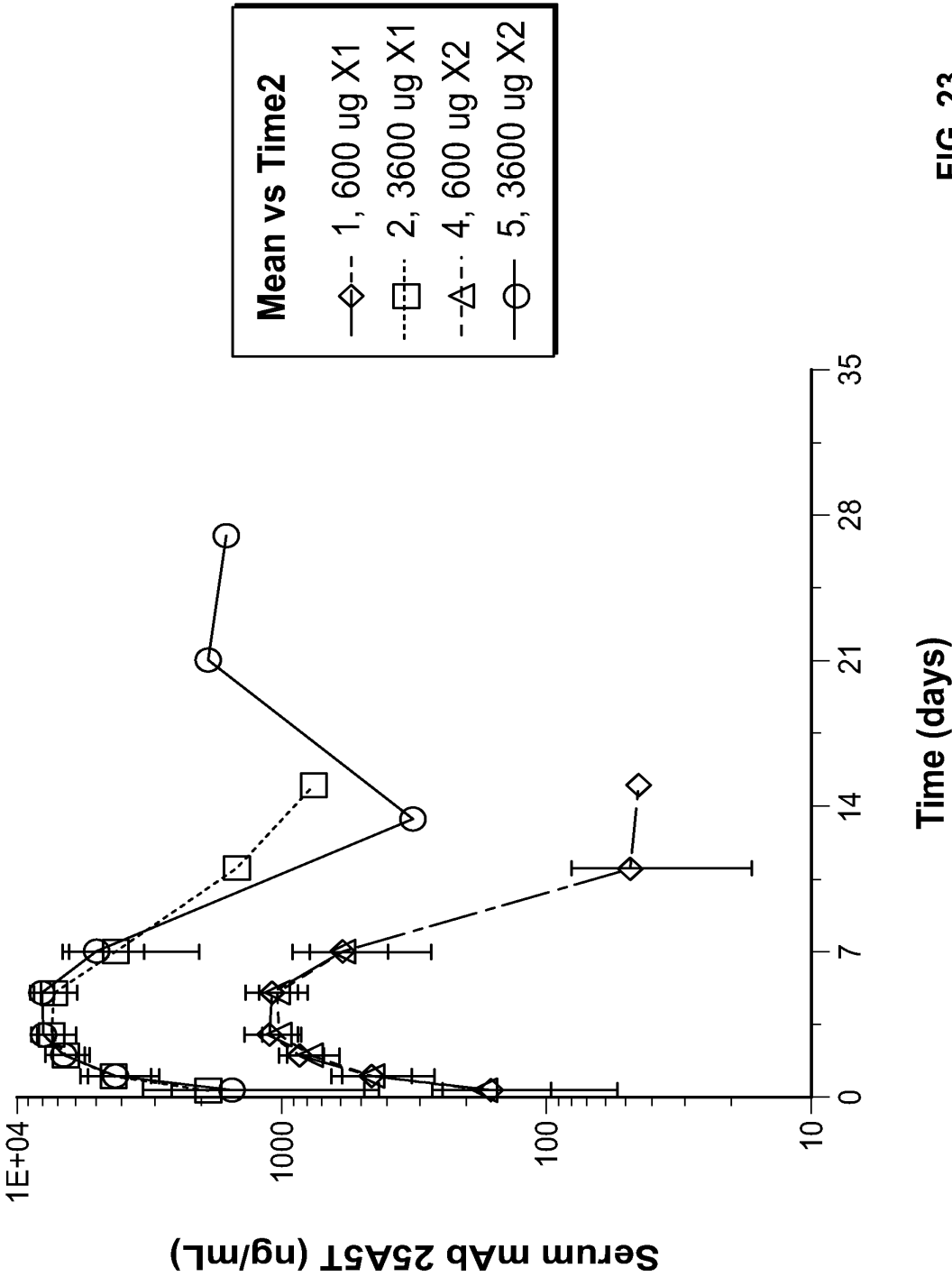


FIG. 23

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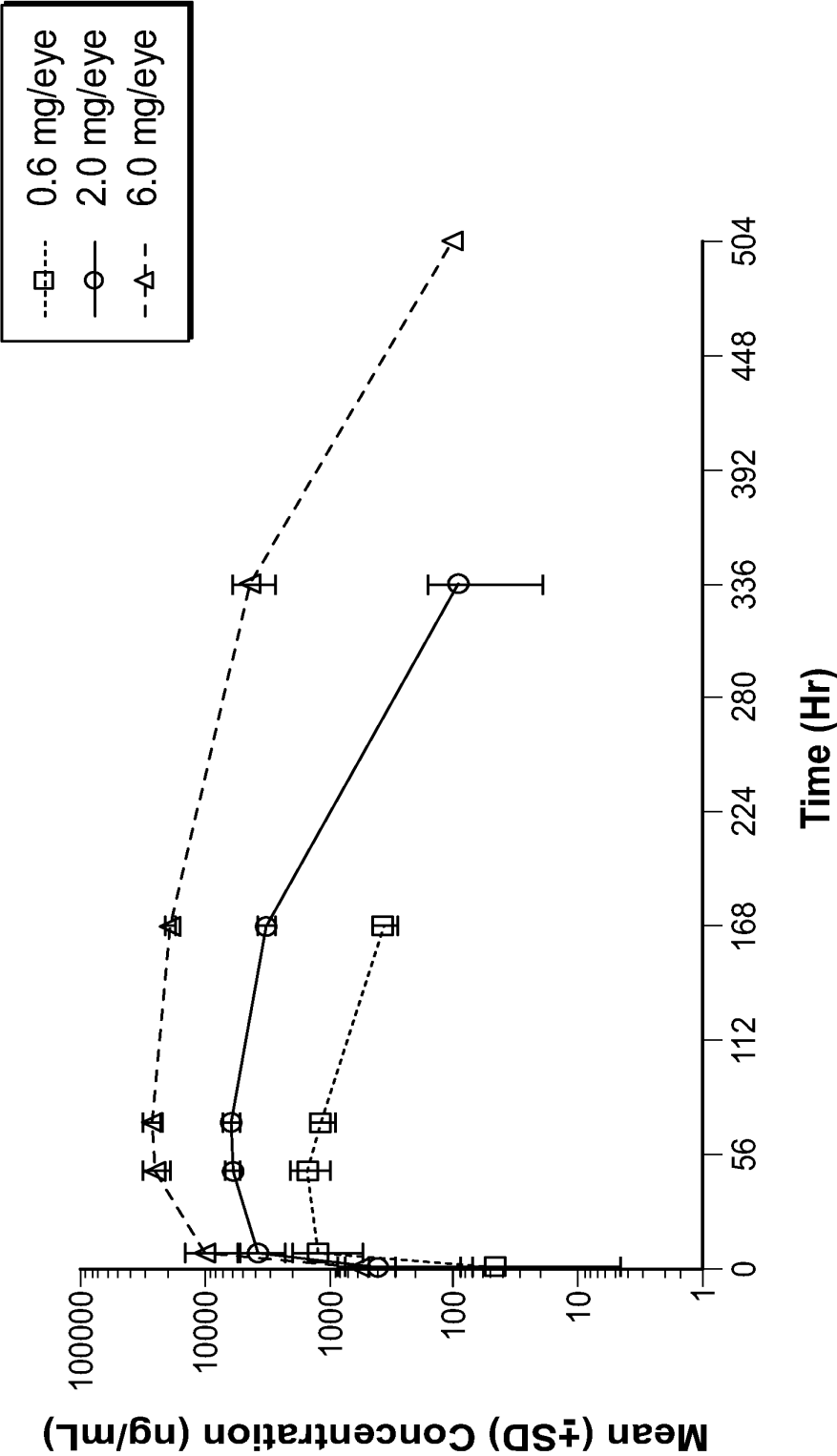


FIG. 24A

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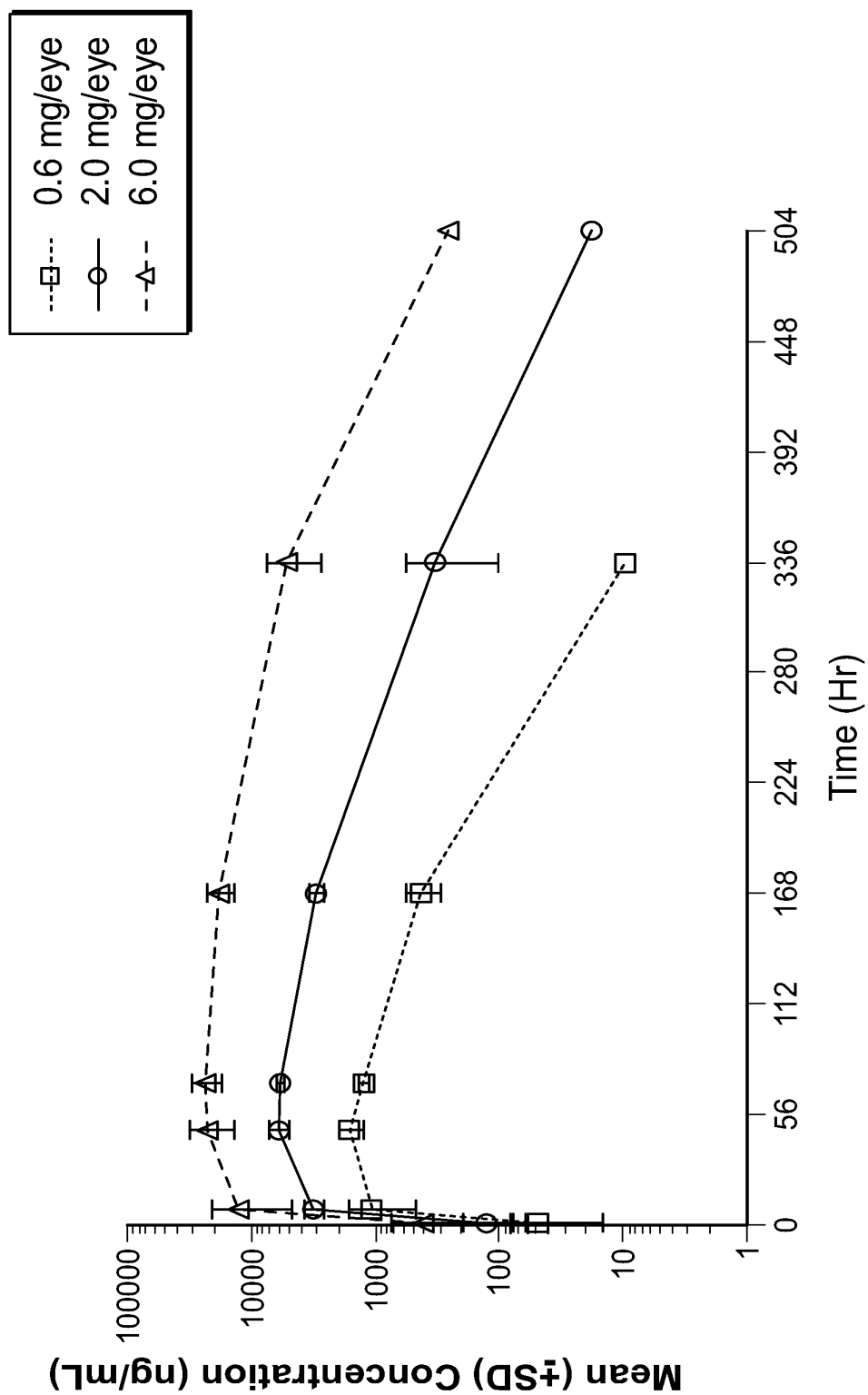


FIG. 24B

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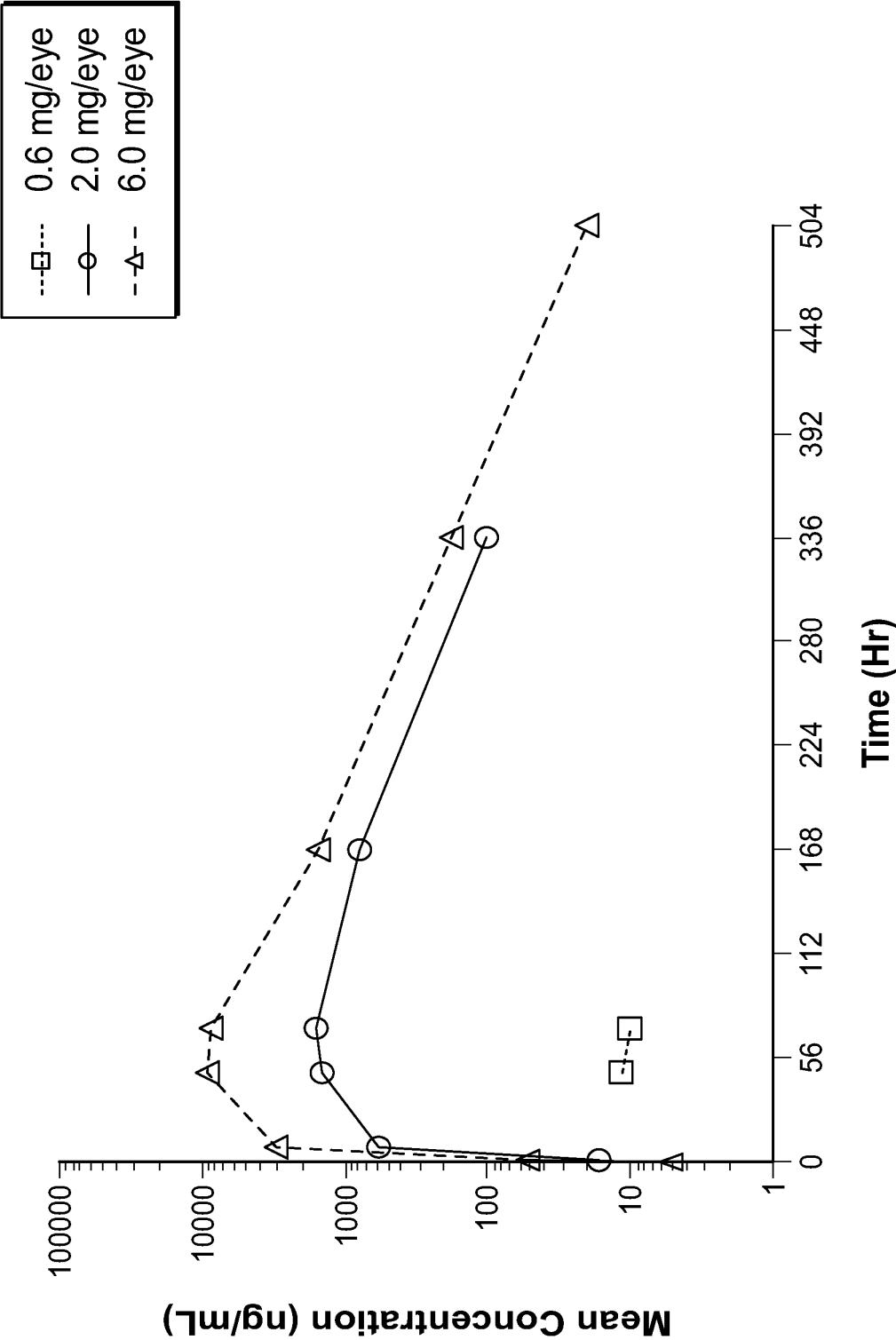


FIG. 25A

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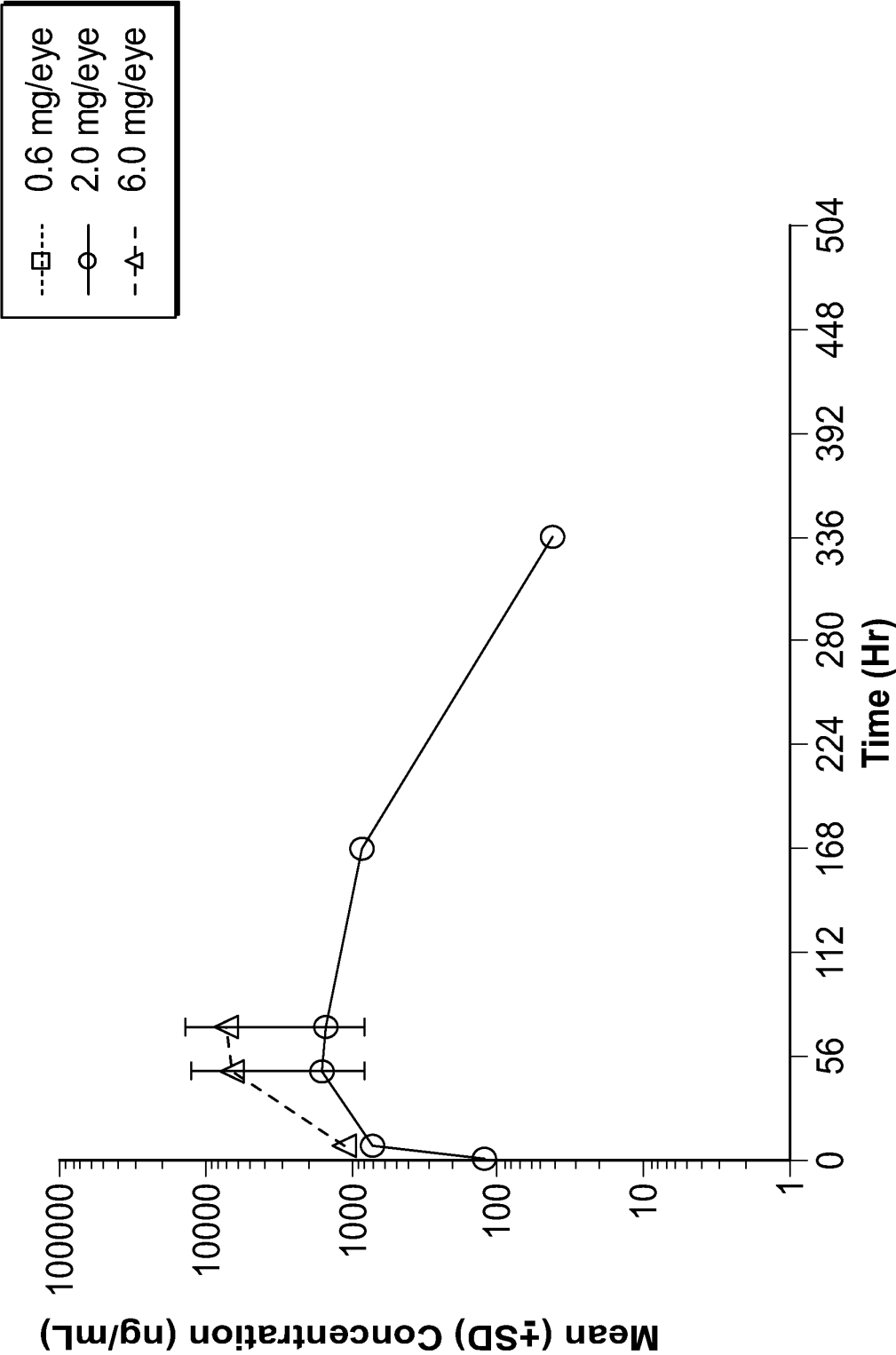


FIG. 25B

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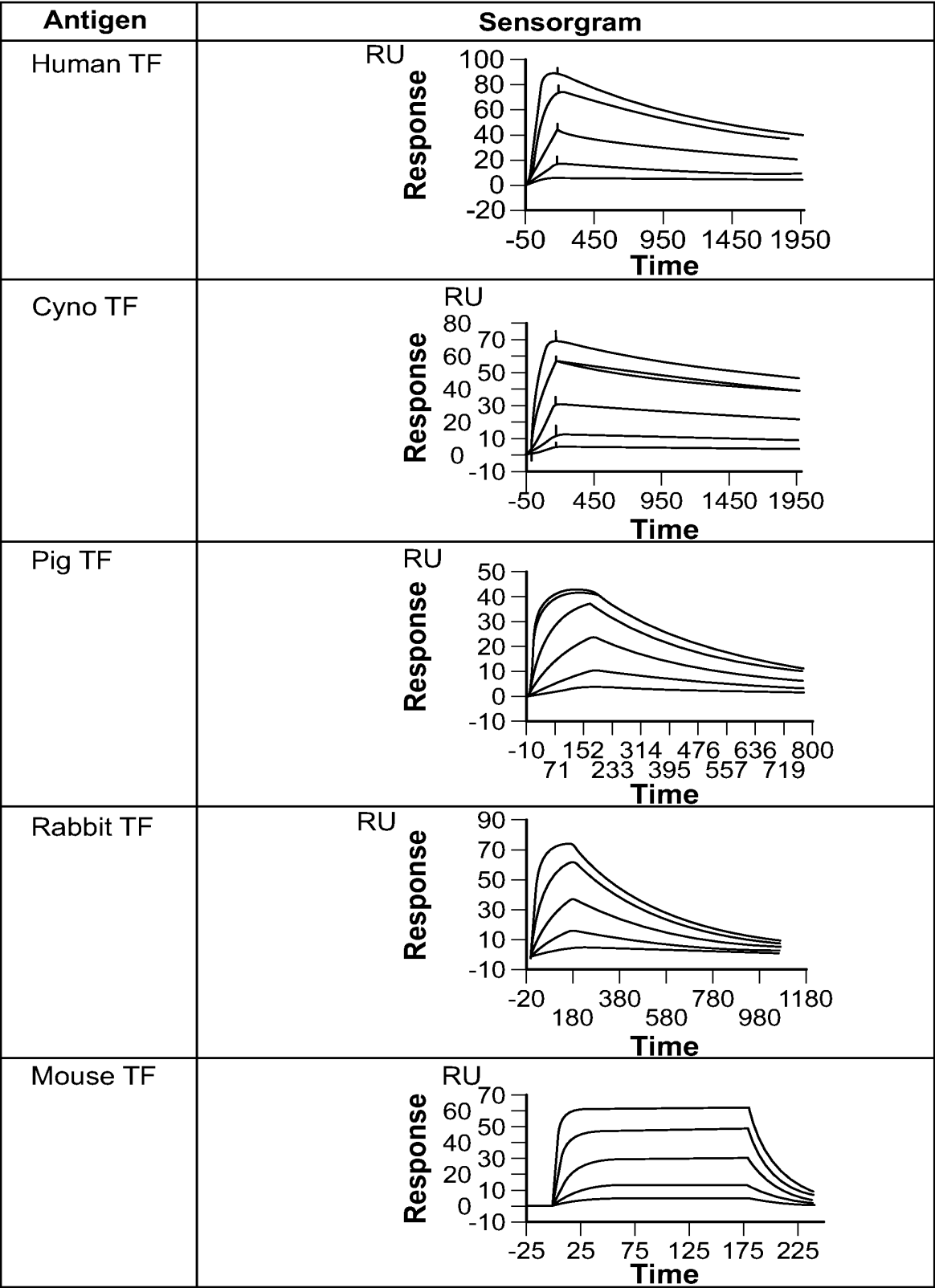


FIG. 26

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/41191

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C07K 16/38, A61P 7/04, C07K 16/40, A61P 27/02 (2021.01)

CPC - A61K 2039/505, A61P 7/00, C07K 16/36, A61K 2039/505

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2012/0237528 A1 (ALMAGRO et al.) 20 September 2012 (20.09.2012) para [0008]-[0010], [0012], [0023], [0041], [0045], [0046], [0079]	1, 6-7 ----- 2-5, 8-10
Y	US 2013/0052672 A1 (VARADI et al.) 28 February 2013 (28.02.2013) para [0009], [0010]	8-10
Y	GAUDREAU et al. Preclinical Pharmacology and Safety of ESBA1008, a Single-chain Antibody Fragment, Investigated as Potential Treatment for Age Related Macular Degeneration. Invest. Ophthalmol. Vis. Sci., March 2012; vol 53, no 14, abstract 3025, entire abstract	2-5, (8-10)/(2-5)

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

15 October 2021

Date of mailing of the international search report

DEC 02 2021

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Form PCT/ISA/210 (second sheet) (July 2019)

PGR2026-00039
Biocon Ex.1002
Page 3596 of 4945

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/41191

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/41191

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 11-144
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

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(10) International Publication Number
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20 January 2022 (20.01.2022)

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A61K 9/08 (2006.01) A61K 47/22 (2006.01)
A61K 9/19 (2006.01) C07K 16/24 (2006.01)
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BRAUN, Stefan; c/o Merck KGaA, Frankfurter Strasse 250, 64293 DARMSTADT (DE).

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(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: VISCOSITY REDUCING EXCIPIENTS AND COMBINATIONS THEREOF FOR HIGHLY CONCENTRATED PROTEIN FORMULATIONS

(57) Abstract: The present invention relates to liquid compositions and formulations comprising a protein having a reduced viscosity and/or increased stability. Furthermore, the invention relates to methods for reducing the viscosity and/or increasing the stability of a protein solution.

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**Viscosity reducing excipients and combinations thereof for highly
concentrated protein formulations**

5 Technical Field

The present invention relates to liquid compositions and formulations comprising a protein having a reduced viscosity and/or increased stability. Furthermore, the invention relates to methods for reducing the viscosity and/or increasing the stability
10 of a protein solution.

Background

15 Since the FDA authorized the first biopharmaceutical in 1982, many other biologic drugs have followed suit. Most of these are monoclonal antibodies (mAB) or related formats such as bi-specific antibodies or antibody fragments. While these drugs offer unique opportunities in terms of efficacy, their structure and size pose various challenges.

20 Antibodies and other protein therapeutics are usually administered parenterally, for example by intravenous (iv), intramuscular (im) or subcutaneous (sc) route. Subcutaneous injection is particularly popular for the delivery of protein therapeutics due to its potential to simplify patient administration (fast, low-volume injection) and reduce treatment costs (shorter medical assistance). To ensure patient compliance,
25 it is desirable that subcutaneous injection dosage forms be isotonic and can be injected in small volumes (< 2.0 ml per injection site). To reduce the injection volume, proteins are often administered with a concentration of 1 mg/ml to 150 mg/ml.

30 At the same time, mAb-based therapies usually require several mg/kg dosing. The combination of high therapeutic dose and low injection volume thus leads to a need for highly concentrated formulations of therapeutic antibodies. However, being large proteins, antibodies possess a multitude of functional groups in addition to a complex three-dimensional structure. This makes their formulation difficult, particularly when a high concentration is required.

5 One of the main problems with high concentration protein solutions is viscosity. At high concentrations, proteins tend to form highly viscous solutions largely due to non-native self-association. Additionally, proteins show an increased rate of aggregation and particle formation at such high concentrations.

10 These problems concern both the manufacturing process and the administration to the patient. In the manufacturing process, highly concentrated protein formulations that are highly viscous present particular difficulties for ultrafiltration and sterile filtration. In addition, tangential flow filtration is often used for the buffer exchange and for the increase of protein concentration. However, because viscous solutions show an increased back pressure and shear stress during injection and filtration, the therapeutic protein is potentially destabilized and/or process times are prolonged. Said increased shear stress frequently results in a loss of product. Both
15 aspects adversely affect process economics.

At the same time, high viscosity is unacceptable when it comes to administration as it significantly limits the injectability of the protein.

20 To solve these problems and/or to improve the stability of the solution, additives and excipients such as sucrose and sodium chloride are usually added in higher concentrations to biopharmaceutical formulations. However, the resulting solutions often cause pain due to high injection forces and resulting tissue damages. Some of these solutions may even be no longer administrable resulting in a lack of
25 therapeutic options for the patient.

As an alternative, different excipients such as salts, camphor-10-sulfonic acid and specific amino acids, e.g. arginine, histidine, lysine and proline, have been explored as a way of reducing the viscosity of certain high-concentration protein therapeutics.
30 Guo et al. suggest that salts having hydrophobic, bulky, and aliphatic ionic constituents may act as potent viscosity-lowering excipients (Guo Z. et al., *Pharmaceutical Research*; 2012, 29(11):3102-9). Furthermore, WO 02/30463, WO 15/196091, WO 15/196187, WO 17/070501 and WO 19/201904 disclose different viscosity reducing excipients.

5 However, formulating proteins like monoclonal antibodies requires a careful selection of formulation additives and/or excipients to avoid protein denaturation and loss of biological activity. In addition, the excipients need to be pharmaceutically safe and physiologically compatible so to avoid any undesired side effects such as allergic reactions.

10 Consequently, the pharmaceutical industry has a strong need for additional pharmaceutically acceptable, viscosity-reducing excipients, especially as an alternative when standard solutions based on NaCl and amino acids such as mentioned above fail.

15 The problem to be solved is therefore the provision of excipients that can effectively reduce the viscosity of a protein solution and/or increase stability thereof. Furthermore, the problem to be solved is the provision of excipient combinations that can effectively reduce the viscosity of a protein solution and/or increase stability thereof. Yet another problem to be solved is that many viscosity reducing excipients used at relevant concentrations can adversely affect protein stability.

20 During the bioprocess, the solutions have to be pumped through tubing and chromatography columns. At high viscosities, the flow rate through such columns is limited by said viscosity which leads to longer processing times, significant protein losses during chromatography or might lead to complete non-processability of the protein solution. Furthermore, when passing through a connector from the narrow
25 tube into a less narrow column, shear forces may occur. Shear stress is a typical reason for proteins to denature and potentially to aggregate and thereby reducing the yield of the process. Obviously, such shear stress induced aggregation has an adverse effect on process economics. Moreover, the gel bed within the chromatography column may be damaged by the high pressure.

30 Additionally, some proteins are formulated into high concentration through tangential flow filtration (TFF). When the viscosity of the solution becomes critical, a gel-like layer may be formed near the membrane. Especially the membrane flux is significantly reduced yielding to an increased processing time and therefore to

significant higher manufacturing costs. As discussed before, also during TFF shear stress may occur yielding to insoluble protein aggregates and a reduced yield.

5 Generally, it has been observed that highly viscous solutions develop a certain stickiness making it difficult to recover the complete solution from containers, out of tubing or to remove the entire substance from processing systems. This loss of substance leads to a significantly reduced product yield with the obvious adverse effect on process economics.

10 Furthermore, a problem to be solved is the provision of excipient combinations that can effectively reduce the viscosity of a protein solution.

15 Yet another problem to be solved is that many viscosity reducing excipients used at relevant concentrations can adversely affect protein stability. Hence a further problem to be solved is the provision of excipient combinations that can effectively reduce the viscosity of a protein solution and that show an improved protein stability compared to one viscosity reducing excipient alone used in a higher concentration that results in a similar viscosity reduction compared to the combination.

20 High viscosity of protein solutions causes numerous difficulties in bioprocessing. Since known additives which hitherto are used for reducing the viscosity in corresponding protein solutions do not lead to sufficient viscosity-reducing effects in many cases, it is an object of the present invention to find new possibilities whereby corresponding viscosity-lowering effects can be improved and adverse effects on
25 process economics can be reduced.

30 An additional subject of the present invention is a method for reducing the viscosity of liquid protein compositions in bioprocess, comprising the step of combining the liquid protein composition with at least one first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid or salts thereof, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol.

Summary of the invention

5 The problem is solved by a liquid composition comprising a protein and at least one first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol. The problem is solved by a liquid composition comprising a protein and at least one first viscosity reducing excipient as described above and at least one second viscosity reducing excipient, preferably selected from 10 the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.

15 The use of at least two viscosity reducing excipients allows to overcome the problem that many viscosity reducing excipients used at relevant concentrations can adversely affect protein stability by allowing for the use of a lower amount of each individual excipient and leveraging the stabilizing effect of a second viscosity reducing excipients.

20 Likewise, the problem is solved by a method for reducing the viscosity of a protein solution, comprising a step of adding at least one first excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol to the protein solution. The problem is solved by a method for reducing the viscosity of a protein solution, comprising a step 25 of adding at least one further second excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.

30 The problem is also solved by a method for increasing the stability of a protein solution, comprising a step of adding at least one first excipient is selected from the group consisting of valine, leucine, ascorbic acid, cyanocobalamin and proline to the solution.

- The problem is further solved by a composition comprising a protein and a viscosity-reducing solution comprising at least one first excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol. The problem is solved by a composition comprising a protein and at least one further second excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.
- 5
- 10 Furthermore, the problem is solved by a liquid formulation of a protein, especially a therapeutic protein, comprising at least one first excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol. The problem is solved by a liquid
- 15 formulation of a protein, especially a therapeutic protein, comprising at least one further second excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.
- 20 Furthermore, the problem is solved by a lyophilized protein formulation of a composition comprising a protein and at least one first excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol. The problem is solved by a lyophilized
- 25 protein formulation of a composition comprising a protein and at least one further second excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.
- 30 Furthermore, the problem is solved by a kit comprising a composition comprising a protein and at least one first excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol. The problem is solved by a kit comprising a

composition comprising a protein and at least one further second excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.

5

An additional subject of the present invention is a method for reducing the viscosity of liquid protein compositions in a bioprocess, comprising the step of combining the liquid protein composition with at least one first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol. Preferably the liquid protein composition further comprises a second viscosity reducing excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.

10

15

Detailed description of the invention

The invention is directed to a pharmaceutical composition or liquid formulation comprising a protein and at least one first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol.

20

A "protein" is herein defined as a polymer of amino acids linked to each other by peptide bonds to form a polypeptide. Proteins can be naturally occurring or non-naturally occurring, synthetic, or semisynthetic. The term "protein" is understood to also cover peptides, oligopeptides, polypeptides and any therapeutic protein as defined below. Preferably the "protein" has a length sufficient to form a detectable tertiary structure.

25

30

Without wanting to be bound by a mechanism, it is believed that the viscosity reducing effect of the compositions and formulations according to the invention is based upon an interaction between the excipients and the amino acid residues of the protein. Because all proteins are built from the same pool of amino acids, the

effects described herein are thus applicable to all proteins. The compositions and formulations according to the invention therefore have an advantageous effect on any protein irrespective of its sequence, size and structure.

- 5 The invention is also directed to liquid formulations of therapeutic proteins comprising cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine hydrochloride, thiamine, thiamine monophosphate, thiamine pyrophosphate, paracetamol, guanidine hydrochloride and quinine hydrochloride. The formulations according to the invention show a reduced viscosity and increased stability of the
10 respective protein.

The term "liquid composition" as used herein, refers to a aqueous protein solution at least containing a viscosity reducing excipient.

- 15 The term "liquid formulation" as used herein, refers to a liquid composition for therapeutic use, wherein the protein is a therapeutic protein that is either supplied in an acceptable pharmaceutical diluent or one that is reconstituted in an acceptable pharmaceutical diluent prior to administration to the patient.

- 20 In a preferred embodiment, the protein contained in the compositions and formulations according to the invention is a therapeutic protein.

- 25 The term "therapeutic proteins" as used herein refers to any protein or polypeptide that is administered to a subject with the aim of treating or preventing a disease or medical condition. In particular, the subject may be a mammal or a human. Therapeutic proteins can be administered for different purposes, such as replacing a protein that is deficient or abnormal, augmenting an existing pathway, providing a novel function or activity, interfering with a molecule or organism and delivering other compounds or proteins, such as a radionuclide, cytotoxic drug, or effector
30 proteins. Therapeutic proteins encompass antibody-based drugs, Fc fusion proteins, anticoagulants, blood factors, bone morphogenetic proteins, engineered protein scaffolds, enzymes, growth factors, hormones, interferons, interleukins, antibody drug conjugates (ADCs) and thrombolytics. Therapeutic proteins can be

naturally occurring proteins or recombinant proteins. Their sequence can be natural or engineered.

5 In a particularly preferred embodiment, the protein in the compositions and formulations according to the invention is an antibody, in particular a therapeutic antibody.

10 In a further particularly preferred embodiment, the protein in the compositions and formulations according to the invention is a plasma derived protein, in particular IgG or hyperIgG. Some pharmaceutical formulations containing plasma proteins comprise of mixtures of different plasma proteins.

15 The term "plasma derived proteins" herein refers to a protein derived from the blood plasma of a donor by plasma fractionation. Said donor can be human or non-human. One example for plasma proteins are immune globulines.

20 The term "IgG" herein refers to an Immune globbuline type G. The term "IgM" herein refers to an Immune globbuline type M. The term "IgA" herein refers to an Immune globbuline type A.

The term "hyper-IgG" herein refers to a formulation of IgGs purified from a donor that has been infected by or vaccinated against a specific disease. Said donor can be human or non-human.

25 The term "antibody" herein refers to monoclonal antibodies (including full length or intact monoclonal antibodies), polyclonal antibodies, multivalent antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments.

30 Antibody fragments comprise only a portion of an intact antibody, generally including an antigen binding site of the intact antibody and thus retaining the ability to bind antigen. Examples of antibody fragments encompassed by the present definition include: Fab fragments, Fab' fragments, Fd fragments, Fd' fragments, Fv fragments, dAb fragments, isolated CDR regions, F(ab')₂ fragments as well as single chain antibody molecules, diabodies and linear antibodies.

In one embodiment, the protein is a biosimilar. A "biosimilar" is herein defined as a biological medicine that is highly similar to another already approved biological medicine. In a preferred embodiment, the biosimilar is a monoclonal antibody.

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In one embodiment, the compositions and formulations according to the invention comprise more than one protein species.

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The compositions and formulations according to the invention comprise a first viscosity reducing excipient selected from the group consisting of cyanocobalamin (CAS-Registry Number 68-19-9), pyridoxine (vitamin B₆, CAS-Registry Number 65-23-6), ascorbic acid (vitamin C, CAS-Registry Number 50-81-7), folic acid (CAS-Registry Number 59-30-3), thiamine monophosphate (CAS-Registry Number 10023-48-0), thiamine pyrophosphate (cocarboxylase, CAS-Registry Number 154-87-0), paracetamol (acetaminophen, CAS-Registry Number 103-90-2), guanidine hydrochloride (carbamidimidoylazanium chloride, CAS-Registry Number 50-01-1) and quinine hydrochloride ((R)-[(1S,2S,4S,5R)-5-ethenyl-1-azabicyclo[2.2.2]octan-2-yl](6-methoxyquinolin-4-yl)methanol dihydrate hydrochloride, CAS-Registry Number 6119-47-7).

20

According to the invention, the viscosity reducing excipient also include salts or solvates of the excipients. Preferred salts in the context of the present invention are physiologically acceptable salts of the compounds according to the invention. Salts which are not themselves suitable for pharmaceutical uses but can be used, for example, for isolation, purification or storage of the compounds according to the invention are also included.

25

Physiologically acceptable salts of the compounds according to the invention include salts of conventional bases, such as, by way of example and preferably, alkali metal salts (e.g. sodium and potassium salts), alkaline earth metal salts (e.g. calcium and magnesium salts) and ammonium salts derived from ammonia or organic amines having 1 to 16 C atoms, such as, by way of example and preferably, ethylamine, diethylamine, triethylamine, N,N-diisopropylethylamine, monoethanolamine, diethanolamine, triethanolamine, dimethylaminoethanol,

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diethylaminoethanol, procaine, dicyclohexylamine, dibenzylamine, N-methylpiperidine, N-methylmorpholine, arginine, lysine and 1,2-ethylenediamine.

Physiologically acceptable salts of the compounds according to the invention include salts of conventional acids, such as, by way of example and preferably, acetate, adipate, alginate, citrate, aspartate, benzoate, benzenesul-
5 fonate, bisulfate, butyrate, camphorate, camphorsulfonate, carbonate, digluconate, glycerophosphate, hemisulfate, heptanoate, hexanoate, formate, fumarate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethansulfonate (isethionate), lactate, maleate, mesitylenesulfonate, methanesulfonate, naphthyl-
10 enesulfonate, nicotinate, 2-naphthalenesulfonate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, sulfate, tartrate, trichloroacetate, trifluoroacetate, phosphate, glutamate, bicarbonate, paratoluenesulfonate, and undecanoate.

Without being limited to specific examples, physiologically acceptable salts can be salts of folic acid, e.g. sodium folate, salts of ascorbic acid, e.g. sodium ascorbate,
15 salts of thiamine, e.g. thiamine hydrochloride, salts of ornithine, e.g. ornithine monohydrochloride or salts of carnithine, e.g. carnitine hydrochloride.

Solvates in the context of the invention are designated as those forms of the compounds according to the invention which form a complex in the solid or liquid
20 state by coordination with solvent molecules. Hydrates are a specific form of solvates, in which the coordination takes place with water. Hydrates are preferred solvates in the context of the present invention.

In one embodiment, the invention comprises at least one first excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid,
25 thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol.

The liquid compositions and formulations according to the invention comprise an amount of the first excipient sufficient to reduce the viscosity of the composition
30 and/or stabilize the protein. For example, the compositions and formulations according to the invention may comprise about 5 mM to about 300 mM, about 5 mM to about 250 mM or about 5 mM to about 150 mM of the first excipient. In exemplary embodiments the concentration of the first excipient is 1, 5, 10, 12, 13, 15, 20, 25,

30, 35, 50, 75, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 210, 220, 250, or 300 mM or greater.

5 In a preferred embodiment, the compositions according to the invention comprise 1 to 9 mM of cyanocobalamin, more preferably 5 mM. In another preferred embodiment, the compositions according to the invention comprise 5 to 500 mM of pyridoxine, more preferably 25 to 300 mM, most preferably 150 mM when used alone and 75 mM when used in combination. In another preferred embodiment, the compositions according to the invention comprise 5 to 500 mM of ascorbic acid,
10 more preferably 25 to 300 mM, most preferably 150 mM. In a preferred embodiment, the compositions according to the invention comprise 5 to 20 mM of folic acid, more preferably 5 to 15 mM, most preferably 13 mM when used alone and 12 mM when used in combination. In another preferred embodiment, the compositions according to the invention comprise 1 to 420 mM of thiamine monophosphate, more preferably
15 25 to 300 mM, most preferably 150 mM when used alone and 75 mM when used in combination. In another preferred embodiment, the compositions according to the invention comprise 1 to 450 mM of thiamine pyrophosphate, more preferably 25 to 300 mM, most preferably 75 mM. In another preferred embodiment, the compositions according to the invention comprise 5 to 500 mM of guanidine
20 hydrochloride, more preferably 25 to 300 mM, most preferably 150 mM. In another preferred embodiment, the compositions according to the invention comprise 1 to 25 mM of quinine hydrochloride, more preferably 5 to 25 mM, most preferably 25 mM. In another preferred embodiment, the compositions according to the invention comprise 5 to 100 mM of paracetamol, more preferably 25 to 100 mM, most
25 preferably 75 mM.

30 The inventors have surprisingly found that the addition of valine, proline, leucine, isoleucine, phenylalanine, cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine hydrochloride, thiamine, thiamine monophosphate, thiamine pyrophosphate, paracetamol, guanidine hydrochloride or quinine hydrochloride significantly reduces the viscosity of a protein solution and increases the stability of said protein in the solution. The present invention thus provides compositions and formulations having reduced viscosity and/or increased stability.

The liquid compositions and formulations according to the invention show an increased stability in comparison to a composition comprising a protein, but not comprising the first and/or second excipient.

5 Cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol are all compounds known to be non-toxic and safe. Thus, their administration is well tolerated.

10 In one aspect of the invention these compositions and formulations may further comprise excipients that are used for purposes other than reducing viscosity, e.g. stabilization, solubilization or preservation.

15 In another aspect of the invention, the compositions and formulations comprise more than one of the first excipients. For example, the compositions and formulations according to the invention may comprise two, three or four of the first excipients, preferably they contain two of the first excipients.

20 The combination of two, three or four first excipients can synergically reduce the viscosity and/or increase stability in the compositions and formulations comprising a protein or in protein solutions. As defined herein, "synergically" refers to the effect that the action of a combination of components is greater than the sum of the action of each of the components alone.

25 In one embodiment, the compositions and formulations of the invention comprise cyanocobalamine and pyridoxine. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamine and ascorbic acid. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamine and folic acid. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamine and thiamine monophosphate. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamine and thiamine pyrophosphate. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamine and guanidine hydrochloride. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamine and

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quinine hydrochloride. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamine and paracetamol.

5 In one embodiment, the compositions and formulations of the invention comprise pyridoxine and ascorbic acid. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and folic acid. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and thiamine monophosphate. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and thiamine pyrophosphate. In one
10 embodiment, the compositions and formulations of the invention comprise pyridoxine and guanidine hydrochloride. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and quinine hydrochloride. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and paracetamol.

15 In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and folic acid. In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and thiamine monophosphate. In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and thiamine pyrophosphate. In one
20 embodiment, the compositions and formulations of the invention comprise ascorbic acid and guanidine hydrochloride. In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and quinine hydrochloride. In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and paracetamol.

25 In one embodiment, the compositions and formulations of the invention comprise folic acid and thiamine monophosphate. In one embodiment, the compositions and formulations of the invention comprise folic acid and thiamine pyrophosphate. In one embodiment, the compositions and formulations of the invention comprise folic acid and guanidine hydrochloride. In one
30 embodiment, the compositions and formulations of the invention comprise folic acid and quinine hydrochloride. In one embodiment, the compositions and formulations of the invention comprise folic acid and paracetamol.

In one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and thiamine pyrophosphate. In one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and guanidine hydrochloride. In one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and quinine hydrochloride. In one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and paracetamol.

In one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and guanidine hydrochloride. In one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and quinine hydrochloride. In one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and paracetamol.

In one embodiment, the compositions and formulations of the invention comprise guanidine hydrochloride and quinine hydrochloride. In one embodiment, the compositions and formulations of the invention comprise guanidine hydrochloride and paracetamol.

In one embodiment, the compositions and formulations of the invention comprise quinine hydrochloride and paracetamol.

In a preferred embodiment, the compositions and formulations of the invention comprise phenylalanine and an excipient selected from the group consisting of pyridoxine, ascorbic acid, folic acid, thiamine hydrochloride, thiamine monophosphate and thiamine pyrophosphate. In a preferred embodiment, the compositions and formulations of the invention comprise thiamine hydrochloride and an excipient selected from the group consisting of pyridoxine or folic acid. A combination of these excipients is particularly useful for reducing the viscosity of a protein solution or composition.

In one embodiment, the compositions and formulations of the invention comprise thiamine hydrochloride and folic acid. In one embodiment, the compositions and formulations of the invention comprise thiamine hydrochloride and pyridoxine. In

one embodiment, the compositions and formulations of the invention comprise phenylalanine and pyridoxine. In one embodiment, the compositions and formulations of the invention comprise phenylalanine and thiamine monophosphate. In one embodiment, the compositions and formulations of the invention comprise phenylalanine and thiamine pyrophosphate. In one embodiment, the compositions and formulations of the invention comprise phenylalanine and folic acid.

When more than one excipient is present in the compositions and formulations of the invention, the concentration of the excipients may be the same or different.

The compositions and formulations according to the invention may further comprise at least one second viscosity reducing excipient.

The term "viscosity reducing excipient", as used herein, refers to any compound at a suitable concentration which is known to reduce the viscosity of a protein solution by at least 5% compared to an identical composition not comprising the viscosity reducing excipient.

The at least one second viscosity reducing excipient is preferably selected from the group consisting of valine (CAS-Registry Number 72-18-4), proline (CAS-Registry Number 147-85-3), leucine (CAS-Registry Number 61-90-5), isoleucine (CAS-Registry Number 73-32-5), phenylalanine (CAS-Registry Number 63-91-2), thiamine hydrochloride (CAS-Registry Number 67-03-8), arginine, ornithine, carnitine, meglumine ((2R,3R,4R,5S)-6-(methylamino)hexane-1,2,3,4,5-pentol, CAS-Registry Number 6284-40-8), benzenesulfonic acid (CAS-Registry Number 98-11-3), caffeine (1,3,7-Trimethylxanthine, CAS-Registry Number 58-08-2) and camphorsulfonic acid ((7,7-dimethyl-2-oxobicyclo[2.2.1]heptan-1-yl)methanesulfonic acid).

The liquid compositions and formulations according to the invention may comprise an amount of at least one second excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid sufficient to further reduce the viscosity of the composition and/or stabilize the protein. For example,

the compositions and formulations according to the invention may comprise about 50 mM to about 300 mM, about 100 mM to about 250 mM or about 140 mM to about 200 mM of the second excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid. In exemplary embodiments the concentration of the at least one second excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid is 5, 10, 15, 20, 25, 30, 35, 50, 75, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 210, 220, 250, or 300 mM or greater. In a preferred embodiment, the compositions according to the invention comprise 150 mM of valine, leucine, isoleucine, proline, phenylalanine, lysine or thiamine hydrochloride. In a preferred embodiment, the compositions and formulations according to the invention comprise 50-100 mM of the second excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.

In another preferred embodiment the compositions and formulations according to the invention comprise folic acid in a concentration of 5-13 mM. In another preferred embodiment the compositions and formulations according to the invention comprise cyanocobalamin in a concentration of 5 mM.

In these embodiments, the first excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol forms a viscosity-reducing solution together with the second excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid that can be added to compositions and formulations comprising a protein or protein solutions in order to reduce the viscosity and/or increase stability thereof.

In another preferred embodiment the compositions and formulations according to the invention comprise at least one first and at least one second viscosity reducing

excipient, wherein the excipients synergistically reduce the viscosity and/or increase stability in the compositions and formulations comprising a protein or in protein solutions.

- 5 According to the invention, a synergistical reduction of the viscosity is given if the viscosity reduction by a combination of two or more excipients is more than the expected sum of the viscosity reduction of each individual excipient. Preferably a synergistical reduction of the viscosity is given if the percentage viscosity reduction by a combination of two or more excipients is more than the expected sum of the
10 percentage viscosity reduction of each individual excipient.

- Additionally, according to the invention, a combination of two or more viscosity reducing excipients is synergistic in case the protein stability reduction by a combination of two or more excipients is less than the expected sum of the stability
15 reduction of each individual excipient.

In one embodiment all combinations mentioned in the present application result in a synergistic reduction of viscosity of a liquid composition comprising a protein.

- 20 In one embodiment the following combinations pyridoxine / arginine, folic acid / ornithine, folic acid / carnithine, pyridoxine / meglumine, thiamine monophosphate / meglumine, pyridoxine / thiamine monophosphate, phenylalanine / camphorsulfonic acid and phenylalanine / benzenesulfonic acid result in a synergistic reduction of viscosity of a liquid composition comprising a protein.

- 25 In one embodiment, the compositions and formulations of the invention comprise cyanocobalamin and arginine. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamin and ornithine. In one embodiment, the compositions and formulations of the invention comprise
30 cyanocobalamin and carnitine. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamin and meglumine. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamin and camphorsulfonic acid. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamin and benzenesulfonic

acid. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamin and caffeine. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamin and valine. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamin and proline. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamin and leucine. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamin and isoleucine. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamin and phenylalanine. In one embodiment, the compositions and formulations of the invention comprise cyanocobalamin and thiamin hydrochloride.

In one embodiment, the compositions and formulations of the invention comprise pyridoxine and arginine. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and ornithine. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and carnitine. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and meglumine. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and camphorsulfonic acid. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and benzenesulfonic acid. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and caffeine. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and valine. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and proline. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and leucine. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and isoleucine. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and phenylalanine. In one embodiment, the compositions and formulations of the invention comprise pyridoxine and thiamin hydrochloride.

In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and arginine. In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and ornithine. In one embodiment, the

compositions and formulations of the invention comprise ascorbic acid and carnitine. In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and meglumine. In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and camphorsulfonic acid. In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and benzenesulfonic acid. In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and caffeine. In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and valine. In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and proline. In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and leucine. In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and isoleucine. In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and phenylalanine. In one embodiment, the compositions and formulations of the invention comprise ascorbic acid and thiamin hydrochloride.

In one embodiment, the compositions and formulations of the invention comprise folic acid and arginine. In one embodiment, the compositions and formulations of the invention comprise folic acid and ornithine. In one embodiment, the compositions and formulations of the invention comprise folic acid and carnitine. In one embodiment, the compositions and formulations of the invention comprise folic acid and meglumine. In one embodiment, the compositions and formulations of the invention comprise folic acid and camphorsulfonic acid. In one embodiment, the compositions and formulations of the invention comprise folic acid and benzenesulfonic acid. In one embodiment, the compositions and formulations of the invention comprise folic acid and caffeine. In one embodiment, the compositions and formulations of the invention comprise folic acid and valine. In one embodiment, the compositions and formulations of the invention comprise folic acid and proline. In one embodiment, the compositions and formulations of the invention comprise folic acid and leucine. In one embodiment, the compositions and formulations of the invention comprise folic acid and isoleucine. In one embodiment, the compositions and formulations of the invention comprise folic acid and phenylalanine. In one

embodiment, the compositions and formulations of the invention comprise folic acid and thiamin hydrochloride.

5 In one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and arginine. In one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and ornithine. In one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and carnitine. In one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and meglumine. In 10 one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and camphorsulfonic acid. In one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and benzenesulfonic acid. In one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and caffeine. In one 15 embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and valine. In one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and proline. In one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and leucine. In one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and isoleucine. In 20 one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and phenylalanine. In one embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and thiamin hydrochloride.

25 In one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and arginine. In one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and ornithine. In one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and carnitine. In one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and meglumine. In 30 one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and camphorsulfonic acid. In one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate

and benzenesulfonic acid. In one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and caffeine. In one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and valine. In one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and proline. In one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and leucine. In one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and isoleucine. In one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and phenylalanine. In one embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and thiamin hydrochloride.

In one embodiment, the compositions and formulations of the invention comprise guanidine hydrochloride and arginine. In one embodiment, the compositions and formulations of the invention comprise guanidine hydrochloride and ornithine. In one embodiment, the compositions and formulations of the invention comprise guanidine hydrochloride and carnitine. In one embodiment, the compositions and formulations of the invention comprise guanidine hydrochloride and meglumine. In one embodiment, the compositions and formulations of the invention comprise guanidine hydrochloride and camphorsulfonic acid. In one embodiment, the compositions and formulations of the invention comprise guanidine hydrochloride and benzenesulfonic acid. In one embodiment, the compositions and formulations of the invention comprise guanidine hydrochloride and caffeine. In one embodiment, the compositions and formulations of the invention comprise guanidine hydrochloride and valine. In one embodiment, the compositions and formulations of the invention comprise guanidine hydrochloride and proline. In one embodiment, the compositions and formulations of the invention comprise guanidine hydrochloride and leucine. In one embodiment, the compositions and formulations of the invention comprise guanidine hydrochloride and isoleucine. In one embodiment, the compositions and formulations of the invention comprise guanidine hydrochloride and phenylalanine. In one embodiment, the compositions and formulations of the invention comprise guanidine hydrochloride and thiamin hydrochloride.

In one embodiment, the compositions and formulations of the invention comprise quinine hydrochloride and arginine. In one embodiment, the compositions and formulations of the invention comprise quinine hydrochloride and ornithine. In one embodiment, the compositions and formulations of the invention comprise quinine hydrochloride and carnitine. In one embodiment, the compositions and formulations of the invention comprise quinine hydrochloride and meglumine. In one embodiment, the compositions and formulations of the invention comprise quinine hydrochloride and camphorsulfonic acid. In one embodiment, the compositions and formulations of the invention comprise quinine hydrochloride and benzenesulfonic acid. In one embodiment, the compositions and formulations of the invention comprise quinine hydrochloride and caffeine. In one embodiment, the compositions and formulations of the invention comprise quinine hydrochloride and valine. In one embodiment, the compositions and formulations of the invention comprise quinine hydrochloride and proline. In one embodiment, the compositions and formulations of the invention comprise quinine hydrochloride and leucine. In one embodiment, the compositions and formulations of the invention comprise quinine hydrochloride and isoleucine. In one embodiment, the compositions and formulations of the invention comprise quinine hydrochloride and phenylalanine. In one embodiment, the compositions and formulations of the invention comprise quinine hydrochloride and thiamin hydrochloride.

In one embodiment, the compositions and formulations of the invention comprise paracetamol and arginine. In one embodiment, the compositions and formulations of the invention comprise paracetamol and ornithine. In one embodiment, the compositions and formulations of the invention comprise paracetamol and carnitine. In one embodiment, the compositions and formulations of the invention comprise paracetamol and meglumine. In one embodiment, the compositions and formulations of the invention comprise paracetamol and camphorsulfonic acid. In one embodiment, the compositions and formulations of the invention comprise paracetamol and benzenesulfonic acid. In one embodiment, the compositions and formulations of the invention comprise paracetamol and caffeine. In one embodiment, the compositions and formulations of the invention comprise paracetamol and valine. In one embodiment, the compositions and formulations of the invention comprise paracetamol and proline. In one embodiment, the

compositions and formulations of the invention comprise paracetamol and leucine. In one embodiment, the compositions and formulations of the invention comprise paracetamol and isoleucine. In one embodiment, the compositions and formulations of the invention comprise paracetamol and phenylalanine. In one embodiment, the compositions and formulations of the invention comprise paracetamol and thiamin hydrochloride.

In a preferred embodiment, the compositions and formulations according to the invention are liquid formulations. In a preferred embodiment, the compositions and formulations according to the invention are liquid formulations and the protein is a therapeutic protein.

In another aspect, the invention provides lyophilized protein formulations comprising a protein and at least one first excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol. Upon reconstitution with a suitable amount of diluent, the formulations exhibit reduced viscosity relative to control formulations with the otherwise same composition but not comprising the excipient. Thus, the excipient is present at an amount effective to reduce viscosity upon reconstitution with diluent.

In another aspect, the invention provides lyophilized protein formulations comprising a protein and at least one first excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol and at least one second viscosity reducing excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.

A lyophilized protein formulation includes a protein and the at least one excipient according to the invention that has been dried and is present as particles in, for example, powder form. In the present context the expression "powder" refers to a

collection of essentially dry particles, i.e. the moisture content being at least below about 10% by weight, 6% by weight, 4% by weight, or lower.

5 As defined herein, "viscosity" refers to the resistance of a substance (typically a liquid) to flow. Viscosity is related to the concept of shear force; it can be understood as the effect of different layers of the fluid exerting shearing force on each other, or on other surfaces, as they move against each other. There are several ways to express viscosity. The units of viscosity are Ns/m^2 , known as Pascal-seconds (Pas). Viscosity can be "kinematic" or "absolute". Kinematic viscosity is a measure of the rate at which momentum is transferred through a fluid. It is measured in Stokes (St).
10 The kinematic viscosity is a measure of the resistive flow of a fluid under the influence of gravity. When two fluids of equal volume and differing viscosity are placed in identical capillary viscometers and allowed to flow by gravity, the more viscous fluid takes longer than the less viscous fluid to flow through the capillary. If, for example, one fluid takes 200 seconds (s) to complete its flow and another fluid takes 400 s, the second fluid is called twice as viscous as the first on a kinematic viscosity scale. The dimension of kinematic viscosity is $\text{length}^2/\text{time}$. Commonly, kinematic viscosity is expressed in centiStokes (cSt). The SI unit of kinematic viscosity is mm^2/s , which is equal to 1 cSt. The "absolute viscosity," sometimes
15 called "dynamic viscosity" or "simple viscosity," is the product of kinematic viscosity and fluid density. Absolute viscosity is expressed in units of centipoise (cP). The SI unit of absolute viscosity is the milliPascal-second (mPas), where 1 cP=1 mPas.

25 Viscosity may be measured by using, for example, a viscometer at a given shear rate or multiple shear rates. An "extrapolated zero-shear" viscosity can be determined by creating a best fit line of the four highest-shear points on a plot of absolute viscosity versus shear rate, and linearly extrapolating viscosity back to zero-shear. Alternatively, for a Newtonian fluid, viscosity can be determined by averaging viscosity values at multiple shear rates. Viscosity can also be measured
30 using a microfluidic viscometer at single or multiple shear rates (also called flow rates), wherein absolute viscosity is derived from a change in pressure as a liquid flows through a channel. Viscosity equals shear stress over shear rate. Viscosities measured with microfluidic viscometers can, in some embodiments, be directly compared to extrapolated zero-shear viscosities, for example those extrapolated

- from viscosities measured at multiple shear rates using a cone and plate viscometer. According to the invention, viscosity of compositions and formulations is reduced when at least one of the methods described above show a stabilizing effect. Preferably, viscosity is measured at 20 °C using a microfluidic viscometer. More preferably the viscosity is measured using a RheoSense mVROC microfluidic viscometer at 20 °C. Most preferably the viscosity is measured at 20 °C using a RheoSense mVROC microfluidic viscometer and using a 500 µl syringe, a shear rate of 3000 s⁻¹ or 2000 s⁻¹ and a volume of 200 µl.
- The person ordinary skilled in the art is familiar with the viscosity measurement using a microfluidic viscometer. As microfluidic viscometer the RheoSense mVROC microfluidic viscometer (mVROC™ Technology), especially with the parameters described above can be used. Detailed specifications, methods and setting can be found in the 901003.5.1-mVROC_User's_Manual.
- “Shear rate” herein refers to the rate of change of velocity at which one layer of fluid passes over an adjacent layer. The velocity gradient is the rate of change of velocity with distance from the plates. This simple case shows the uniform velocity gradient with shear rate $(v_1 - v_2)/h$ in units of (cm/sec)/(cm)=1/sec. Hence, shear rate units are reciprocal seconds or, in general, reciprocal time. For a microfluidic viscometer, change in pressure and flow rate are related to shear rate. “Shear rate” is to the speed with which a material is deformed. Formulations containing proteins and viscosity-lowering agents are typically measured at shear rates ranging from about 0.5 s⁻¹ to about 200 s⁻¹ when measured using a cone and plate viscometer and a spindle appropriately chosen by one skilled in the art to accurately measure viscosities in the viscosity range of the sample of interest (i.e., a sample of 20 cP is most accurately measured on a CPE40 spindle affixed to a DV2T viscometer (Brookfield)); greater than about 20 s⁻¹ to about 3,000 s⁻¹ when measured using a microfluidic viscometer.
- For classical “Newtonian” fluids, as generally used herein, viscosity is essentially independent of shear rate. For “non-Newtonian fluids,” however, viscosity either decreases or increases with increasing shear rate, e.g., the fluids are “shear thinning” or “shear thickening”, respectively. In the case of concentrated (i.e., high-

concentration) protein solutions, this may manifest as pseudoplastic shear-thinning behavior, i.e., a decrease in viscosity with shear rate.

5 In one embodiment, the compositions and formulations of the invention show a reduction of viscosity of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70% or 75% compared to an identical composition not comprising the at least one first excipient.

10 In one embodiment, the compositions and formulations of the invention show a reduction of viscosity of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70% or 75% compared to an identical composition not comprising the at least one first and at least one second excipient.

15 As used herein, the term "stability" encompasses both chemical and physical stability.

20 The term "chemical stability" herein refers to the ability of the protein components in a formulation to resist degradation via chemical pathways, such as oxidation, deamidation or hydrolysis. A protein formulation is typically considered chemically stable if less than about 5% of the components are degraded after 24 months at 4 °C. According to the present invention the protein formulation is typically considered chemically stable if less than about 5% of the components are degraded after 24 weeks at 25 °C with a relative humidity of 60%.

25 Stability can be assessed in many ways known to the skilled person, including monitoring conformational change over a range of temperatures (thermostability) and/or time periods (shelf-life) and/or after exposure to stressful handling situations (e.g. physical shaking). Stability of formulations containing varying concentrations of formulation components can be measured using a variety of methods. For
30 example, the amount of protein aggregation can be measured by visual observation of turbidity, by measuring absorbance at a specific wavelength, by size exclusion chromatography (in which aggregates of a protein will elute in different fractions compared to the protein in its native active state), HPLC, or other chromatographic methods. Other methods of measuring conformational change can be used,

including differential scanning calorimetry (DSC), e.g. to determine the temperature of denaturation, or circular dichroism (CD), which measures the molar ellipticity of the protein. Fluorescence can also be used to analyze the composition. Fluorescence encompasses the emission of light subsequent to absorption of light, which requires a suitable wavelength. Potential readouts are changes in the polar properties of light, light intensity, or emission wavelength. Fluorescence emission can be intrinsic to a protein or can be due to a fluorescence reporter molecule that for example binds to the hydrophobic pockets of partially unfolded proteins. An increase in binding of reporter molecules can be monitored by detection of the fluorescence signal of a protein sample. Other means for measuring stability can be used and are well known to persons skilled in the art. According to the invention, stability of compositions and formulations is increased when at least one of the methods described above show a stabilizing effect.

In one embodiment, the compositions and formulations of the invention show an increase in stability of the protein of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70% or 75% compared to compositions not comprising the at least one first viscosity reducing excipient or not comprising the at least one first and at least one second viscosity reducing excipient.

In one embodiment, the compositions and formulations according to the invention comprise a viscosity reducing excipient selected from the group consisting of valine, leucine, ascorbic acid, cyanocobalamin and proline and show an increased protein stability characterized by an elevated T_m and/or T_{agg} .

In one embodiment, the compositions and formulations according to the invention comprise a viscosity reducing excipient selected from the group consisting of valine, leucine, ascorbic acid, cyanocobalamin and proline and show both (i) an increased protein stability characterized by an elevated T_m and/or T_{agg} and (ii) a reduced viscosity.

In a preferred embodiment the compositions and formulations of the invention comprise phenylalanine, camphorsulfonic acid or benzenesulfonic acid as the second excipient.

- In a preferred embodiment, the compositions and formulations of the invention comprise phenylalanine and pyridoxine. In a preferred embodiment, the compositions and formulations of the invention comprise phenylalanine and thiamine hydrochloride. In a preferred embodiment, the compositions and formulations of the invention comprise phenylalanine and folic acid. In a preferred embodiment, the compositions and formulations of the invention comprise phenylalanine and thiamine monophosphate. In a preferred embodiment, the compositions and formulations of the invention comprise phenylalanine and thiamine pyrophosphate.
- In a preferred embodiment, the compositions and formulations of the invention comprise phenylalanine and arginine. In a preferred embodiment, the compositions and formulations of the invention comprise phenylalanine and ornithine. In a preferred embodiment, the compositions and formulations of the invention comprise phenylalanine and carnitine. In a preferred embodiment, the compositions and formulations of the invention comprise phenylalanine and meglumine. In a preferred embodiment, the compositions and formulations of the invention comprise phenylalanine and benzenesulfonic acid. In a preferred embodiment, the compositions and formulations of the invention comprise phenylalanine and camphorsulfonic acid.
- In a preferred embodiment, the compositions and formulations of the invention comprise pyridoxine and arginine. In a preferred embodiment, the compositions and formulations of the invention comprise pyridoxine and ornithine. In a preferred embodiment, the compositions and formulations of the invention comprise pyridoxine and carnitine. In a preferred embodiment, the compositions and formulations of the invention comprise pyridoxine and meglumine. In a preferred embodiment, the compositions and formulations of the invention comprise pyridoxine and thiamine hydrochloride.
- In a preferred embodiment, the compositions and formulations of the invention comprise folic acid and arginine. In a preferred embodiment, the compositions and formulations of the invention comprise folic acid and ornithine. In a preferred embodiment, the compositions and formulations of the invention comprise folic acid and carnitine. In a preferred embodiment, the compositions and formulations of the

invention comprise folic acid and meglumine. In a preferred embodiment, the compositions and formulations of the invention comprise folic acid and thiamine hydrochloride.

5 In a preferred embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and arginine. In a preferred embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and ornithine. In a preferred embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and carnitine. In a preferred
10 embodiment, the compositions and formulations of the invention comprise thiamine monophosphate and meglumine.

In a preferred embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and arginine. In a preferred embodiment, the
15 compositions and formulations of the invention comprise thiamine pyrophosphate and carnitine. In a preferred embodiment, the compositions and formulations of the invention comprise thiamine pyrophosphate and meglumine.

In a preferred embodiment, the composition comprises a combination of two
20 viscosity reducing excipients selected from the group consisting of phenylalanine and benzenesulfonic acid, phenylalanine and camphorsulfonic acid, thiamine hydrochloride and benzenesulfonic acid , thiamine hydrochloride and camphorsulfonic acid, pyridoxine and arginine, pyridoxine and ornithine, pyridoxine and carnitine, pyridoxine and meglumine, folic acid and arginine, folic acid and
25 ornithine, folic acid and carnitine, folic acid and meglumine, thiamine monophosphate and arginine, thiamine monophosphate and ornithine, thiamine monophosphate and carnitine, thiamine monophosphate and meglumine, thiamine pyrophosphate and arginine, thiamine pyrophosphate and ornithine, thiamine pyrophosphate and carnitine, thiamine pyrophosphate and meglumine.

30 In a further embodiment, the liquid composition comprises a protein, at least one first viscosity reducing excipient selected from the group consisting of pyridoxine, folic acid, thiamine monophosphate and phenylalanine and at least one second

viscosity reducing excipient selected from the group consisting of arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.

5 In a further preferred embodiment, the liquid composition comprises a combination of a first and a second viscosity reducing excipient selected from the list consisting of pyridoxine and arginine, folic acid and ornithine, folic acid and carnithine, pyridoxine and meglumine, thiamine monophosphate and meglumine, pyridoxine and thiamine monophosphate, phenylalanine and camphorsulfonic acid and phenylalanine and benzenesulfonic acid.

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In a further preferred embodiment, the combination of a first and a second viscosity reducing excipient is pyridoxine and arginine. In a further preferred embodiment, the combination of a first and a second viscosity reducing excipient is folic acid and ornithine. In a further preferred embodiment, the combination of a first and a second viscosity reducing excipient is folic acid and carnithine. In a further preferred embodiment, the combination of a first and a second viscosity reducing excipient is pyridoxine and meglumine. In a further preferred embodiment, the combination of a first and a second viscosity reducing excipient is thiamine monophosphate and meglumine. In a further preferred embodiment, the combination of a first and a second viscosity reducing excipient is pyridoxine and thiamine monophosphate. In a further preferred embodiment, the combination of a first and a second viscosity reducing excipient is phenylalanine and camphorsulfonic acid. In a further preferred embodiment, the combination of a first and a second viscosity reducing excipient is phenylalanine and benzenesulfonic acid.

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In a further preferred embodiment, the liquid composition comprises a combination of a first and a second viscosity reducing excipient selected from the list consisting of pyridoxine and arginine, pyridoxine and meglumine and pyridoxine and thiamine monophosphate.

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In a further preferred embodiment, the liquid composition comprises a combination of a first and a second viscosity reducing excipient selected from the list consisting of folic acid and ornithine and folic acid and carnithine.

In a further preferred embodiment, the liquid composition comprises a combination of a first and a second viscosity reducing excipient selected from the list consisting of pyridoxine and meglumine and thiamine monophosphate and meglumine.

5 In a further preferred embodiment, the liquid composition comprises a combination of a first and a second viscosity reducing excipient selected from the list consisting of thiamine monophosphate and meglumine and pyridoxine and thiamine monophosphate.

10 In a further preferred embodiment, the liquid composition comprises a combination of a first and a second viscosity reducing excipient selected from the list consisting of phenylalanine and camphorsulfonic acid and phenylalanine and benzenesulfonic acid.

15 In one embodiment, the compositions and formulations according to the invention have a pH between 2 and 10, preferably between 4 and 8, more preferably between 5 and 7.2. In one embodiment, the compositions and formulations have a pH of exactly 5 or exactly 7.2.

20 The compositions and formulations according to the invention may additionally comprise pharmaceutically acceptable diluents, solvents, carriers, adhesives, binders, preservatives, solubilizers, surfactants, penetration enhancers, emulsifiers or bioavailability enhancers. The skilled person knows how to choose suitable additives for liquid compositions that are safe and well tolerated.

25 The compositions and formulations according the invention which comprise combinations of first and/or second viscosity reducing excipients may further comprise excipients that are used for purposes other than reducing viscosity, e.g. stabilization, solubilization or preservation.

30 In a preferred embodiment, the compositions and formulations according to the invention comprise a stabilizer such as a sugar and/or a surfactant. Suitable sugars as stabilizers are known in the literatur, e.g. sucrose or trehalose. In a preferred embodiment, the sugar is sucrose. Suitable surfactants are known in the literatur,

e.g. polysorbate 20 or polysorbate 80 or poloxamer 188. In another preferred embodiment, the surfactant is polysorbate 80. The addition of a further stabilizers additionally enhances the stabilizing effect of the compositions according to the inventions.

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The liquid compositions and formulations according to the invention comprise an amount of the first and optionally second excipient sufficient to reduce the viscosity of the composition and/or stabilize the protein. For example, the compositions and formulations according to the invention may comprise about 5 mM to about 300 mM, about 5 mM to about 250 mM or about 5 mM to about 150 mM of each first and optionally second excipient. In exemplary embodiments the concentration of each first and optionally second excipient is 1, 5, 10, 12, 13, 15, 20, 25, 30, 35, 50, 75, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 210, 220, 250, or 300 mM or greater. Preferably the concentration of each first and optionally second excipient is 75 or 150 mM.

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In one embodiment the liquid compositions comprise a combination of two excipients, wherein the molar concentration of the excipients can be identical or different. Preferably the concentrations of each first and second excipient is between 1 mM and 200 mM, more preferably between 25 and 150 mM, most preferably between 50 and 100 mM. The molar ratio of the first and second excipient is between 1:100 and 100:1, preferably between 1:10 and 10:1, more preferably between 1:5 and 5:1, most preferably between 1:2 and 2:1. In a particular embodiment the molar concentration of the first and second excipient is identical.

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The invention further provides a liquid composition according to the invention whereas the protein has a molecular weight from 120 kDa to 250 kDa, preferably from 130 kDa to 180 kDa.

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In a preferred embodiment, the protein concentration in the compositions and formulations according to the invention is at least 1 mg/ml, at least 50 mg/ml, preferably at least 75 mg/ml and more preferably at least 100 mg/ml. In another preferred embodiment, the protein concentration is between 90 mg/ml and 300 mg/ml, more preferably the protein concentration is between 100 and 250 mg/ml, even more preferable between 120 and 210 mg/ml. The present invention is

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particularly useful for these high-concentration compositions, formulations and solutions.

5 The invention further provides a liquid composition according to the invention further comprising a buffer at a concentration of 10 mM to 50 mM. The buffer can be a suitable acetate- or phosphate salt and provide a pH of 5 to 7.2.

10 The invention further provides a liquid composition according to the invention further comprising a sugar as stabilizer, preferably 50 to 100 mg/ml sucrose.

The invention further provides a liquid composition according to the invention further comprising a surfactant, preferably 0.01 to 0.2 mg/ml, more preferably 0.05 mg/ml of polysorbate 80.

15 The invention further provides a liquid composition according to the invention whereas the viscosity is between 1 mPas and 60 mPas, preferably between 1 mPas and 50 mPas, more preferably between 1 mPas and 30 mPas, most preferably between 1 mPas and 20 mPas. Preferably the viscosity is measured at 20 °C, using a microfluidic viscometer. More preferably the viscosity is measured using a
20 RheoSense mVROC microfluidic viscometer at 20 °C. Most preferably the viscosity is measured using a RheoSense mVROC microfluidic viscometer at 20 °C, using a 500 µl syringe, a shear rate of 3000 s⁻¹ or 2000 s⁻¹ and a volume of 200 µl.

25 The invention further provides a liquid composition according to the invention whereas the protein has a molecular weight from 120 kDa to 250 kDa at a concentration between 90 mg/ml to 300 mg/ml, preferably further comprising an acetate buffer or phosphate buffer at a concentration between 10 mM to 50 mM, wherein the formulation has a pH between 5 and 7.2, and a viscosity between 1 mPas and 60 mPas when measured at 20 °C, preferably using a microfluidic
30 viscometer, more preferably using a RheoSense mVROC microfluidic viscometer at 20 °C, most preferably using a RheoSense mVROC microfluidic viscometer at 20 °C with a 500 µl syringe, a shear rate of 3000 s⁻¹ or 2000 s⁻¹ and a volume of 200 µl.

Preferably, liquid composition according to the invention wherein a first and a second viscosity reducing excipient are present, have a protein concentration between 90 mg/ml to 300 mg/ml, more preferably between 100 mg/ml and 200 mg/ml and a concentration of the first and second viscosity reducing excipient
5 between 50 and 200 mM each, more preferably between 50 and 100 mM each, most preferably 75 mM each. Preferably the protein is an antibody and has a molecular weight from 120 kDa to 250 kDa. Preferably the formulation has a pH between 4 and 8, more preferably between 5 and 7.2 and the viscosity is between 1 mPas and 60 mPas when measured at 20 °C using a microfluidic viscometer.

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The invention further provides a liquid composition according to the invention whereas the protein is Infliximab. Preferably the Infliximab concentration in the liquid compositions is between 122 mg/ml and 185 mg/ml.

15 Infliximab (REMICADE®) developed by Janssen Biotech, Inc. and its biosimilar drugs (FUXABI®) developed by Biogen, (Inflectra) by Celltrion, is used in treatment of rheumatoid arthritis, adult ulcerative colitis, plaque psoriasis, psoriatic arthritis, ankylosing spondylitis, adult & pediatric Crohn's disease (Dose/Dosage: 5 mg/kg). Infliximab is a mAb against tumor necrosis factor alpha (TNF-α) used to treat
20 autoimmune diseases. Infliximab neutralizes the biological activity of TNFα by binding with high affinity to the soluble and transmembrane forms of TNFα and inhibits binding of TNFα with its receptors. It is marketed under the trade name REMICADE® by Janssen Global Services, LLC ("Janssen") in the U.S., Mitsubishi Tanabe Pharma in Japan, Xian Janssen in China, and Merck Sharp & Dohme
25 ("MSD"); elsewhere. In some embodiments, the formulations contain a biosimilar of REMICADE®, such as REMSIMA™ or INFLECTRA™. Both REMSIMA™, developed by Celltrion, Inc. ("Celltrion"), and INFLECTRA™, developed by Hospira Inc., UK. Infliximab is currently administered via iv infusion at doses ranging from about 3 mg/kg to about 10 mg/kg.

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The invention further provides a liquid composition according to the invention whereas the protein is Evolocumab. Preferably the Evolocumab concentration in the liquid compositions is between 163 mg/ml and 204 mg/ml.

Evolocumab (REPATHA®) developed by Amgen is used in treatment of HeFH, CVD, reducing of low density lipoprotein cholesterol (LDL-C) by targeting PCSK9 (proprotein convertase subtilisin kexin type 9) (Dose/Dosage: 420 mg monthly).

5 The invention further provides a kit comprising compositions and formulations according to the invention. The kit may additionally comprise instructions for administration as well as a container, a syringe and/or other device for administration.

10 The invention is also directed to a kit comprising a lyophilized protein formulation of the invention, optionally in a container, and instructions for its reconstitution and administration, optionally with a vial of sterile diluent, and optionally with a syringe or other administration device. Exemplary containers include vials, tubes, bottles, single or multi-chambered pre-filled syringes, or cartridges, but also a 96-well plate comprising ready-to-use freeze-dried or spray-dried formulations sitting in the wells.

15 Exemplary administration devices include syringes with or without needles, infusion pumps, jet injectors, pen devices, transdermal injectors, or other needle-free injectors.

20 The invention is also directed to methods for reducing the viscosity of a protein solution for the purpose of the manufacture of all aforementioned liquid compositions and formulations. All embodiments regarding the combinations and concentrations of excipients, proteins, concentrations and molecular weight of proteins, pH, buffer and buffer concentrations as mentioned for the liquid composition above are also applicable for the methods for reducing the viscosity of

25 a protein solution.

30 The invention further provides a method for reducing the viscosity of a protein solution comprising a step of adding at least one first excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol or salts or solvates thereof to the protein solution.

5 The invention further provides a method for reducing the viscosity of a protein solution comprising a step of adding at least one first excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol or salts or solvates thereof and a step of adding a second viscosity reducing excipient.

10 The invention further provides a method for reducing the viscosity of a protein solution comprising a step of adding at least one first excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol or salts or solvates thereof and a step of adding a second excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, 15 camphorsulfonic acid and benzenesulfonic acid or salts or solvates thereof to the protein solution.

20 The invention further provides a method for reducing the viscosity of a protein solution, comprising a step of adding at least one first viscosity reducing excipient as mentioned above or salts or solvates thereof and a second viscosity reducing excipient as mentioned above or salts or solvates thereof.

25 The invention further provides a method for reducing the viscosity of a protein solution, comprising a step of adding at least one first viscosity reducing excipient selected from the group consisting of pyridoxine, folic acid, thiamine monophosphate and phenylalanine or salts or solvates thereof and a second viscosity reducing excipient selected from the group consisting of arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid or salts or solvates thereof to the protein solution.

30 The invention further provides a method for reducing the viscosity of a protein solution, wherein the combination of a first and a second viscosity reducing excipient is selected from the list consisting of pyridoxine and arginine, folic acid and ornithine, folic acid and carnithine, pyridoxine and meglumine, thiamine monophosphate and

meglumine, pyridoxine and thiamine monophosphate, phenylalanine and camphorsulfonic acid and phenylalanine and benzenesulfonic acid.

5 The invention further provides a method for reducing the viscosity of a protein solution as described above, whereas the protein in the protein solution has a molecular weight from 120 kDa to 250 kDa, preferably from 130 kDa to 180 kDa.

10 The invention further provides a method for reducing the viscosity of a protein solution as described above, whereas the protein concentration in the protein solution is at least 1 mg/ml, at least 50 mg/ml, preferably at least 75 mg/ml and more preferably at least 100 mg/ml. In another preferred embodiment, the protein concentration is between 90 mg/ml and 300 mg/ml, more preferably the protein concentration is between 100 and 200 mg/ml. The present invention is particularly useful for these high-concentration protein solutions.

15 The invention further provides a method for reducing the viscosity of a protein solution as described above, whereas the protein solution is further comprising a buffer at a concentration of 5 mM to 50 mM, preferably 10 mM to 50 mM. The buffer can be a suitable acetate- or phosphate salt and provide a pH of 5 to 7.2.

20 The invention further provides a method for reducing the viscosity of a protein solution as described above, whereas the protein solution is further comprising a sugar as stabilizer, preferably 50 to 100 mg/ml sucrose.

25 The invention further provides a method for reducing the viscosity of a protein solution as described above, whereas the protein solution is further comprising a surfactant, preferably 0.01 to 0.2 mg/ml, more preferably 0.05 mg/ml of polysorbate 80.

30 The invention further provides a method for reducing the viscosity of a protein solution as described above, whereas the resulting viscosity of the solution is between 1 mPas and 60 mPas, preferably between 1 mPas and 50 mPas, more preferably between 1 mPas and 30 mPas, most preferably between 1 mPas and 20 mPas. Preferably the viscosity is measured at 20 °C, using a microfluidic viscometer. More preferably the viscosity is measured using a RheoSense mVROC

microfluidic viscometer at 20 °C. Most preferably the viscosity is measured using a RheoSense mVROC microfluidic viscometer at 20 °C, using a 500 µl syringe, a shear rate of 3000 s⁻¹ or 2000 s⁻¹ and a volume of 200 µl.

5 The invention further provides a method for reducing the viscosity of a protein solution as described above, whereas viscosity is reduced by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70% or 75% compared to an identical composition not comprising the at least one first excipient or not comprising the at least one first and at least one second excipient.

10 The invention further provides a method for reducing the viscosity of a protein solution as described above, whereas the protein in the protein solution has a molecular weight from 120 kDa to 250 kDa at a concentration between 90 mg/ml to 300 mg/ml, the solution is further comprising an acetate buffer or phosphate buffer
15 at a concentration between 10 mM to 50 mM, wherein the solution formulation has a pH between 5 and 7.2, and a viscosity between 1 mPas and 60 mPas when measured at 20 °C, preferably using a microfluidic viscometer, more preferably using a RheoSense mVROC microfluidic viscometer at 20 °C, most preferably using a RheoSense mVROC microfluidic viscometer at 20 °C with a 500 µl syringe, a
20 shear rate of 3000 s⁻¹ or 2000 s⁻¹ and a volume of 200 µl.

The invention further provides a method for reducing the viscosity of a protein solution as described above, wherein a first and a second viscosity reducing excipient are present as mentioned above. Preferably, such embodiments of the
25 invention have a protein concentration between 90 mg/ml to 300 mg/ml, more preferably between 100 mg/ml and 200 mg/ml and a concentration of the first and second viscosity reducing excipient between 50 and 200 mM each, more preferably between 50 and 100 mM each, most preferably 75 mM each. Preferably the protein is an antibody and has a molecular weight from 120 kDa to 250 kDa. Preferably the
30 formulation has a pH between 5 and 7.2 and the viscosity is between 1 mPas and 60 mPas when measured at 20 °C, preferably using a microfluidic viscometer.

Likewise, the invention is directed to methods for increasing the stability of a protein solution comprising a step of adding at least one viscosity reducing excipient

selected from the group consisting of valine, leucine, ascorbic acid, cyanocobalamin and proline or salts or solvates thereof to the solution.

5 The invention further provides a method of preventing self-association of a protein in a solution by adding at least one first excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol or salts or solvates thereof to the solution or a combination of at least two viscosity reducing excipients as mentioned above.

10 In one aspect, combinations of first excipients are added in the methods of the invention. The combinations used in the methods of invention may be the same ones as defined for the compositions and formulations of the invention.

15 In one aspect, the methods of the invention further comprise a step of adding a second excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid to the solution. Herein, the same combinations of first and second excipients as defined for the compositions and formulations of the invention may be added.

20 In the methods according to the invention, the excipients may be added in any way known to the artisan to the protein solution. When more than one excipient is added, the excipient may be mixed together to form a viscosity-reducing solution which is then added to the protein solution. Likewise, the excipients may be added separately
25 to the protein solution.

In the methods according to the invention, the protein may be a therapeutic protein. In a preferred embodiment, the protein is an antibody as defined above. In the methods according to the invention, the protein may be a plasma derived protein. In
30 a further preferred embodiment, the protein is an IgG or hyper-IgG as defined above.

In a preferred embodiment of the invention the liquid composition is a pharmaceutical composition. The invention is also directed to a pharmaceutical

composition as described above comprising a therapeutic protein for the treatment of disease.

5 In particular pharmaceutical compositions as described above comprising a therapeutic protein are suitable for the treatment of cancer, rheumatoid arthritis, morbus crohn, colitis ulcerose, ankylosing spondylitis, psoriasis-arthritis, psoriasis, hypercholesterolemia, mixed dyslipidemia, homozygous familial hypercholesterolemia, myocardial infaction, peripheral arterial disease or immune deficiency disorders.

10 The invention is also directed to methods of treatment, wherein the treatment comprises administration of a pharmaceutical composition as described above comprising a therapeutic protein.

15 In one aspect, the method of treatment is a method of treating cancer. That is, the compositions and formulations according to the invention are useful for the treatment of cancer, rheumatoid arthritis, morbus crohn, colitis ulcerose, ankylosing spondylitis, psoriasis-arthritis, psoriasis, hypercholesterolemia, mixed dyslipidemia, homozygous familial hypercholesterolemia, myocardial infaction, peripheral arterial
20 disease or immune deficiency disorders.

At a protein concentration of 122 mg/ml as determined via absorption spectroscopy the protein viscosity has exceeded the limit of convenient injectability in the absence of excipients. The limit of convenient injectability is dependent on many factors, such
25 as the length and inner diameter of the needle, the inner diameter of the syringe barrel. It is known, that drug products with a viscosity of up to 100 mPas can be injected to the patient. However, a viscosity of 60 mPas is preferred. Lower viscosities of below 30 mPas or below 20 mPas are even more preferred due to increased patient convenience.

30 Addition of valine, leucine, phenylalanine or proline reduced the viscosity of the formulation. At a higher concentration of 143 mg/ml the viscosity of the formulation in the absence of excipient is dramatically increased to values above 80 mPas. Addition of leucine, phenylalanine and proline reduced viscosity of the formulation.

In this experiment phenylalanine was found to be the most efficient viscosity reducing excipient, in particular for higher protein concentration.

5 At a protein concentration of 149 mg/ml addition of 150 mM pyridoxine, thiamine hydrochloride or thiamine monophosphate was found to reduce the viscosity of the formulation. Also, addition of 5 mM cyanocobalamin, 13 mM folic acid or 75 mM of thiamine pyrophosphate was found to reduce the viscosity of the formulation. Especially thiamine hydrochloride show a high viscosity reducing potential.

10 At an even higher protein concentration of 174 mg/ml a viscosity reducing effect of 150 mM pyridoxine, ascorbic acid, thiamine hydrochloride or thiamine phosphate was observed. Also, addition of 5 mM cyanocobalamin, 13 mM folic acid or 75 mM of thiamine pyrophosphate was found to reduce the viscosity of the formulation compared to a formulation without viscosity reducing excipients. Especially thiamine hydrochloride, thiamine monophosphate showed a high viscosity reducing potential.

15 Thiamine pyrophosphate and thiamine hydrochloride, thiamine monophosphate were assessed at even higher protein concentrations of 152 mg/ml and 185 mg/ml. 75 mM thiamine pyrophosphate and 150 mM thiamine hydrochloride, thiamine monophosphate were found to have a strong viscosity reducing effect on the Infliximab solution.

20 An increase of the thermodynamic transition temperature characterizing heat induced protein unfolding (T_m) was observed upon the addition of 150 mM valine, leucine, ascorbic acid, or proline. Likewise addition of 5 mM cyanocobalamin resulted in an increase of T_m . Additionally, addition of 150 mM valine, leucine,

25 ascorbic acid, and proline led to an increase of the aggregation onset temperature T_{agg} .

Using excipient combinations the thermodynamic unfolding temperature can likewise be increased. We observe an increase of T_m for combinations comprising

30 of each 75 mM Cation/Anion when using ornithine/pyridoxine, ornithine/thiamine monophosphate, ornithine/thiamine pyrophosphate, arginine/pyridoxine, arginine/thiamine monophosphate, arginine/thiamine pyrophosphate, carnitine/pyridoxine, carnitine /thiamine monophosphate, phenylalanine/thiamine monophosphate, and, phenylalanine/thiamine pyrophosphate.

At a protein concentration of 192 mg/ml, as measured using absorption spectroscopy, a reduced viscosity for Evolocumab formulations upon addition of 150 mM leucine, isoleucine, phenylalanine, lysine, and guanidine hydrochloride was observed. When a higher protein concentration of 192 mg/ml was used, a reduced viscosity using 150 mM leucine, isoleucine, phenylalanine, proline, lysine, and guanidine hydrochloride as excipients was observed. leucine, lysine and guanidine hydrochloride, showed the most pronounced viscosity reducing effect.

At a protein concentration of 180 mg/ml determined using a Bradford assay, viscosity of a Evolocumab formulation was reduced upon addition of 150 mM pyridoxine or thiamine hydrochloride. Likewise, addition of 5 mM cyanocobalamin, 25 mM quinine hydrochloride or 75 mM paracetamol reduced viscosity of the formulation.

At an even higher protein concentration of 196 mg/ml addition of 150 mM pyridoxine, thiamine hydrochloride or ascorbic acid reduced viscosity of the formulation. Also, the addition of 5 mM cyanocobalamin, 25 mM quinine hydrochloride or 75 mM Paracetamol reduced the viscosity of the formulation. In this data set thiamine hydrochloride was found to be the most efficient viscosity reducing excipient.

In a third data set viscosity reducing capacity of 75 mM thiamine pyrophosphate and 150 mM thiamine hydrochloride, thiamine monophosphate were compared. Both excipients reduced the viscosity of a Evolocumab formulation. 75 mM thiamine pyrophosphate is highly efficient when used for formulations with 179 mg/ml and 204 mg/ml Evolocumab. 150 mM thiamine monophosphate was only tested at a Evolocumab concentration of 180 mg/ml.

It has been found that combinations of two viscosity reducing excipients can synergistically reduce the viscosity of a protein solution. The percentual viscosity of an Infliximab formulation at 150 mg/ml, pH 7.2 was synergistically reduced upon addition of a combination of ornithine and folic acid and a combination of carnitine and folic acid.

5 The percentual viscosity of an Evolocumab formulation at 190 mg/ml, pH 5.0 was synergistically reduced upon addition of a combination of phenylalanine and camphorsulfonic acid, a combination of phenylalanine and benzenesulfonic acid, a combination of arginine and pyridoxine, a combination of meglumine and pyridoxine and a combination of meglumine and thiamine monophosphate.

10 Furthermore, it has been found that the excipients and excipient combinations as mentioned above are beneficial in the bioprocess. Experiments according to the present invention suggest that excipients and excipient combinations are suitable to reduce the backpressure on chromatography columns and allow for larger flow rates. This leads to less shear forces straining the proteins in the solution and therefore, aggregation will be reduced. Altogether a higher yield can be obtained. Beyond this, when the process can be run using higher flow rates, the process time will be reduced significantly.

15 In bioprocesses as meant here, the addition of these excipients, which are found to serve as viscosity-reducing additives, lead to an improved process economy, in that on the one hand the yield of intact protein can be improved, and on the other hand the duration of the process can be reduced.

20 In the present invention the foundation of the experiments conducted is laid by Amicon® centrifugal filtration studies. These experiments resemble any process step, where a solution is passed through a filter membrane by centrifugal force. The resistance to the centrifugal force is dependent on the properties of the filter, which is assumed to be constant within the context of this experiments, and the viscosity of the solution. Excipients and excipient combinations that provide the most favorable solutions are selected for more complex experiments using stirred cells.

25 In experiments using Amicon® stirred cells nitrogen gas is used to apply pressure to the solution that is being pressed through a filter. The resistance of the pressure is dependent on the properties of the filter, which is assumed to be constant within the context of this experiments, and the viscosity of the solution. The excipient combination that gives most favorable results is used in an experiment using a laboratory scale tangential flow filtration (TFF) system.

These experiments highlight the beneficial effects of viscosity reducing agents during dead-end filtration approaches. Additionally, it becomes clear that in technical approaches, where a solution is passed through a filter or medium, like a gel bed, by a force applied by back-end pressure, e.g. during chromatographic purifications, the disclosed viscosity reducing excipients have beneficial effects. While said filtration steps are mainly used in the downstream process, viscosity reducing excipients can also be beneficial in the upstream process. When protein concentrations elevate to levels where they cause viscosity, with the described negative effects of pressure limitations and shear forces when the solution is passed through a tubing or a filter to remove cellular material and debris the presented invention will obviously have beneficial effects. To measure process efficiency in tangential flow filtration, where in contrast to previously used method the majority of the field flow travels tangentially across the surface of the filter, rather than passing through the filter, a laboratory scale TFF system was used. While the filtration principle is different than in the methods described previously, also here the filtration efficiency depends on the resistance of the membrane, which also here remains constant, and the solution viscosity, which is modified by the invention. Filtration methods are typical unit operation used to exchange a formulation buffer or to bring the concentration of a biomolecule to the desired level. The stirred cells used herein are representation of dead-end filters, where a feed is passed through a filtering material that withholds larger molecules on top of the material releasing the filtrate on the other end of the device.

A frequent method to exchange buffers and to concentrate proteins is tangential flow filtration, where in contrast to previously used methods the majority of the field flow travels tangentially across the surface of the filter, rather than passing through the filter. Like when stirred cells are used in tangential flow filtration the large molecules are separated from smaller molecules by passing said smaller molecules through a suitable filter material. In contrast to stirred cells, which represent one form of dead end filtration, in tangential flow filtration the flow geometry of the feed is different to avoid the formation of a filter cake and allowing for a continuous process. When stirred cells are used the formation of a filter cake is likewise prevented by the use of a stirring device. Therefore, the stirred cells closely

resemble a tangential flow filtration device in spite of the differences in filter geometry. The efficiency of both methods is critically depending on the membrane resistance. Also, a high viscosity is known to reduce the flux rate that can be used and therefore increasing processing time resulting in higher production costs. It is therefore expected that a reduced viscosity allows for a more efficient filtration process while shear forces remain low yielding to a higher protein concentration in the filtrate. This is highlighted by the work of Hung et al. who state: "During production of concentrated monoclonal antibody formulations by tangential flow ultrafiltration (TFF), high viscosities and aggregation often cause extensive membrane fouling, flux decay and low product yields" (Journal of Membrane Science Volume 508, 15 June 2016, Pages 113-126)

Experiments have shown that viscosity reducing excipients selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol or salts or solvates thereof are suitable to improve bioprocess economics as described before. Furthermore, viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol or salts or solvates thereof in combination with a second viscosity reducing excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid improve bioprocess economics. Especially combinations in various ratios depending on the protein solutions improve bioprocess economics as described.

Therefore, another aspect of the present invention is to provide a method for reducing the viscosity of a protein solution in a bioprocess, comprising a protein in a concentration in the range of at least 90 mg/ml up to 300 mg/ml, comprising the step of combining the protein solution with first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol. In another aspect, the

protein solution is combined with a first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol and a second viscosity
5 reducing excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.

Another aspect of the present invention is the use of the method for reducing the
10 viscosity of a protein solution as described above in a bioprocess.

According to the present invention all parameters mentioned above; e.g. combinations of the excipients, concentrations of the excipients, concentrations the protein, ratios of the excipients, further elements of the composition such as buffers
15 or stabilizers, pH values, viscosity reductions, specifications of the protein, molecular weight of the protein also apply to the use in bioprocess.

Preferably the at least one first viscosity reducing excipient is selected from the group consisting of pyridoxine, folic acid, thiamine monophosphate and
20 phenylalanine and at least one second viscosity reducing excipient is selected from the group consisting of arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.

Preferably a combination is used comprising a first and a second viscosity reducing
25 excipient selected from the list consisting of pyridoxine and arginine, folic acid and ornithine, folic acid and carnithine, pyridoxine and meglumine, thiamine monophosphate and meglumine, pyridoxine and thiamine monophosphate, phenylalanine and camphorsulfonic acid and phenylalanine and benzenesulfonic acid.

30 Preferably the first viscosity reducing excipients is thiamine, thiamine monophosphate, thiamine pyrophosphate or pyridoxine, more preferably thiamine and thiamine pyrophosphate. Preferably a concentration of 75 – 500 mM, more preferably 75 – 150 mM, most preferably 75 mM or 150 mM is used.

5 Preferably the second viscosity reducing excipients is arginine or ornithine. Preferred combinations are thiamine and arginine as well as thiamine and ornithine. Preferably the concentration of each excipient is 75 – 500 mM, more preferably 75 – 150 mM, most preferably 75 mM or 150 mM.

10 When a first viscosity reducing excipient is used in combination with a second viscosity reducing excipient, the ratio is in the range of 1:3 to 3:1, more preferred 1:2 to 2:1, most preferred 1:1.

15 Depending on the protein solution and the bioprocess carried out, different buffer systems may be used as buffers. Acetates, like ammonium acetate, or sodium acetate, carbonates like ammonium bicarbonate or sodium bicarbonate, or phosphates, like sodium phosphate or Tris-phosphate may be used here, depending on the conditions during the bioprocess.

20 Another aspect of the present invention is to provide a method for reducing the viscosity of a protein solution in a bioprocess as mentioned above, wherein the permeate flux of the protein solution in a filtration step is increased compared to an identical protein solution not comprising camphorsulfonic acid or compared to an identical protein solution not comprising at least one first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol or compared to an identical protein solution not comprising at least one first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol and a second viscosity reducing excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.

Another aspect of the present invention is the use of the method in a bioprocess as mentioned above, wherein the permeate flux of the protein solution in a filtration

- step is increased compared to an protein solution composition not comprising camphorsulfonic acid or compared to an identical protein solution not comprising at least one first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol or compared to an identical protein solution not comprising at least one first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol and a second viscosity reducing excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.
- Another aspect of the present invention is to provide a method for reducing the viscosity of a protein solution in a bioprocess as mentioned above, wherein the permeate flux of the protein solution in a filtration step is increased compared to an identical protein solution not comprising at least one first viscosity reducing excipient selected from the group consisting of pyridoxine, folic acid, thiamine monophosphate and phenylalanine and at least one second viscosity reducing excipient selected from the group consisting of arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.
- Another aspect of the present invention is to provide a method for reducing the viscosity of a protein solution in a bioprocess as mentioned above, wherein the permeate flux of the protein solution in a filtration step is increased compared to an identical protein solution not comprising a combination of a first and a second viscosity reducing excipient selected from the list consisting of pyridoxine and arginine, folic acid and ornithine, folic acid and carnithine, pyridoxine and meglumine, thiamine monophosphate and meglumine, pyridoxine and thiamine monophosphate, phenylalanine and camphorsulfonic acid and phenylalanine and benzenesulfonic acid.

Increase of permeate flux means a percentage increase of at least 2%, preferably at least 5%, more preferably at least 10%, most preferred 10% to 100%.

- 5 Another aspect of the present invention is to provide a method for reducing the viscosity of a protein solution in a bioprocess as mentioned above, wherein the protein recovery after buffer exchange and volume reduction in filters is increased compared to an identical protein solution not comprising camphorsulfonic acid or compared to an identical protein solution not comprising at least one first viscosity
- 10 reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol or compared to an identical protein solution not comprising at least one first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine
- 15 pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol and a second viscosity reducing excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.
- 20 Another aspect of the present invention is the use of the method in a bioprocess as mentioned above, wherein the protein recovery after buffer exchange and volume reduction in filters is increased compared to an identical protein solution not comprising camphorsulfonic acid or compared to an identical protein solution not comprising at least one first viscosity reducing excipient selected from the group
- 25 consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol or compared to an identical protein solution not comprising at least one first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine,
- 30 thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol and a second viscosity reducing excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.

Another aspect of the present invention is the use of the method in a bioprocess as mentioned above, wherein the protein recovery after buffer exchange and volume reduction in filters is increased compared to an identical protein solution not comprising at least one first viscosity reducing excipient selected from the group consisting of pyridoxine, folic acid, thiamine monophosphate and phenylalanine and at least one second viscosity reducing excipient selected from the group consisting of arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.

Another aspect of the present invention is the use of the method in a bioprocess as mentioned above, wherein the protein recovery after buffer exchange and volume reduction in filters is increased compared to an identical protein solution not comprising a combination of a first and a second viscosity reducing excipient selected from the list consisting of pyridoxine and arginine, folic acid and ornithine, folic acid and carnithine, pyridoxine and meglumine, thiamine monophosphate and meglumine, pyridoxine and thiamine monophosphate, phenylalanine and camphorsulfonic acid and phenylalanine and benzenesulfonic acid.

Increase of protein recovery after buffer exchange and volume reduction in filters means a percentage increase of protein recovery of at least 1%, preferably at least 2%, more preferably at least 5%, most preferred 5% to 20%.

Another aspect of the present invention is to provide a method for reducing the viscosity of a protein solution in a bioprocess as mentioned above, wherein the process time for a filtration step, preferably a filtration step wherein the protein is concentrated, is reduced compared to an identical protein solution not comprising camphorsulfonic acid or compared to an identical protein solution not comprising at least one first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol or compared to an identical protein solution not comprising at least one first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride,

quinine hydrochloride and paracetamol and a second viscosity reducing excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.

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Another aspect of the present invention is the use of the method in a bioprocess as mentioned above, wherein the process time for a filtration step, preferably a filtration step wherein the protein is concentrated, is reduced compared to an identical protein solution not comprising camphorsulfonic acid or compared to an identical protein solution not comprising at least one first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol or compared to an identical protein solution not comprising at least one first viscosity reducing excipient selected from the group consisting of cyanocobalamin, pyridoxine, ascorbic acid, folic acid, thiamine, thiamine monophosphate, thiamine pyrophosphate, guanidine hydrochloride, quinine hydrochloride and paracetamol and a second viscosity reducing excipient selected from the group consisting of valine, proline, leucine, isoleucine, phenylalanine, arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.

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Another aspect of the present invention is the use of the method in a bioprocess as mentioned above, wherein the process time for a filtration step, preferably a filtration step wherein the protein is concentrated, is reduced compared to an identical protein solution not comprising at least one first viscosity reducing excipient selected from the group consisting of pyridoxine, folic acid, thiamine monophosphate and phenylalanine and at least one second viscosity reducing excipient selected from the group consisting of arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.

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Another aspect of the present invention is the use of the method in a bioprocess as mentioned above, wherein the process time for a filtration step, preferably a filtration step wherein the protein is concentrated, is reduced compared to an identical protein solution not comprising a combination of a first and a second viscosity reducing

5 excipient selected from the list consisting of pyridoxine and arginine, folic acid and ornithine, folic acid and carnithine, pyridoxine and meglumine, thiamine monophosphate and meglumine, pyridoxine and thiamine monophosphate, phenylalanine and camphorsulfonic acid and phenylalanine and benzenesulfonic acid.

10 Reduction of process time for a filtration step means a percentage reduction of at least 5%, preferably at least 10%, more preferably at least 25%, most preferred 25% to 100%.

10 In a particular embodiment of the invention, the filtration step is a tangential flow filtration (TFF).

15 The term "bioprocess" refers to therapeutic cell manufacturing processes, which can be separated into upstream processes and downstream processes. The upstream process is defined as the entire process prior to separating protein from cellular compounds. The upstream process comprises early cell isolation and cultivation, to cell banking and culture expansion of the cells until final harvest. The downstream part of a bioprocess refers to the part where the target protein is purified from the feed of the upstream and is processed to meet purity and quality requirements. 20 Some type of cells need to be disrupted when entering the downstream process. Yet other cells may secrete the target protein into the media and need to be removed via filtration. Further downstream processing is usually divided into the main sections: a purification section and a polishing section. A bioprocess can be a batch process or a semi-continuous or a continuous process. 25

The term "permeate flux" refers to the volume passing through a defined filter within a certain period of time, typically on the order of minutes.

30 The term "filtration step" refers to a process step where a liquid is passed through a material with a defined pore size allowing for the separation of materials based on their size. For some filters the pore size is defined in nanometers. Yet for other filters, the pore size is not directly defined, but the weight of a molecule to be withheld is given. Filtering materials can be placed in a way that they block the cross-

section of the filtration device (dead-end filtration). Yet filtering materials can be placed in a way that the solution to be filtered is tangentially flowing across the surface of said material, e.g. tangential flow filtration. The filtering material can be a membrane, a glass filter, a metallic filter or a resin. The resin can be held in a chromatography column. The resin can be a cationic or anion exchange resin, an affinity resin, like a Protein A or glutathione resin, or a hydrophobic or hydrophilic resin.

The term "protein recovery" after buffer exchange and volume reduction refers to the fraction of protein to be retrieved after a process step.

The term "tangential flow filtration" or "TFF" refers to a method of filtration where a solution passes over a defined filter tangentially. Substances smaller than the filter pores are forced out of the solution through the filter by the pressure resulting from solution flow rate, viscosity, temperature and other factors.

Examples

1. Viscosity measurements

1.1 Viscosity reducing effect of valine, leucine, phenylalanine, proline, ascorbic acid, pyridoxine, cyanocobalamin, thiamine hydrochloride, folic acid, thiamine pyrophosphate and thiamine monophosphate on Infliximab formulated in phosphate buffer at pH 7.2

Buffer Preparation

5 mM phosphate buffer was prepared by appropriately mixing sodium dihydrogenphosphate and di-sodium hydrogenphosphate to yield a pH of 7.2 and dissolving the mixture in ultrapure water. The ratio was determined using the Henderson-Hasselbalch equation. pH was adjusted using HCl and NaOH where necessary.

50 mg/ml sucrose and 0.05 mg/ml polysorbate 80 were added as stabilizers.

Sample Preparation

Individual excipient solutions of 150 mM valine, leucine, phenylalanine, ascorbic acid, pyridoxine, proline and thiamine monophosphate were prepared in phosphate buffer pH 7.2. In the same buffer, a 5 mM solution of cyanocobalamin was prepared.

5 Folic acid was prepared in a 13 mM concentration in phosphate puffer pH 7.2. Thiamine pyrophosphate was prepared in a 75 mM concentration in phosphate puffer pH 7.2. The pH was adjusted using HCl or NaOH where necessary.

10 A concentrated Infliximab solution containing the desired excipients was prepared using centrifugal filters (Amicon, 30 kDa MWCO) to exchange the original buffer with a buffer containing the respective excipient and to reduce the volume of the solution. The protein was subsequently diluted to 122 mg/ml and 143 mg/ml, respectively.

Protein Concentration Measurements

15 Protein Concentration was determined using absorption spectroscopy applying Lambert-Beer's law. When excipients themselves had a strong absorbance at 280 nm, a Bradford assay was used.

Concentrated protein solutions were diluted so that their expected concentration would lie between 0.3 and 1.0 mg/mL in the measurement.

20 For absorption spectroscopy, the absorbance at 280 nm was measured using a BioSpectrometer® kinetic (Eppendorf, Hamburg, Germany) with a protein extinction coefficient of $A_{0.1\%, 280\text{nm}} = 1.428$.

25 Some excipients have themselves a strong absorption at 280 nm, which makes it necessary to use a Bradford assay for concentration determination.

For the Bradford assay, a kit as well as Bovine Gamma Globulin Standard from Thermo Scientific™ (Thermo Fisher, Waltham, Massachusetts, USA) were used. Absorption was measured at 595 nm using a Multiskan™ Wellplatereader (Thermo Fisher, Waltham, Massachusetts, USA). Protein concentrations were determined by

30 linear regression of a standard curve from 125 to 1500 µg/ml.

Viscosity Measurements

The mVROC™ Technology (Rheo Sense, San Ramon, California, USA) was used for viscosity measurements.

5 Measurements were performed at 20 °C using a 500 µl syringe and a shear rate of 3000 s⁻¹. A volume of 200 µl was used. All samples were measured as triplicates.

Results of viscosity measurements regarding these experiments can be seen in Fig. 1, Fig. 2 and Fig. 3.

10 1.2 *Viscosity reducing effect of leucine, isoleucine, phenylalanine, proline, lysine, guanidine hydrochloride, ascorbic acid, pyridoxine, cyanocobalamin, quinine hydrochloride, thiamine hydrochloride, paracetamol, thiamine pyrophosphate and thiamine monophosphate on Evolocumab formulated in acetate buffer pH 5.0*

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Buffer Preparation

20 mM Acetate buffer was prepared by mixing 1,2 mg/ml glacial acetic acid with ultrapure water. pH was adjusted to 5.0 using HCl and NaOH if necessary. 0.1 mg/ml Polysorbate 80 was added as stabilizer

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Sample Preparation

Excipient solutions of 150 mM leucine, isoleucine, phenylalanine, ascorbic acid, pyridoxine, proline, lysine, guanidine hydrochloride, thiamine hydrochloride, thiamine monophosphate were prepared in acetate buffer pH 5.0, respectively. Similarly, 25 mM quinine hydrochloride and 5 mM cyanocobalamin were prepared, respectively. Paracetamol and thiamine pyrophosphate were prepared with a concentration of 75 mM. The pH was adjusted using HCl or NaOH, if necessary. A concentrated Evolocumab solution containing the desired excipients was prepared using centrifugal filters (Amicon, 30 kDa MWCO) to exchange the original buffer with a buffer containing the relevant excipients and to reduce the volume of the solution. The protein was subsequently diluted to 172 mg/ml and 192 mg/ml respectively for experiments regarding leucine, isoleucine, phenylalanine, proline, lysine and guanidine hydrochlorid, 180 and 196 mg/ml for experiments regarding ascorbic acid, pyridoxine, cyanocobalamin, quinine hydrochloride, thiamine

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hydrochloride and paracetamol, and 179 and 204 mg/ml for experiments regarding thiamine pyrophosphate and thiamine monophosphate.

Protein Concentration Measurements

5 Protein Concentration was determined using absorption spectroscopy applying Lambert-Beer's-Law. When excipients themselves have a strong absorbance at 280 nm a Bradford-Assay was used.

Concentrated protein solutions were diluted so that their expected concentration would lie between 0.3 and 1.0 mg/mL in the measurement.

10 For absorption spectroscopy the absorbance at 280 nm was measured at 280 nm using a BioSpectrometer® kinetic (Eppendorf, Hamburg, Germany) with a protein extinction coefficient of $A_{0.1\%, 280\text{nm}} = 1.428$.

For Bradford-Assay a kit as well as a Bovine Gamma Globulin Standard from Thermo Scientific™ (Thermo Fisher, Waltham, Massachusetts, USA) was used.

15 Absorption was measured at 595 nm using a Multiskan™ Wellplatereader (Thermo Fisher, Waltham, Massachusetts, USA). Protein Concentrations were determined by linear regression of a standard curve from 125 to 1500 µg/mL.

Viscosity Measurements

20 The mVROC™ Technology (Rheo Sense, San Ramon, California USA) was used for viscosity measurements.

Measurements were performed at 20 °C using a 500 µl syringe and a shear rate of 3000 s⁻¹ for protein solutions from 160-179 mg/ml and of 2000 s⁻¹ for protein solutions from 180-210 mg/ml. A volume of 200 µl was used. All samples were measured as triplicates.

25

Results of viscosity measurements regarding these experiments can be seen in Fig. 4, Fig. 5 and Fig. 6.

30

1.3 Viscosity reducing effect of combinations of thiamine hydrochloride, pyridoxine, folic acid, phenylalanine, thiamine monophosphate and thiamine pyrophosphate on Infliximab formulated in phosphate buffer, pH 7.2.

Buffer Preparation

5 mM Phosphate buffer was prepared by appropriately mixing sodium dihydrogenphosphate and di-sodium hydrogenphosphate to yield a pH of 7.2 and dissolving the said mixture in ultrapure water. The said ratio was determined using the Henderson-Hasselbalch equation. pH was adjusted using HCl and NaOH if necessary.

50 mg/ml Sucrose and 0.05 mg/ml Polysorbate 80 were added as stabilizers.

Sample Preparation

Excipient solutions of 75 mM thiamine hydrochloride or 75 mM phenylalanine dissolved in phosphate buffer, pH 7.2 were supplemented with 75 mM pyridoxine, 12mM folic acid, 75 mM thiamine monophosphate or thiamine pyrophosphate. A concentrated Infliximab solution containing the desired excipients was prepared using centrifugal filters (Amicon, 30 kDa MWCO) to exchange the original buffer with a buffer containing the relevant excipients and to reduce the volume of the solution. The protein was subsequently diluted to 122 mg/ml and 154 mg/ml respectively.

Protein Concentration Measurements

Protein Concentration was determined using a Bradford-Assay. Therefore a kit from Thermo Scientific™ (Thermo Fisher, Waltham, Massachusetts, USA) as well as a Infliximab-Standard prepared by using absorption spectroscopy applying Lambert-Beer's-Law were used. Absorption was measured at 595 nm using a Multiskan™ Wellplaterreader (Thermo Fisher, Waltham, Massachusetts, USA). Protein Concentrations were determined by an appropriate polynomial regression of a standard curve from 125 to 1500 µg/mL.

Viscosity Measurements

Viscosity Measurements were performed according to the method described under 1.1.

Results of viscosity measurements regarding these experiments can be seen in Fig. 7.

- 1.4 Viscosity reducing effect of combinations of a first set consisting of L-ornithine, L-arginine, L-carnitine, camphorsulfonic acid and benzenesulfonic acid and a second set consisting of thiamine hydrochloride, phenylalanine, pyridoxine, folic acid, thiamine monophosphate and thiamine pyrophosphate on Infliximab formulated in phosphate buffer, pH 7.2.

Buffer Preparation

Buffer Preparation was performed according to the method described under 1.3.

10 Sample Preparation

Excipient solutions of 75 mM L-ornithine monohydrochloride, L-arginine, L-carnitine hydrochloride or phenylalanine dissolved in phosphate buffer, pH 7.2 were supplemented with 75 mM pyridoxine, 12mM folic acid, 75 mM thiamine monophosphate or 75 mM thiamine pyrophosphate. 75 mM thiamine hydrochloride or phenylalanine were supplemented with 75 mM camphorsulfonic acid or benzenesulfonic acid.

A concentrated Infliximab solution containing the desired excipients was prepared as described under 1.3.

20 Protein Concentration Measurements

Protein Concentration Measurements were performed according to the method described under 1.3.

Viscosity Measurements

Viscosity Measurements were performed according to the method described under 1.1.

Results of viscosity measurements regarding these experiments can be seen in Fig. 8.

- 1.5 Viscosity reducing effect of sodium chloride, arginine and combinations of a first set consisting of L-ornithine, L-arginine, L-carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid and a second set consisting of thiamine hydrochloride, pyridoxine, thiamine monophosphate and thiamine pyrophosphate on Evolocumab formulated in acetate buffer, pH 5.0.

Buffer Preparation

20 mM Acetate buffer was prepared by mixing 1,2 mg/ml glacial acetic acid with ultrapure water. pH was adjusted to 5.0 using HCl and NaOH if necessary.

5 0.1 mg/ml Polysorbate 80 was added as stabilizer

Sample Preparation

10 Excipient solutions of 75 mM L-ornithine monohydrochloride, L-arginine, L-carnitine hydrochloride or meglumine dissolved in acetate buffer, pH 5.0 were supplemented with 75 mM pyridoxine, 75 mM thiamine monophosphate or 75 mM thiamine pyrophosphate. 75 mM thiamine hydrochloride were supplemented with 75 mM pyridoxine, camphorsulfonic acid or benzenesulfonic acid.

15 A concentrated Evolocumab solution containing the desired excipients was prepared using centrifugal filters (Amicon, 30 kDa MWCO) to exchange the original buffer with a buffer containing the relevant excipients and to reduce the volume of the solution. The protein was subsequently diluted to 163 mg/ml and 180 mg/ml, respectively.

Protein Concentration Measurements

20 Protein Concentration was determined using a Bradford-Assay
Therefore a kit from Thermo Scientific™ (Thermo Fisher, Waltham, Massachusetts, USA) as well as a Evolocumab-Standard prepared by using absorption spectroscopy applying Lambert-Beer's-Law were used. Absorption was measured at 595 nm using a Multiskan™ Wellplatereader (Thermo Fisher, Waltham, Massachusetts, USA). Protein Concentrations were determined by an appropriate
25 polynomial regression of a standard curve from 125 to 1500 µg/mL.

Viscosity Measurements

30 Viscosity Measurements were performed according to the method described under 1.2.

Results of viscosity measurements regarding these experiments can be seen in Fig. 9.

1.6 *Synergies in viscosity reduction for a combination of arginine / pyridoxine, ornithine / folic acid, carnithine / folic acid, meglumine / pyridoxine, meglumine / thiamine monophosphate, pyridoxine / thiamine monophosphate, phenylalanine / camphorsulfonic acid and phenylalanine / benzenesulfonic acid*

5

Samples of Infliximab at 150 mg/mL containing 75mM L-ornithine monohydrochloride, L-carnitine hydrochloride, pyridoxine or thiamine monophosphate were prepared and analyzed as described in 1.1.

10

Samples of Evolocumab at 190 mg/mL containing 75mM meglumine, camphorsulfonic acid, benzenesulfonic acid, pyridoxine or thiamine monophosphate were prepared and analyzed as described in 1.2.

15

For these data and data obtained in 1.1 to 1.5 the percentual reduction compared to a respective control sample not containing investigated viscosity reducing excipients (viscosity of control) was calculated. Using these percentual viscosity reductions an expected viscosity reduction could be calculated if one would combine those.

20

Expected values can not be calculated based on absolute values, since two combined excipients which reduce the viscosity by 50% each would result in a viscosity of 0 mPas. From a scientific point this is not feasible, especially in case the excipients reduce the viscosity by more than 50% resulting in negative viscosity values. Hence, the expected viscosity reduction of both excipients was determined

25

based on a consecutive calculation:

Expected viscosity =

Viscosity of control * (100% - viscosity reduction [%] 1st excipient) * (100% - viscosity reduction [%] 2nd excipient)

30

Example calculation for a combination of two excipients reducing the viscosity by (i) 50% each and (ii) 75% each in a solution with a viscosity of 100 mPas:

(i) Expected viscosity = 100 mPas * (100% - 50%) * (100% - 50%) = 25 mPas

(i) Expected viscosity = 100 mPas * (100% - 75%) * (100% - 75%) = 6,25 mPas

If for a combination of two excipients a lower viscosity, i.e. higher viscosity reduction is observed compared to the expected one, the combination is found to be synergistic.

5

Figure 21 shows a synergistic viscosity reduction for the combinations ornithine / folic acid, carnitine / folic acid and pyridoxine / thiamine monophosphate. Figure 22 shows a synergistic viscosity reduction for the combinations phenylalanine / camphorsulfonic acid, phenylalanine / benzenesulfonic acid and arginine / pyridoxine. Figure 23 shows a synergistic viscosity reduction for the combinations meglumine / pyridoxine and meglumine / thiamine monophosphate.

10

2. Thermal stability measurements

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2.1 Thermal stability of Infliximab formulated in phosphate buffer pH 7.2 containing valine, leucine, ascorbic acid, cyanocobalamin and proline as single excipients

Buffers and samples were prepared as described under 1.1.

20

Thermal stability measurements

The nano-DSF Technology of a Prometheus system (NanoTemper Technologies GmbH, Munich, Germany) was used to determine melting points and aggregation points.

25

Fluorescence was measured at 330 and 350 nm as well as the backscattering intensity over a temperature range from 20 to 95 °C with an incline of 1 °C/min.

Results of thermal stability measurements regarding these experiments can be seen in Fig. 10.

30

2.2 *Thermal stability of Infliximab formulated in phosphate buffer pH 7.2 containing combinations of a first set consisting of L-ornithine, L-arginine, L-carnitine, phenylalanine and benzenesulfonic acid and a second set consisting of pyridoxine, thiamine monophosphate and thiamine pyrophosphate.*

Buffers and samples were prepared as described under 1.4 and 1.5.

Thermal stability measurements were performed as described under 2.1.

Results of thermal stability measurements regarding these experiments can be seen in Fig. 11.

3. Storage stability measurements

3.1 *Storage conditions*

The use of excipient combinations is beneficial to aid in managing the stability of a pharmaceutical formulation. To evaluate stability of said formulation, samples are stored at standardized conditions in terms of temperature and relative humidity. Typically assessed temperatures include 4 °C, 20 °C, 25 °C, and 40 °C. The relative moisture is typically 40%, 60% or 75%. Stability of protein formulations can be assessed using different criteria. Methods to measure said criteria need to be optimized and validated for the specific protein studied, a task that is obvious to a person skilled in the art.

We prepare pharmaceutical formulations of proteins at high concentrations >70 mg/ml and store them at 25 °C with a relative humidity of 60%. During the following 24 weeks samples are on 6 time points removed from storage and analyzed with respect to the following parameters: Fragmentation, aggregation/monomer content, content, conformational stability, particle content, and pH.

3.2 Protein fragmentation measurement

Fragmentation can be measured using an HPLC system with an appropriate size exclusion column that is sensitive to substances smaller than the antibody in question. Molecules are separated based on their size and the elution time of fragments shall be higher than the elution time of the intact protein. Fragments and protein are typically detected by absorption at 214 nm. Another method to detect fragmentation is denaturing gel electrophoresis, typically performed using a polyacrylamide gel containing sodium dodecyl sulphate (SDS-PAGE). SDS solubilizes proteins and their fragments masking potential charges and thereby allows for the separation of molecules by size within the gel matrix.

3.3 Protein aggregation measurement

Aggregation or monomer content can be measured using HPLC-SEC using a column suitable to determine the molecular weight of the target protein. When absorption at 214 nm is used, the numerical integral of the target protein peak can be used to determine the protein content of the sample. The protein content of a sample after incubation divided by a reference sample yields to the monomer content. In contrast to the HPLC method to determine fragmentation, the column used herein is suitable to detect molecular species that are larger than the target protein.

3.4 Protein content measurement

Protein content can be measured by the HPLC method described in the previous paragraph or alternatively using absorption spectroscopy applying the Lambert-Beer Equation. Additionally, a Bradford Assay may be used. To probe for conformational stability a thermal shift assay using fluorescence spectroscopy can be employed. By measuring the heat denaturation mid-point T_m changes in the protein conformation can be discovered, since changes in T_m would report on changes in the structure of the measured protein.

3.5 Particle analytical method

5 Particles can be visualized by light obscuration methods e.g. turbidity measurements, flow Imaging techniques, and dynamic light scattering dependent on their respective size.

pH can be monitored by using a pH-electrode.

Generally, deviations of < 10% of each parameter can be accepted. However, particle analytical method, in particular those focused on single particle counting, inherently have a comparatively high error of up to 30% due to the use of Poissonian statistics.

4. Benefits for centrifugal filter units

15 Buffer preparation

A 10 mM citrate buffer was prepared using citric acid monohydrate and dissolving it in ultrapure water. pH was adjusted using HCl and NaOH to 5.5 if necessary. 0,25 mg/mL Polysorbate 80 was added as stabilizer. Excipient solutions of Phenylalanine (Phe), Ornithine (OM), Arginine (Arg) Thiamine HCl, Thiamine monophosphate, and Thiamine pyrophosphate were prepared in citrate buffer, pH 5.5 with a concentration of 150 mM. Combinations containing two of these excipients were prepared with a concentration of 75 mM for each or 150 mM for each.

Sample Preparation

25 A Cetuximab solution containing approximately 14.7 mg/ml was used as starting material. Thereof, a sufficient volume was calculated to achieve a final concentration of more than 120 mg/ml in 500 µL sample assuming up to 20% sample loss.

Protein concentration measurements

30 Protein concentration was determined using absorption spectroscopy applying Lambert-Beer's-Law. When excipients themselves have a strong absorbance at 280 nm a Bradford-Assay was used.

Concentrated protein solutions were diluted so that their expected concentration would lie between 0.3 and 1.0 mg/mL in the measurement.

For absorption spectroscopy the absorbance at 280 nm was measured at 280 nm using a BioSpectrometer® kinetic (Eppendorf, Hamburg, Germany) with a protein extinction coefficient of A0.1%, 280nm=1.4.

- 5 For excipients that absorbed light at 280 nm protein concentration was determined using a Bradford-Assay. Therefore, a kit from Thermo Scientific™ (Thermo Fisher, Waltham, Massachusetts, USA) as well as a Cetuximab-Standard prepared by using absorption spectroscopy applying Lambert-Beer's-Law were used. Absorption was measured at 595 nm using a Multiskan™ Wellplatereader (Thermo
10 Fisher, Waltham, Massachusetts, USA). Protein Concentrations were determined by an appropriate polynomial regression of a standard curve from 125 to 1500 µg/mL.

Volume measurement

- 15 Permeate volume was measured using a volumetric flask of an appropriate size.

For Amicon® centrifugal filter units the permeate was transferred to the volumetric flask after centrifugation. For Amicon® stirred cell experiments, the permeate was directly collected in a such one.

- 20 Volume of concentrated protein solutions were measured using a Multipette® E3X (Eppendorf, Hamburg, Germany) and Combitips advanced® of an appropriate size.

Buffer exchange and volume reduction

- 25 An Amicon® centrifugal filter with a 30 kDa MWCO was used to exchange the original buffer with a buffer containing the relevant excipients and to reduce the volume of the solution. Five diavolumes were used to exchange the original buffer with a buffer containing the relevant excipients.

- To measure permeate flux, Amicon® centrifugal filters were centrifuged at 2000 xg for 15 minutes and volume measured as described above (repeated four times and
30 average calculated).

To achieve the final concentration, Amicon® centrifugal filters were centrifuged in small timesteps and cumulated duration denoted upon reaching the 500 µL mark.

Fig. 12 to Fig. 16 below highlight the process improvement indicated by an increased permeate flux.

5. Benefits for stirred cells

5 Excipients and combinations that had a positive effect in processing with Amicon® centrifugal filters were used in Amicon® stirred Cell filtration. While Amicon® spin column concentrators are driven by centrifugal force, stirred cells are operated by back pressure that is applied to the solution in form of nitrogen or air flow. This model system is frequently used to test processability of a solution and is a more closer model system compared to the previously used Amicon® centrifugal filters.

10

Buffer were prepared according to example 4.

Sample preparation

15 The volume of antibody stock solution was calculated to yield at least 10 mL of a solution comprising 25 mg/mL cetuximab assuming a loss of up to 20%.

Protein concentration measurements and volume measurements were performed according to example 4.

20

Buffer exchange and volume reduction

For stirred cell setup a model containing up to 50 mL was equipped with a Ultrafiltration disc filter with a NMWL of 30 kDa and an active membrane area of 13.4 cm². The respective volume of cetuximab stock solution was filled in the stirred cell and respective buffer added to the 50 mL mark. Five diavolumes were used to exchange the original buffer with a buffer containing the relevant excipient or combination.

25

To measure permeate flux, a pressure of 4 bar was applied on the Amicon® stirred cell at a mixing speed of 200 rpm (using magnetic stirrer plate) for 30 minutes (four times). For final concentration time, the cell was filled again to 50 mL mark with the respective buffer and 4 bar pressure applied at 200 rpm stirrer speed. Upon reaching the 10 mL mark, duration was denoted, process stopped and volume as well as concentration of the resulting antibody solution measured as described above.

30

As described in example 4, the effect on mean permeate flux of the formulation is assessed first. Results are depicted in Fig. 17 to Fig. 19.

6. Benefits for processing with TFF

5

Based on previous experiments using Amicon® Filters and Stirred Cell, one excipient combination was selected to evaluate filtration benefits in a tangential flow filtration system. This system represents the closest model to a large scale bioprocessing step. As the excipient combination used herein showed a beneficial effect on the process mimicked by the two previous steps it is assumed that other excipients with positive effects on process efficiency and recovery prior would have a similar effect in this setting as well.

10

Buffer were prepared according to example 4.

15

Sample preparation

The volume of antibody stock solution was calculated to yield at least 30 mL of a solution comprising 80 mg/mL Cetuximab assuming a loss of up to 20%.

20

Protein concentration measurements and volume measurements were performed according to example 4.

Buffer exchange and volume reduction

25

An ÄKTA flux S cross flow filtration system (GE Healthcare, Marlborough, Massachusetts USA) equipped with a Pellicon® XL cassette, Biomax® 30 kDa, device size: 50 cm² and a 500 ml reservoir was used to assess process benefits.

30

An above specified amount of a cetuximab solution was given into the reservoir and filled to the 450 ml mark. Using a transfer pump four further diavolumes were added stepwise while the sample was circulated in the system. For sample concentration, feed flow rate was adjusted to 30 ml/min and stirrer speed to 60 rpm. Process was either stopped by minimal tank level of 20 g or by reaching a feed pump pressure of 4 bar. Concentrated antibody solution was recovered using in-line outlets of the system.

5 In Fig. 20 the tank level and the pressure is plotted vs. the process time. A formulation without excipient vs. a formulation where 75 mM Ornithine and 75 mM ThiamineHCl are used to manage viscosity are compared. When using viscosity reducing excipients the processing time can be decreased from about 2 hours to about 1.4 hours. At the same time when using viscosity reducing excipients the system pressure remains lower than the system pressure for a comparable formulation lacking said excipients. It is clear that a further reduction of processing time is feasible if a higher system pressure is well tolerated by the protein and a higher flow rate can be used. Moreover, a 30% higher concentration was reached using viscosity reducing excipients.

Figure legends

15 Fig. 1 shows the viscosity of Infliximab at pH 7.2, with the protein concentration determined using Lambert-Beer's law, using valine, leucine, phenylalanine and proline as excipients.

20 Fig. 2 shows the viscosity of Infliximab at pH 7.2, with the protein concentration determined using Bradford assay, using ascorbic acid, cyanocobalamin, thiamine hydrochloride, folic acid, thiamine pyrophosphate and thiamine monophosphate as excipients.

25 Fig. 3 shows the viscosity of Infliximab at pH 7.2, with the protein concentration determined using Bradford assay, using thiamine monophosphate and thiamine pyrophosphat as excipients.

30 Fig. 4 shows the viscosity of a Evolocumab solution at pH 5.0, with the protein concentration determined using Lambert-Beer's law, using leucine, isoleucine, phenylalanine, proline, lysine and guanidine hydrochloride as excipients.

Fig. 5 shows the viscosity of a Evolocumab solution at pH 5.0, with the protein concentration determined using Bradford assay, using ascorbic acid, pyridoxine, cyanocobalamin, quinine hydrochloride dihydrate, thiamine hydrochloride and paracetamol as excipients.

Fig. 6 shows the viscosity of a Evolocumab solution at pH 5.0, with the protein concentration determined using Bradford assay, using thiamine pyrophosphate and thiamine hydrochloride, thiamine monophosphate as excipients.

5

Fig. 7 shows the viscosity of a Infliximab solution at pH 7.2, with the protein concentration determined using Bradford assay, using combinations of thiamine hydrochloride, pyridoxine, folic acid, thiamine monophosphate and thiamine pyrophosphate as excipients.

10

Fig. 8 shows the viscosity of a Infliximab solution at pH 7.2, with the protein concentration determined using Bradford assay, using combinations of a first set consisting of L-ornithine, L-arginine, L-carnitine, camphorsulfonic acid and benzenesulfonic acid and a second set consisting of thiamine hydrochloride, phenylalanine, pyridoxine, folic acid, thiamine monophosphate and thiamine pyrophosphate as excipients.

15

Fig. 9 shows the viscosity of a Evolocumab solution at pH 5.0, with the protein concentration determined using Bradford assay, using combinations of a first set consisting of L-ornithine, L-arginine, L-carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid and a second set consisting of thiamine hydrochloride, pyridoxine, thiamine monophosphate and thiamine pyrophosphate as excipients.

20

Fig. 10 shows the change of Tm/Tagg of compositions using valine, leucine, ascorbic acid, cyanocobalamin and proline as excipients, compared to a control of a Infliximab solution at pH 7.2.

25

Fig. 11 shows the change of Tm/Tagg of compositions using combinations of a first set consisting of L-ornithine, L-arginine, L-carnitine, phenylalanine and benzenesulfonic acid and a second set consisting of pyridoxine, thiamine monophosphate and thiamine pyrophosphate compared to a control of a Infliximab solution at pH 7.2.

30

Fig 12 and 13. show the process improvement of viscosity reducing excipients and combinations thereof indicated by an increased permeate flux.

5 Fig 14 and 15 show the decrease in process time that can be achieved by the viscosity reducing excipients and combinations thereof.

Fig. 16 shows the effect of viscosity reducing excipients on protein recovery.

10 Fig. 17 shows the process improvement of viscosity reducing excipients and combinations thereof on indicated by an increased permeate flux in Amicon® stirred Cell filtration.

15 Fig. 18 shows the decrease in process time that can be achieved by the viscosity reducing excipients and combinations thereof in Amicon® stirred Cell filtration.

Fig. 19 shows the effect of viscosity reducing excipients and combinations thereof on protein recovery in Amicon® stirred Cell filtration.

20 Fig. 20 shows the effect of viscosity reducing excipients and combinations thereof on the tank level with the pressure plotted vs. the process time in a tangential flow filtration system.

25 Fig. 21 shows the percentual viscosity reduction of an Infliximab formulation at 150 mg/mL, pH 7.2 upon addition of Ornithine, Folic acid and combination thereof, Carnitine, Folic acid and combination thereof as well as of Pyridoxine, Thiamine monophosphate and combination thereof. The expected reduction was calculated as described in 1.6.

30 Fig. 22 shows the percentual viscosity reduction of an Evolocumab formulation at 190 mg/mL, pH 5.0 upon addition of Phenylalanine, Camphorsulfonic acid and combination thereof, Phenylalanine, Benzenesulfonic acid and combination thereof as well as of Arginine, Pyridoxine and combination thereof. The expected reduction was calculated as described in 1.6.

Fig. 23 shows the percentual viscosity reduction of an Evolocumab formulation at 190 mg/mL, pH 5.0 upon addition of Meglumine, Pyridoxine and combination thereof, Meglumine as well as of Meglumine, Thiamine monophosphate and combination thereof. The expected reduction was calculated as described in 1.6.

5

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25

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Claims

1. A liquid composition comprising a protein, at least one first viscosity reducing excipient selected from the group consisting of pyridoxine, folic acid, thiamine monophosphate and phenylalanine and at least one second viscosity reducing excipient selected from the group consisting of arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid.
2. The liquid composition according to claim 1, comprising a combination of a first and a second viscosity reducing excipient selected from the list consisting of pyridoxine and arginine, folic acid and ornithine, folic acid and carnitine, pyridoxine and meglumine, thiamine monophosphate and meglumine, pyridoxine and thiamine monophosphate, phenylalanine and camphorsulfonic acid and phenylalanine and benzenesulfonic acid.
3. The liquid composition according to claim 1 or 2, having a reduced viscosity compared to an identical composition not comprising the at least one first viscosity reducing excipient and at least one second viscosity reducing excipient.
4. The liquid composition according to any of claims 1 to 3, wherein the concentration of the protein is between 90 mg/ml and 300 mg/ml.
5. The liquid composition according to any of claims 1 to 4, wherein the concentration of the at least one first and second viscosity reducing excipient is between 5 mM and 300 mM each.
6. The liquid composition according to any of claims 1 to 5, wherein the liquid composition has a pH between 4 and 8.
7. The liquid composition according to any of claims 1 to 6, wherein the liquid composition further comprises a phosphate buffer or an acetate buffer at a concentration between 5 mM and 50 mM and a stabilizer.

8. The liquid composition according to any of claims 1 to 7, wherein the liquid composition has a viscosity between 1 mPas and 60 mPas at 20 °C as measured using a microfluidic viscometer.
- 5 9. The liquid composition according to any of claims 1 to 8, wherein the protein has a molecular weight from 120 kDa to 250 kDa.
- 10 10. The liquid composition according to any of claims 1 to 9, wherein the protein is a antibody.
- 10 11. A lyophilized protein formulation of the liquid composition according to any of claims 1 to 10.
- 15 12. A method for reducing the viscosity of a protein solution, comprising a step of adding at least one first viscosity reducing excipient selected from the group consisting of pyridoxine, folic acid, thiamine monophosphate and phenylalanine or salts or solvates thereof and a second viscosity reducing excipient selected from the group consisting of arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and benzenesulfonic acid or salts or solvates thereof to the protein solution.
- 20 13. A method for reducing the viscosity of a protein solution according to claim 12, wherein the combination of a first and a second viscosity reducing excipient is selected from the list consisting of pyridoxine and arginine, folic acid and ornithine, folic acid and carnithine, pyridoxine and meglumine, thiamine monophosphate and meglumine, pyridoxine and thiamine monophosphate, phenylalanine and camphorsulfonic acid and phenylalanine and benzenesulfonic acid.
- 25 14. Use of the method according to claim 12 or 13 in a bioprocess.
- 30 15. Use of the method according to claim 12 or 13, wherein the permeate flux of the protein solution in a filtration step is increased compared to an identical protein solution not comprising the least one first viscosity reducing excipient

selected from the group consisting of pyridoxine, folic acid, thiamine
monophosphate and phenylalanine or salts or solvates thereof and the at least
one second viscosity reducing excipient selected from the group consisting of
arginine, ornithine, carnitine, meglumine, camphorsulfonic acid and
5 benzenesulfonic acid or salts or solvates thereof.

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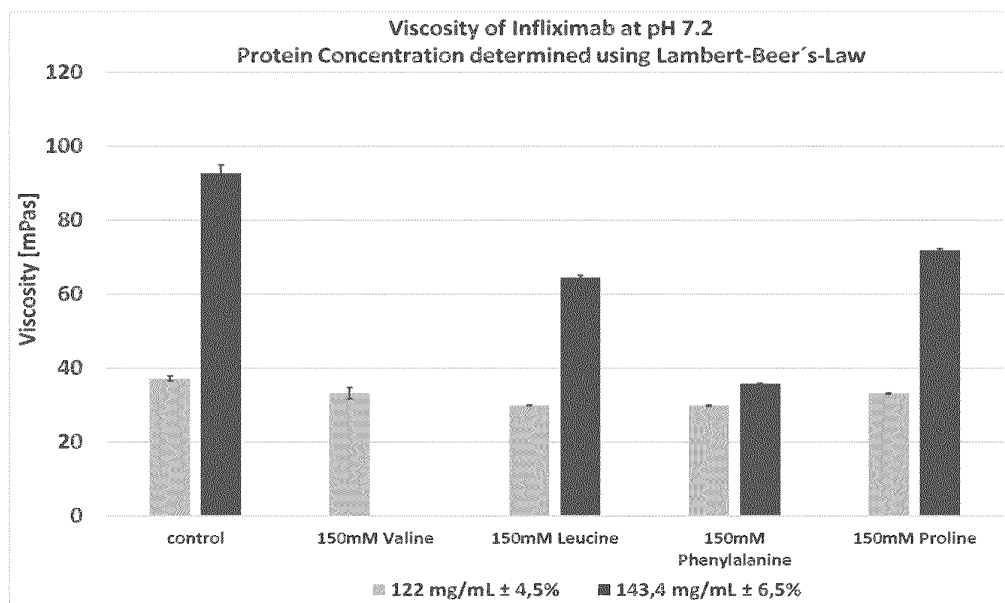
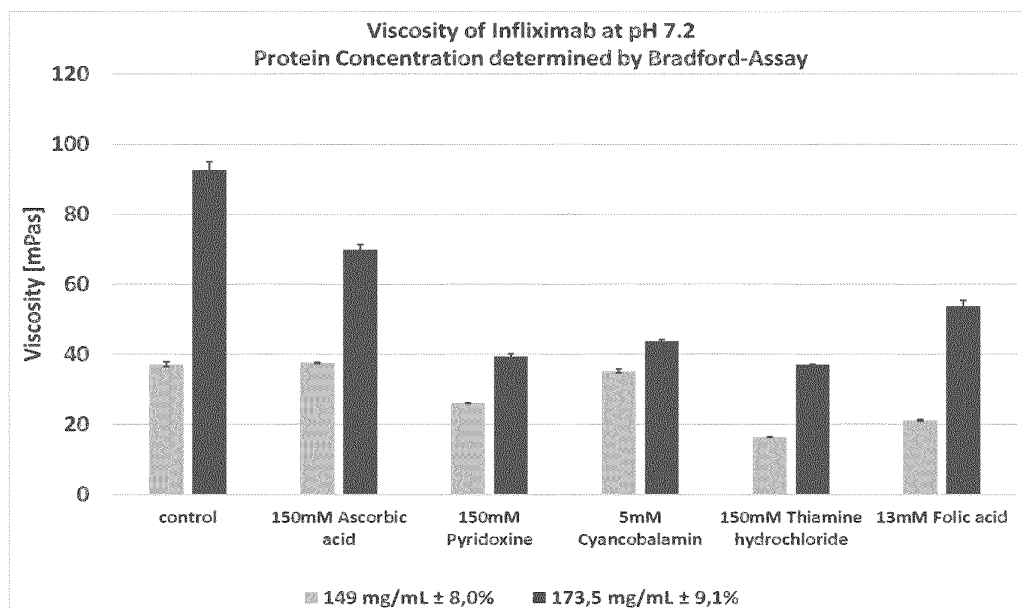
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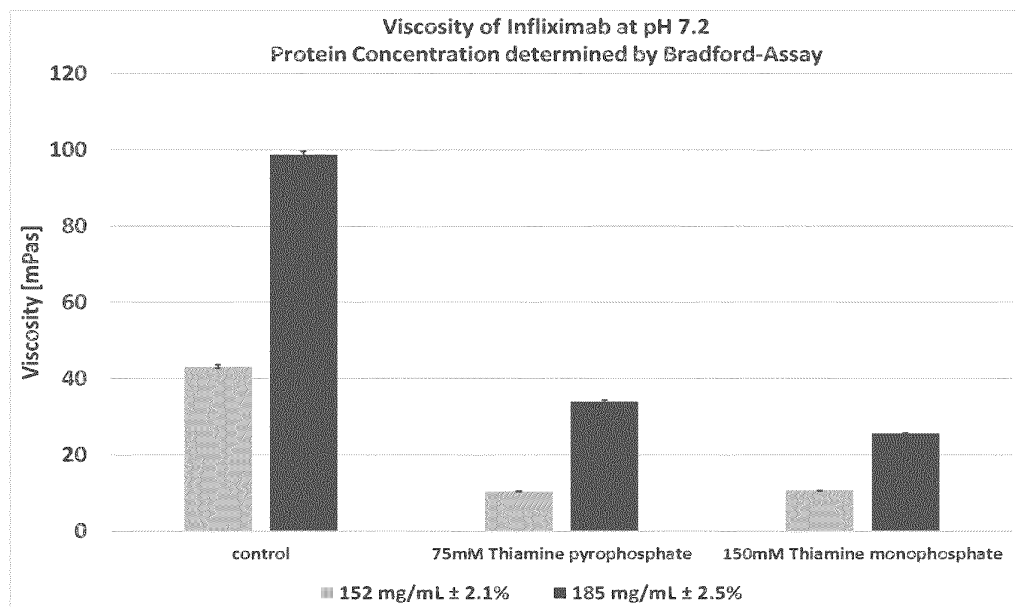
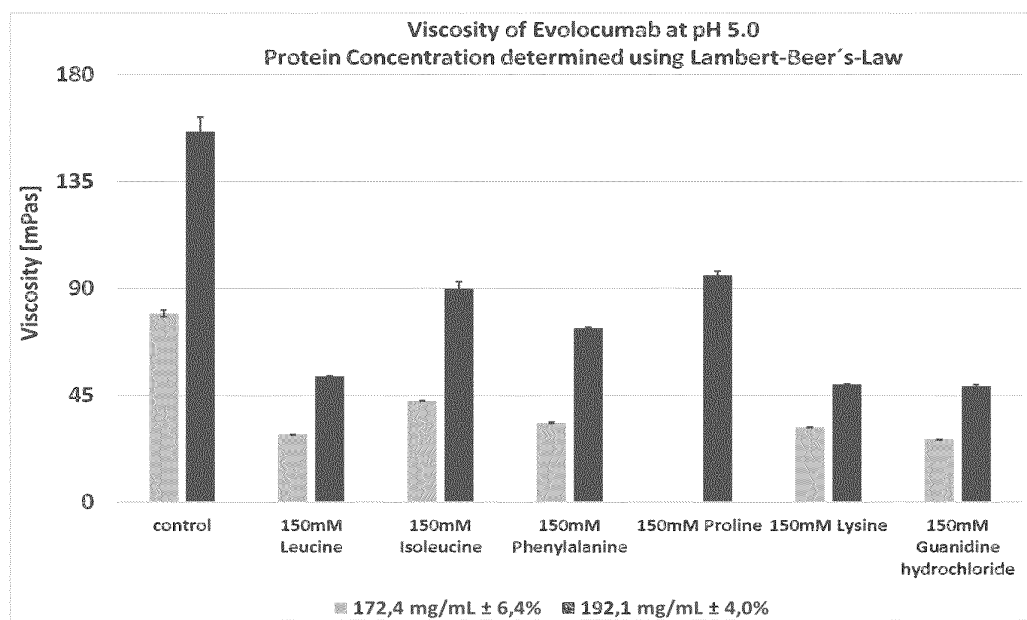
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Figures

**Fig. 1****Fig. 2**

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**Fig. 3****Fig. 4**

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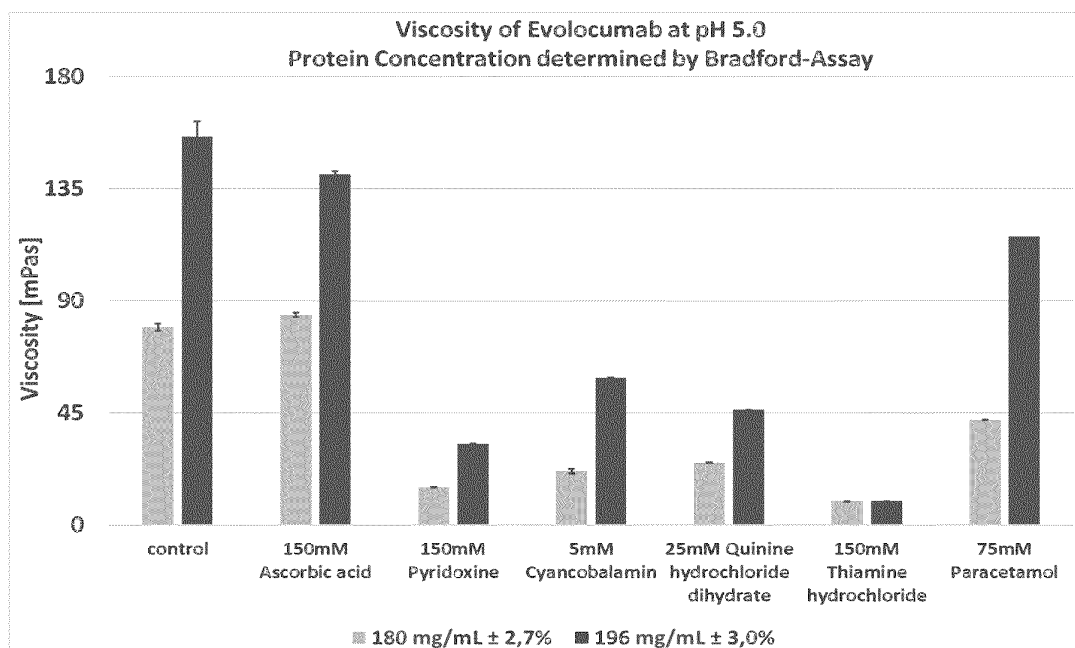


Fig. 5

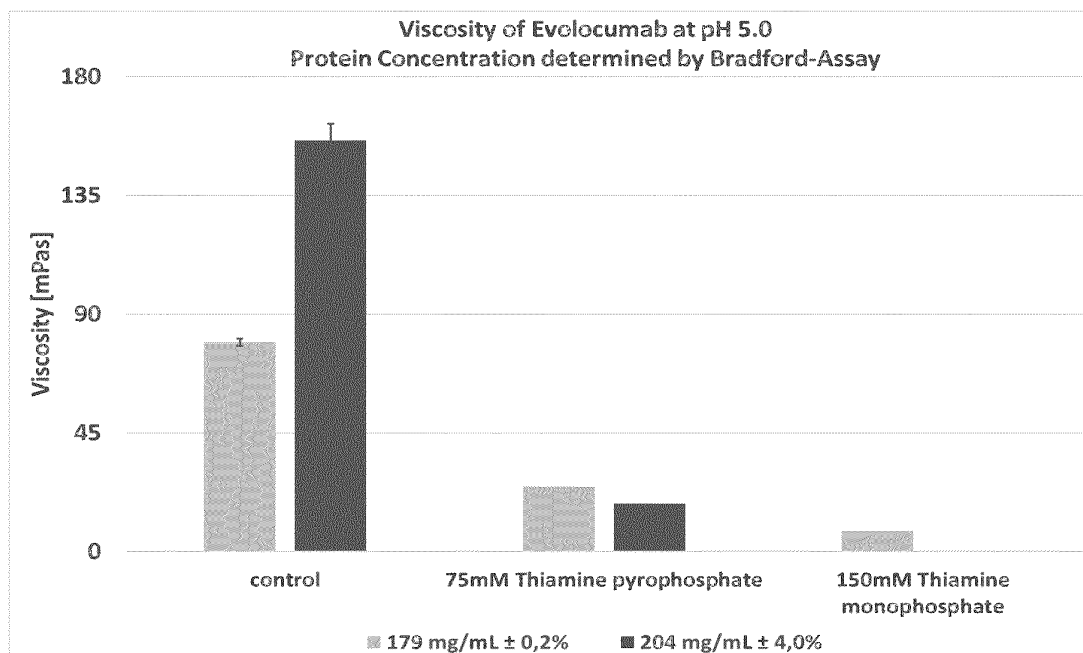
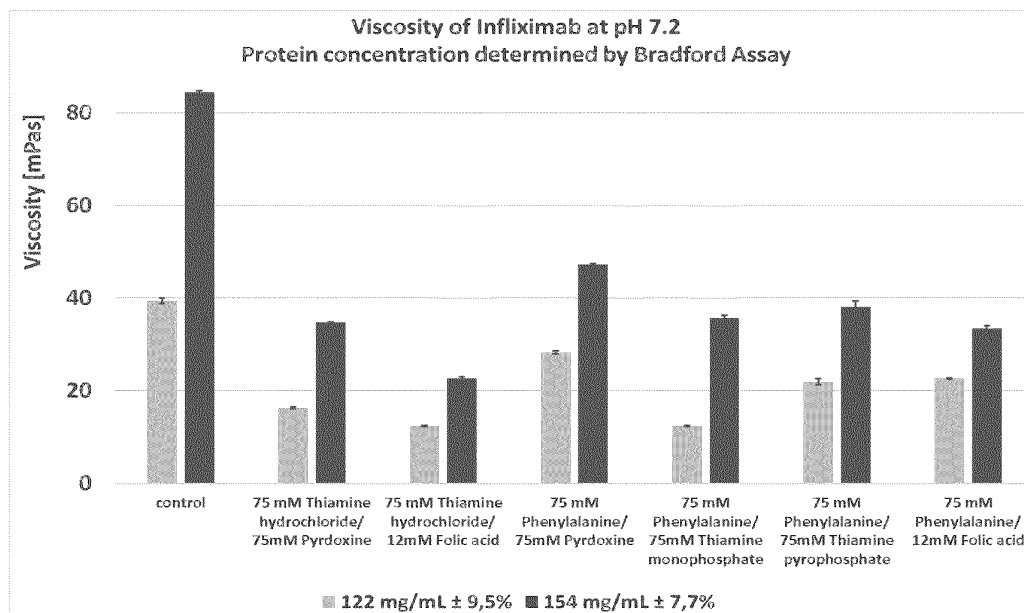


Fig. 6

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**Fig. 7**

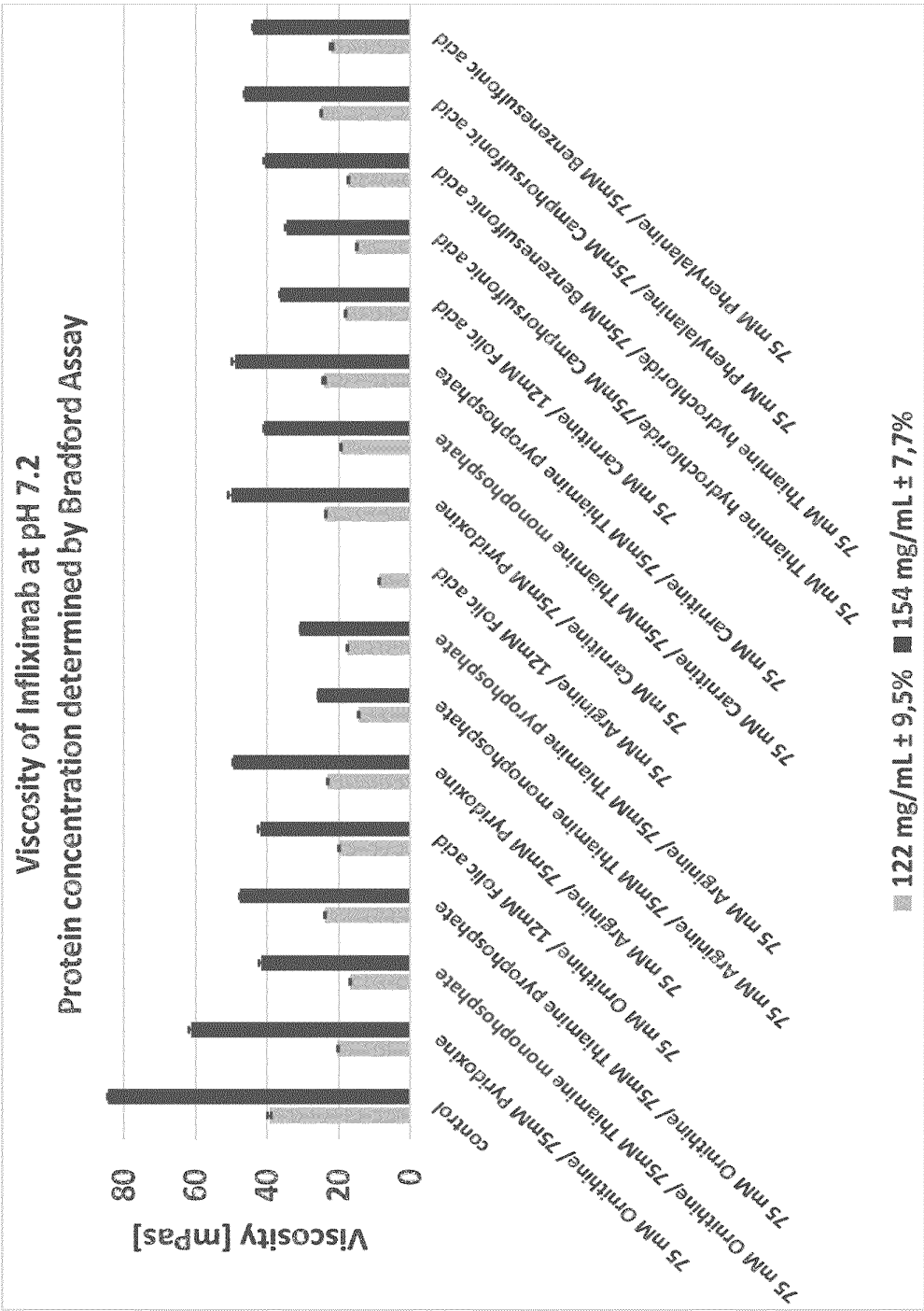


Fig. 8

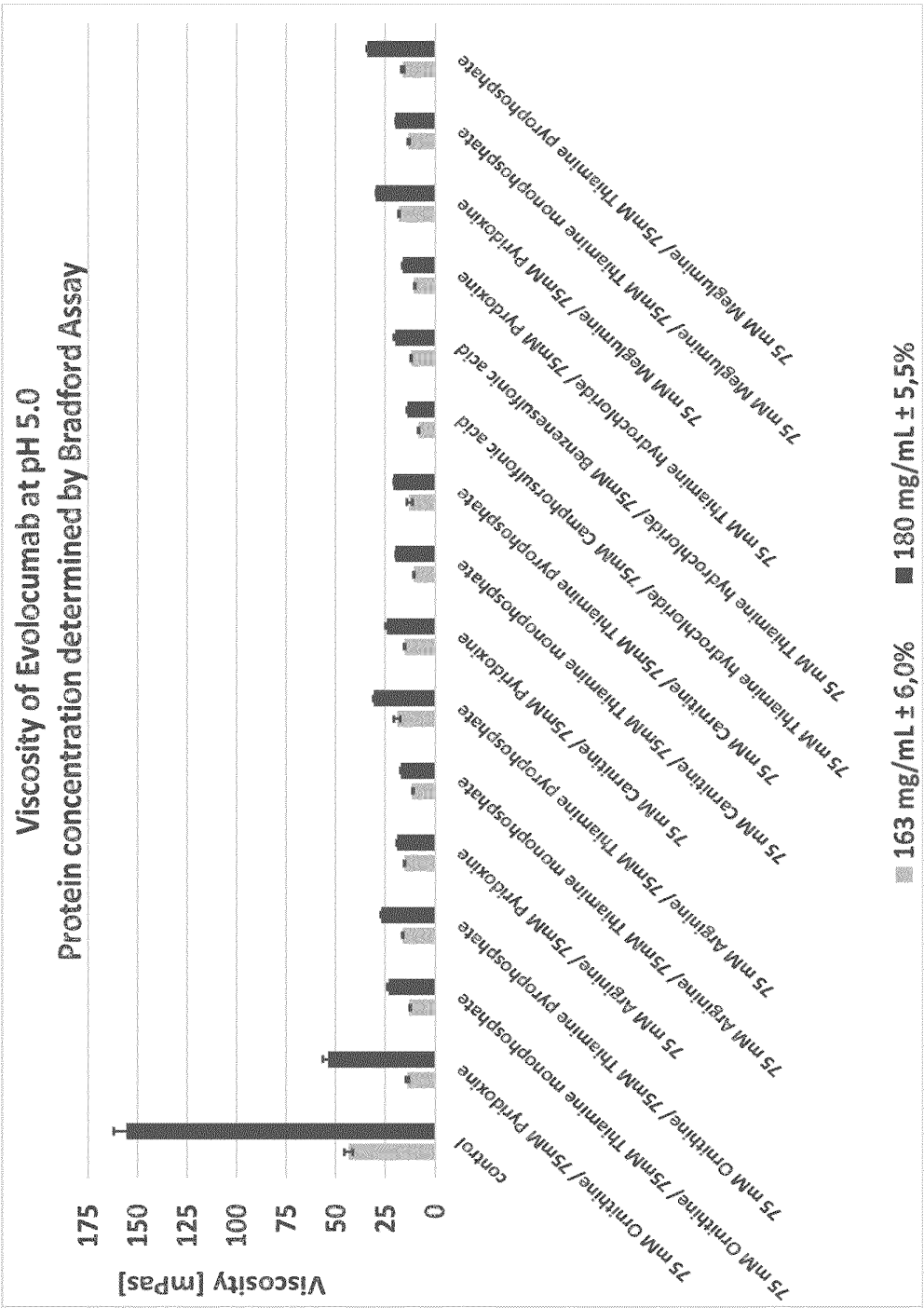


Fig. 9

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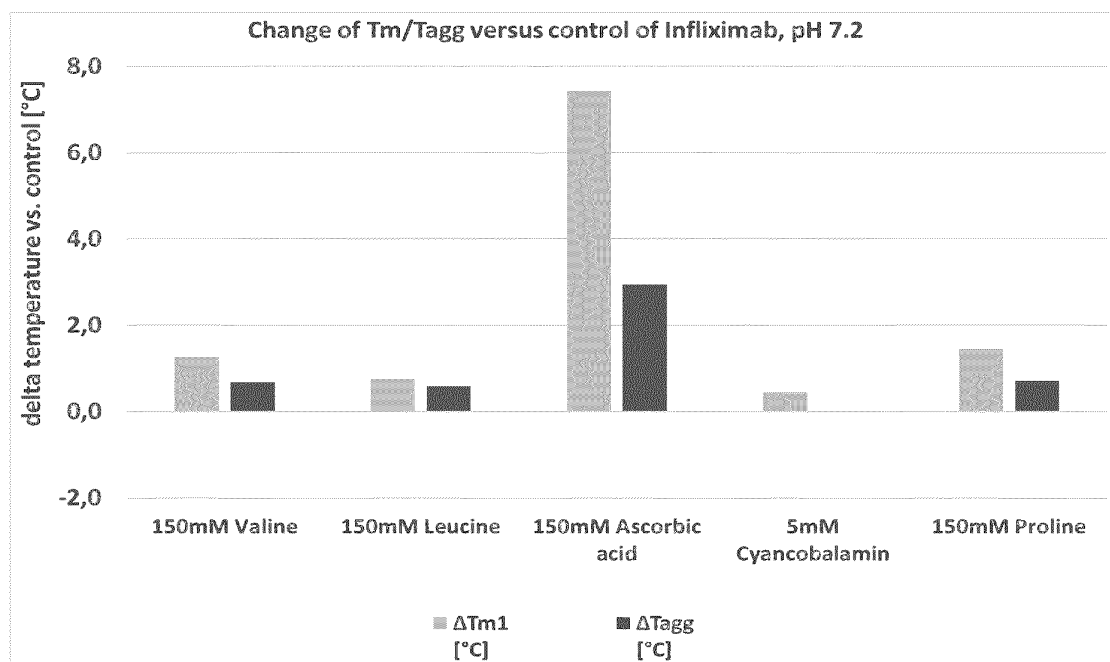


Fig. 10

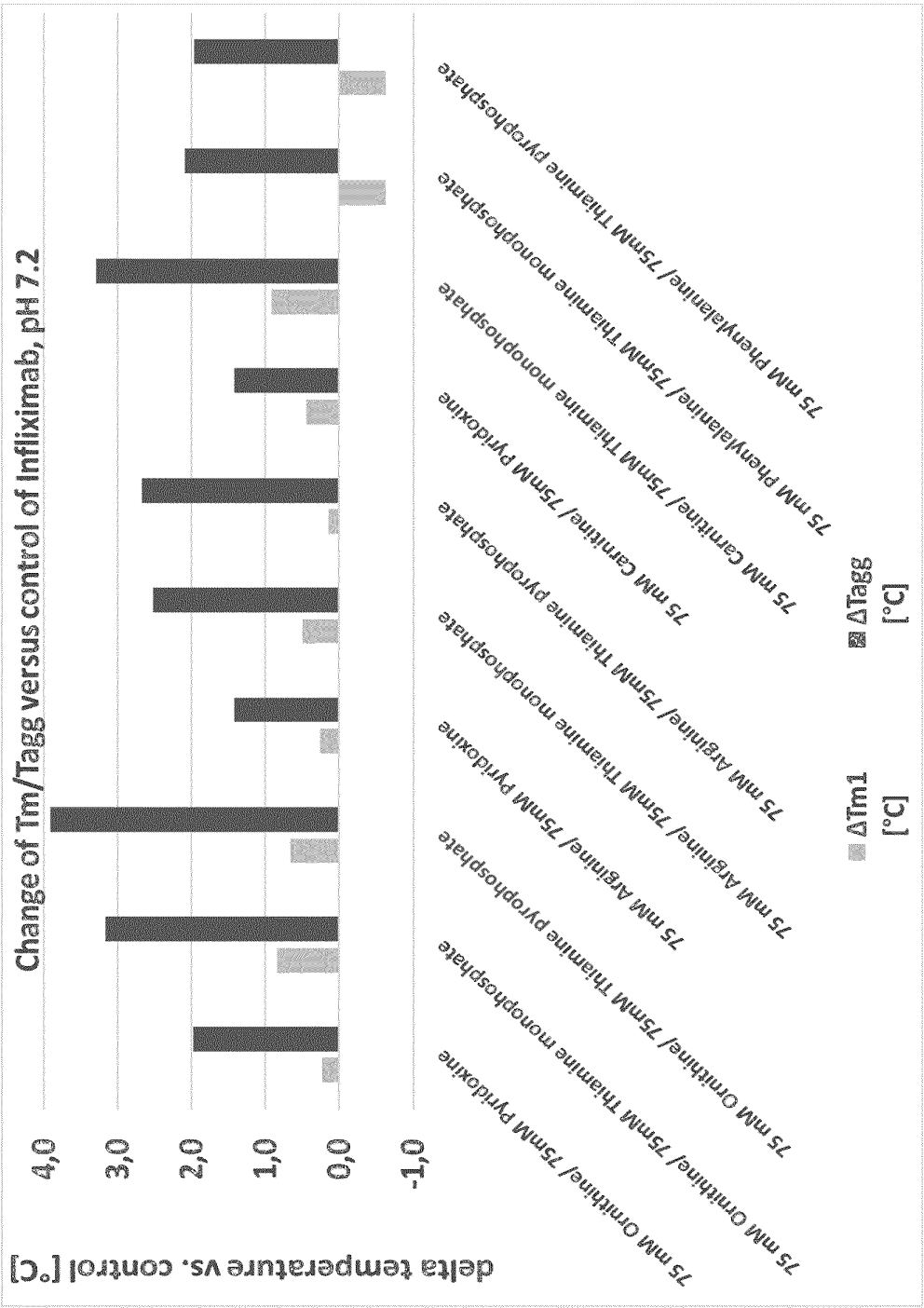


Fig. 11

- 9/16 -

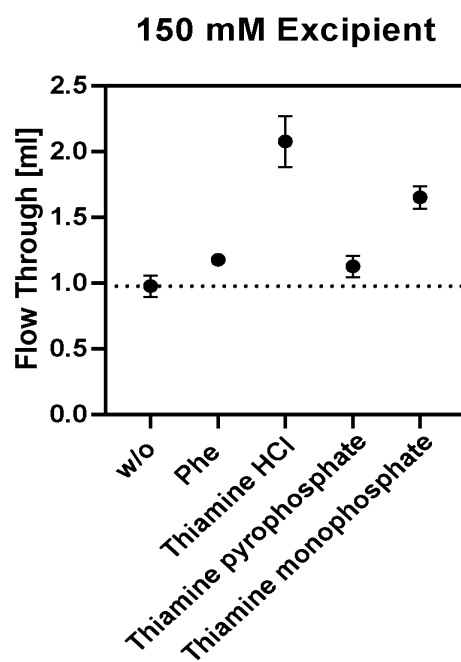


Fig. 12

Combinations 75 mM Each

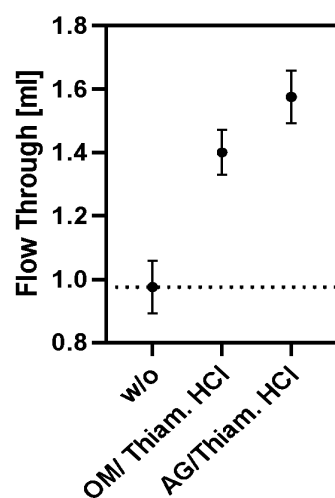


Fig. 13

- 10/16 -

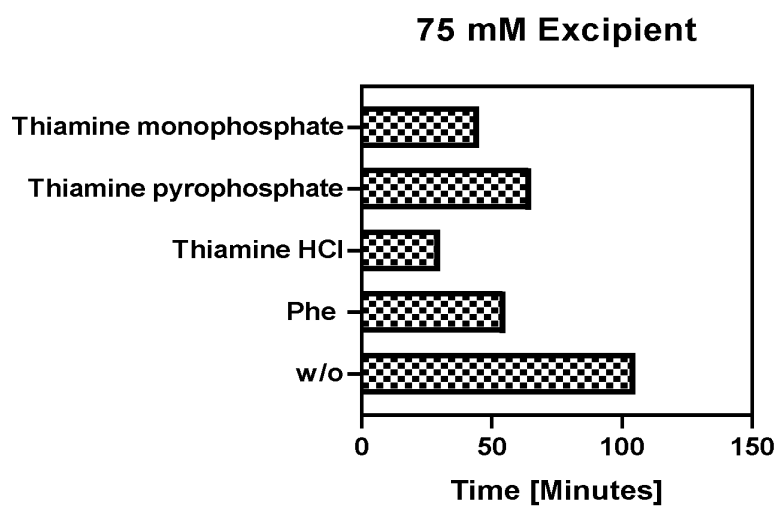


Fig. 14

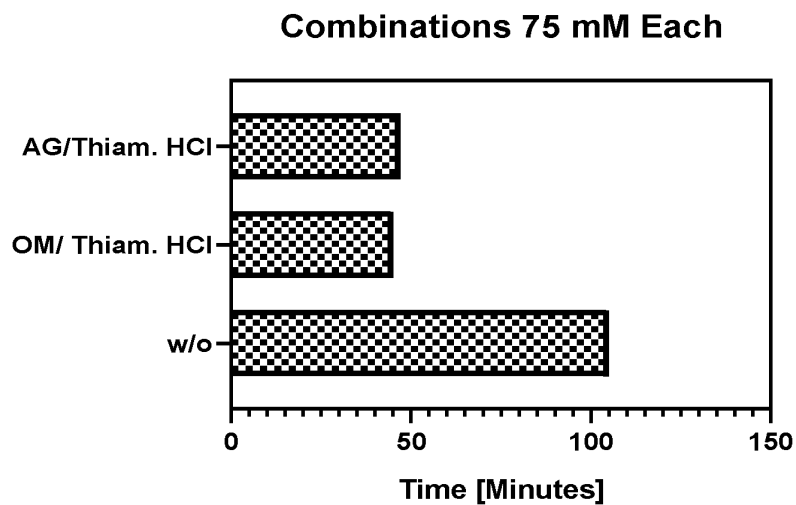


Fig. 15

- 11/16 -

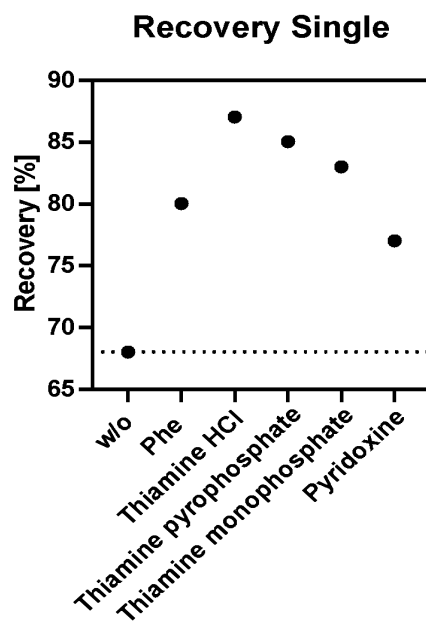


Fig. 16

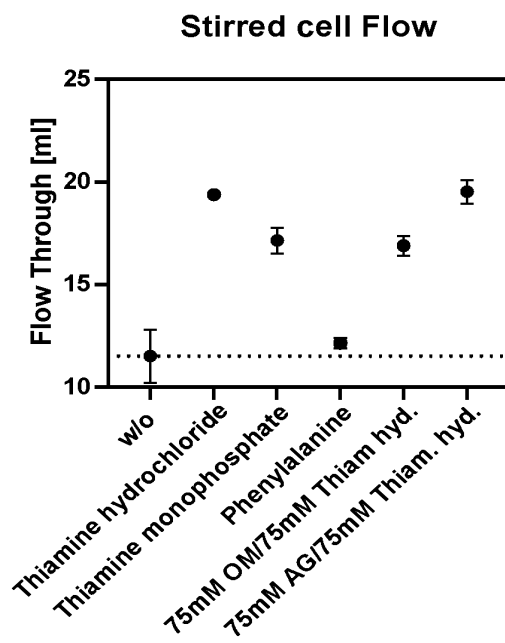


Fig. 17

- 12/16 -

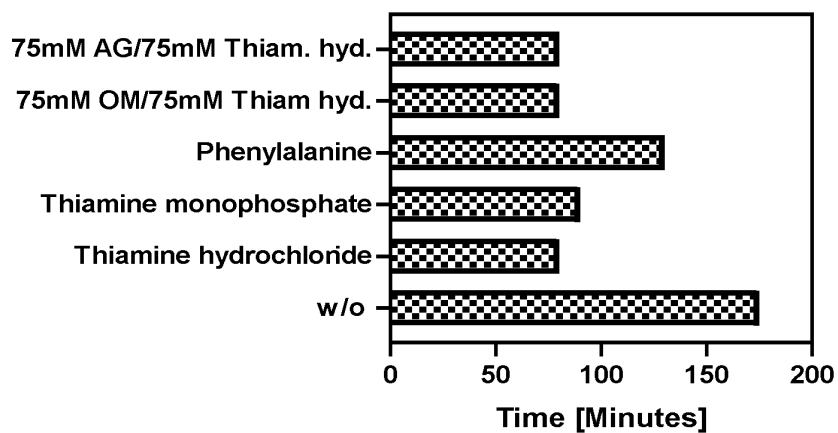


Fig. 18

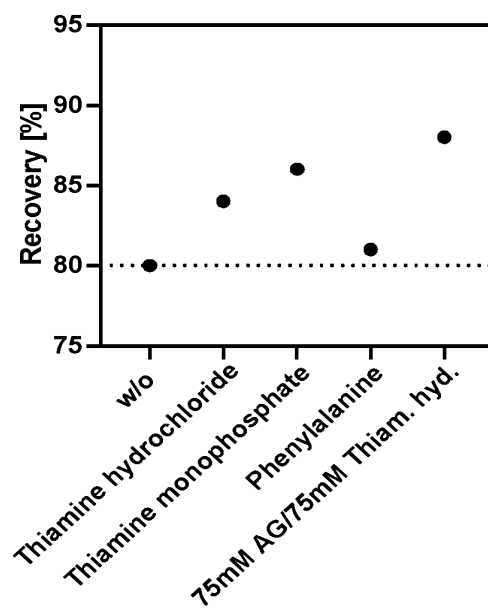


Fig. 19

- 13/16 -

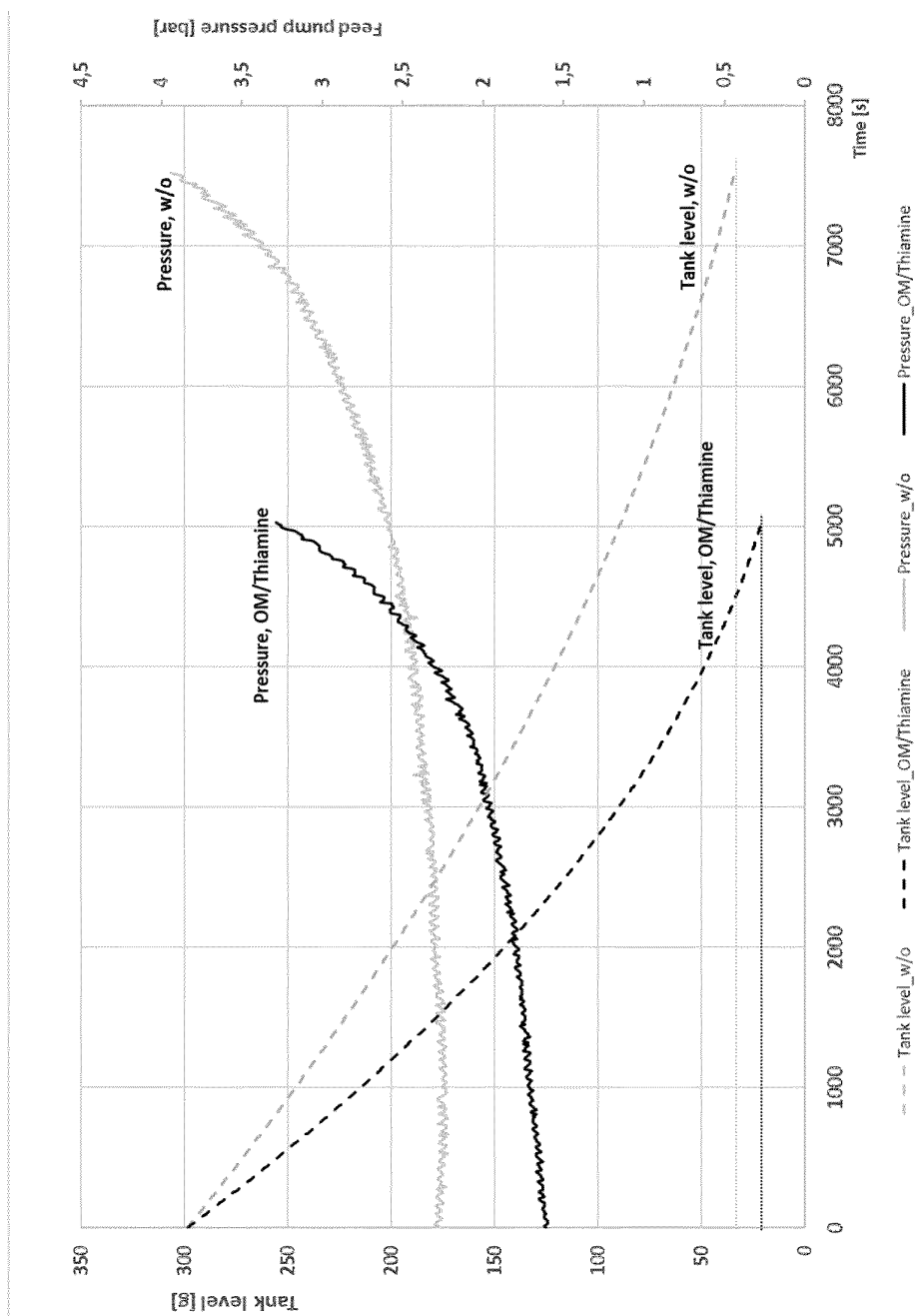


Fig. 20

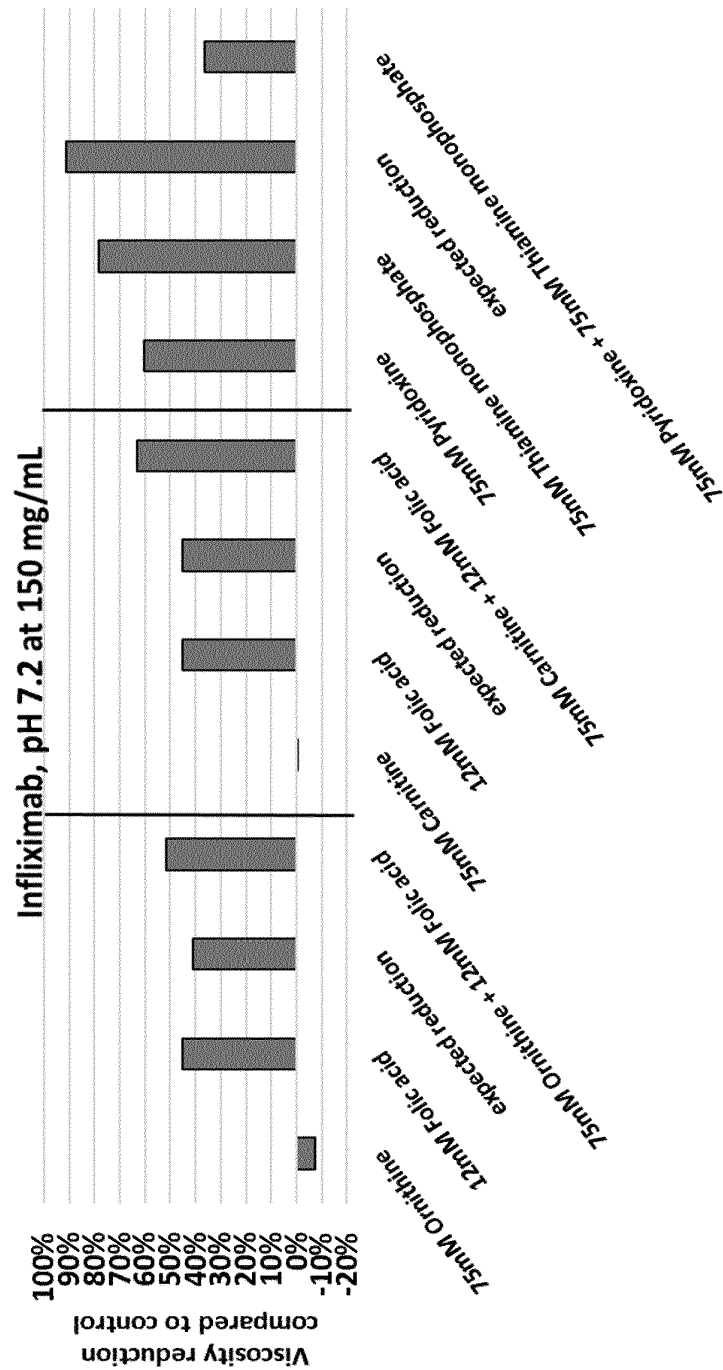


Fig. 21

PGR2026-00039
Biocon Ex.1002
Page 3689 of 4945

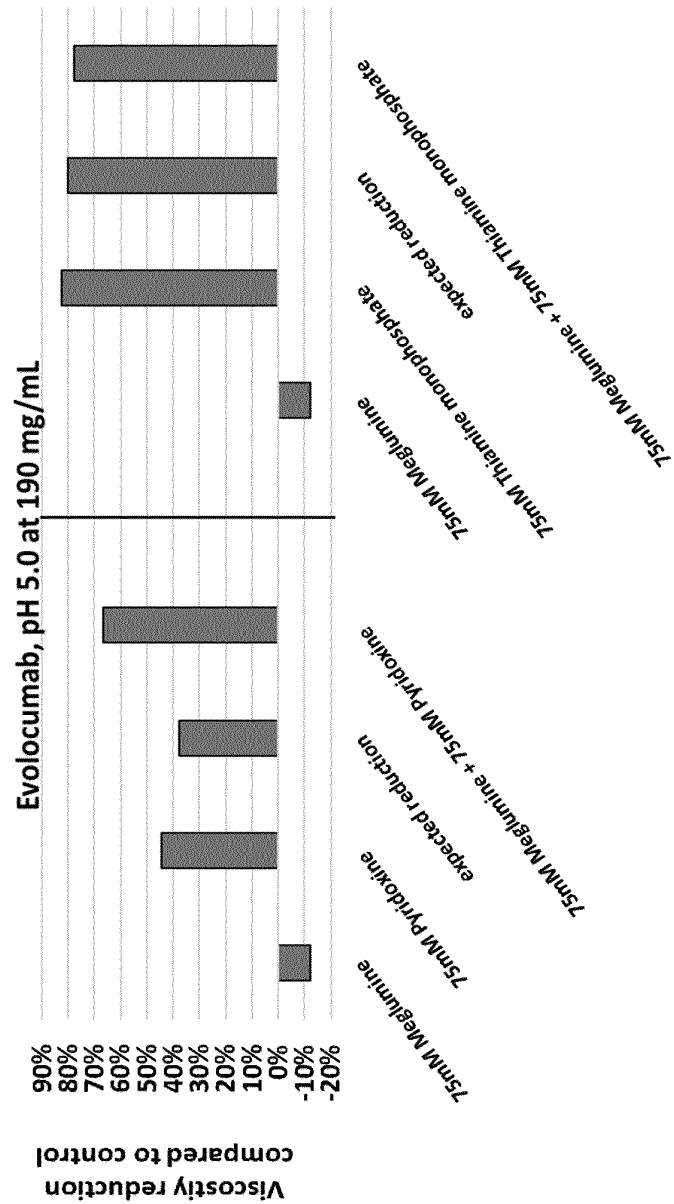


Fig. 23

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/069374

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K9/00 A61K9/08 A61K9/19 A61K39/395 A61K47/12
A61K47/18 A61K47/22 C07K16/24 C07K16/40

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EP0-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 7 172 999 B2 (ROCHE DIAGNOSTICS GMBH [DE]) 6 February 2007 (2007-02-06) comparative example E; column 7, line 63-65; example 8	1-15
X	----- WO 2018/200533 A1 (AMGEN INC [US]) 1 November 2018 (2018-11-01) claims 1-20	1-15
X	----- WO 2019/201904 A1 (MERCK PATENT GMBH [DE]) 24 October 2019 (2019-10-24) cited in the application claims 1-33	1-15
	----- -/-	

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

10 September 2021

Date of mailing of the international search report

21/09/2021

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Konter, Jörg

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2021/069374

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	BRIAND CONNOLLY ET AL: "Weak Interactions Govern the Viscosity of Concentrated Antibody Solutions: High-Throughput Analysis Using the Diffusion Interaction Parameter", BIOPHYSICAL JOURNAL, ELSEVIER, AMSTERDAM, NL, vol. 103, no. 1, 24 April 2012 (2012-04-24), pages 69-78, XP028398737, ISSN: 0006-3495, DOI: 10.1016/J.BPJ.2012.04.047 [retrieved on 2012-05-07] abstract -----	1-15
A	WO 2011/139718 A1 (GENENTECH INC [US]; HOFFMANN LA ROCHE [CH] ET AL.) 10 November 2011 (2011-11-10) page 57, lines 14-24 -----	1-15

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Information on patent family members

International application No

PCT/EP2021/069374

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2021/069374

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(74) Agent: BATES, Caleb A. et al.; Fish & Richardson P.C., P.O. Box 1022, Minneapolis, Minnesota 55440-1022 (US).

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(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))
- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))

(54) Title: METHODS OF TREATING HER2 POSITIVE CANCER WITH TUCATINIB IN COMBINATION WITH TRASTUZUMAB, A TAXANE, AND A VEGFR-2 ANTAGONIST

(57) Abstract: The present disclosure relates to a method of treating a HER2 positive cancer in a subject in need thereof, the method comprising: administering to the subject a therapeutically effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist.

WO 2022/026510 A1

METHODS OF TREATING HER2 POSITIVE CANCER WITH TUCATINIB IN COMBINATION WITH TRASTUZUMAB, A TAXANE, AND A VEGFR-2 ANTAGONIST

CLAIM OF PRIORITY

[0001] This application claims the benefit of U.S. Provisional Application Serial No. 63/058,146, filed on July 29, 2020. The entire contents of the foregoing are incorporated herein by reference.

BACKGROUND

[0002] Encoded by the ERBB2 gene, human epidermal growth factor receptor 2 (HER2) is part of a family of 4 related receptor tyrosine kinases, which include HER1 (also known as epidermal growth factor receptor [EGFR]), HER2, HER3, and HER4. HER1-4 are single-pass transmembrane glycoprotein receptors containing an extracellular ligand binding region and an intracellular signaling domain. HER2 has no known ligand, but it is the preferred dimerization partner for the other HER family receptors. When overexpressed in tumors, HER2 forms ligand-independent homodimeric complexes that autophosphorylate. HER2 homo- or heterodimerization results in the activation of multiple signaling cascades, including the Ras/Raf/MEK/MAPK, PI3K/AKT, Src, and STAT pathways. These signaling pathways lead to cell proliferation, inhibition of apoptosis, and metastasis.

[0003] The treatment and prevention of HER2 positive cancers represents an unmet need. Cancers that are characterized by the overexpression of HER2 (referred to as HER2 positive cancers) are often correlated with poor prognosis or are resistant to many standard therapies. Accordingly, there is a need for new therapies that are effective for the treatment of cancers such as HER2 positive cancers or metastatic HER2 positive cancers.

[0004] All references cited herein, including patent applications, patent publications, and scientific literature, are herein incorporated by reference in their entirety, as if each individual reference were specifically and individually indicated to be incorporated by reference.

SUMMARY

[0005] Provided herein are methods of treating cancer in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a vascular endothelial growth factor receptor 2 (VEGFR-2) antagonist.

[0006] Also provided herein are methods of treating cancer in a subject in need thereof, the method comprising: (a) identifying the subject as having a HER2 positive cancer; and (b) administering to the subject a therapeutically effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a vascular endothelial growth factor receptor 2 (VEGFR-2) antagonist.

[0007] In some embodiments, the trastuzumab is administered to the subject at a dose of about 6 mg/kg. In some embodiments, the trastuzumab is administered to the subject at a dose of about 4 mg/kg. In some embodiments, the tucatinib is administered to the subject at a dose of about 150 mg to about 650 mg. In some embodiments, the tucatinib is administered twice daily. In some embodiments, the tucatinib is administered to the subject orally.

[0008] In some embodiments, the VEGFR-2 antagonist is selected from the group consisting of bevacizumab, ramucirumab, aflibercept, cetuximab, panitumumab, regorafenib, sunitinib, sorafenib, pazopanib, vandetanib, axitinib, cediranib, vatalanib, motesanib, lucatinib, intedanib, semaxanib, apatinib, lenvatinib, carbozantinib, and a combination thereof. In some embodiments, the VEGFR-2 antagonist is a monoclonal antibody selected from the group consisting of bevacizumab, ramucirumab, aflibercept, cetuximab, panitumumab, and combinations thereof. In some embodiments, the VEGFR-2 antagonist is ramucirumab. In some embodiments, the ramucirumab is administered to the subject at a dose of about 8 mg/kg.

[0009] In some embodiments, the taxane is selected from the group consisting of paclitaxel, docetaxel, cabazitaxel, larotaxel, BMS-184476, BMS-188797, BMS-275183, milataxel, ortaxel, TL-310, docosahexaenoic acid-paclitaxel (DHA-paclitaxel), *nab* paclitaxel, EndoTAG+paclitaxel, XRP9881, polymeric-micellar paclitaxel, RPR-109881A, a pharmaceutically acceptable salt or solvate thereof, and a combination thereof. In some embodiments, the taxane is selected from the group consisting of paclitaxel, docetaxel, and cabazitaxel. In some embodiments, the taxane is paclitaxel. In some embodiments, the paclitaxel is administered to the subject at a dose of about

50 mg/m² to about 100 mg/m². In some embodiments, the paclitaxel is administered to the subject at a dose of about 80 mg/m².

[0010] In some embodiments, the HER2 positive cancer is selected from the group consisting of gastric adenocarcinoma, gastroesophageal junction (GEC) adenocarcinoma, esophageal adenocarcinoma, colorectal carcinoma (CRC), cholangiocarcinoma, gallbladder carcinoma, gastric cancer, lung cancer, biliary cancers, bladder cancer, esophageal cancer, melanoma, ovarian cancer, liver cancer, prostate cancer, pancreatic cancer, small intestine cancer, non-small cell lung cancer, head and neck cancer, uterine cancer, cervical cancer, brain cancer, and breast cancer. In some embodiments, the HER2 positive cancer is selected from the group consisting of gastric adenocarcinoma, gastroesophageal junction (GEC) adenocarcinoma, esophageal adenocarcinoma, colorectal carcinoma (CRC), cholangiocarcinoma, gallbladder carcinoma, gastric cancer, lung cancer, biliary cancers, bladder cancer, esophageal cancer, melanoma, ovarian cancer, liver cancer, prostate cancer, pancreatic cancer, small intestine cancer, non-small cell lung cancer, head and neck cancer, uterine cancer, cervical cancer, and brain cancer. In some embodiments, the HER2 positive cancer is gastric adenocarcinoma. In some embodiments, the HER2 positive cancer is gastroesophageal junction (GEC) adenocarcinoma. In some embodiments, the HER2 positive cancer is unresectable or metastatic.

[0011] In some embodiments, the subject has been previously treated with a HER2-directed antibody. In some embodiments, the subject has not been previously treated with an anti-HER2 and/or an anti-EGFR tyrosine kinase inhibitor. In some embodiments, the subject has not been previously treated with a HER2-directed antibody-drug conjugate. In some embodiments, the wherein the anti-HER2/EGFR tyrosine kinase inhibitor is selected from the group consisting of tucatinib, lapatinib, neratinib, or afatinib. In some embodiments, the antibody-drug conjugate is selected from the group consisting of ado-trastuzumab (T-DM1) or trastuzumab deruxtecan (DS8201a).

[0012] In some embodiments, the subject has not been previously treated with an anthracycline. In some embodiments, the anthracycline is selected from the group consisting of doxorubicin, epirubicin, mitoxantrone, idarubicin, liposomal doxorubicin, and combinations thereof.

[0013] In some embodiments, the subject was previously treated with at least one anticancer therapy. In some embodiments, the at least one anticancer therapy is selected from the group consisting of trastuzumab, lapatinib, trastuzumab and a taxane, pertuzumab, and combinations thereof. In some embodiments, the subject is refractory to the at least one anticancer therapy. In some embodiments, the subject developed a brain metastasis during the previous treatment with the at least one anticancer therapy.

[0014] Provided herein are methods of treating a HER2 positive cancer in a subject that has exhibited an adverse event after starting treatment with a combination therapy comprising tucatinib, trastuzumab, a taxane, and a vascular endothelial growth factor receptor 2 (VEGFR-2) antagonist at an initial dosage level, comprising administering to the subject at least one component of the combination therapy at a reduced dosage level.

[0015] In some embodiments, the taxane is selected from the group consisting of paclitaxel, docetaxel, cabazitaxel, larotaxel, BMS-184476, BMS-188797, BMS-275183, milataxel, ortaxel, TL-310, docosahexaenoic acid-paclitaxel (DHA-paclitaxel), *nab* paclitaxel, EndoTAG+paclitaxel, XRP9881, polymeric-micellar paclitaxel, RPR-109881A, a pharmaceutically acceptable salt or solvate thereof, and a combination thereof. In some embodiments, the taxane is paclitaxel.

[0016] In some embodiments, paclitaxel is administered to the subject at an initial dose of about 50 mg/m² to about 100 mg/m². In some embodiments, the paclitaxel is administered to the subject at an initial dose of about 80 mg/m². In some embodiments, the paclitaxel is administered to the subject at a reduced dose of about 50 mg/m² to about 75 mg/m². In some embodiments, the paclitaxel is administered to the subject at a reduced dose of about 70 mg/m². In some embodiments, the paclitaxel is administered to the subject at a reduced dose of about 60 mg/m².

[0017] In some embodiments, the tucatinib is administered to the subject at an initial dose of about 150 mg to about 650 mg. In some embodiments, the tucatinib is administered to the subject at an initial dose of about 300 mg. In some embodiments, the tucatinib is administered to the subject at a reduced dose of about 125 mg to about 275 mg.

[0018] In some embodiments, the VEGFR-2 antagonist is selected from the group consisting of bevacizumab, ramucirumab, aflibercept, cetuximab, panitumumab, regorafenib, sunitinib, sorafenib, pazopanib, vandetanib, axitinib, cediranib, vatalanib, motesanib, lucatinib, intedanib,

semaxanib, apatinib, lenvatinib, carbozantinib, and a combination thereof. In some embodiments, the VEGFR-2 antagonist is ramucirumab. In some embodiments, the ramucirumab is administered to the subject at an initial dose of about 8 mg/kg. In some embodiments, the ramucirumab is administered to the subject at a reduced dose of about 6 mg/kg. In some embodiments, the ramucirumab is administered to the subject at a reduced dose of about 5 mg/kg.

[0019] Also provided herein are methods of treating a HER2 positive cancer in a subject in need thereof, the method comprising: (a) administering to the subject a therapeutically effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a vascular endothelial growth factor receptor 2 (VEGFR-2) antagonist; and (b) administering an effective amount of an anti-diarrheal agent.

[0020] Also provided herein are methods of reducing the severity or incidents of diarrhea, or preventing diarrhea in a subject having a HER2 positive cancer and being treated with an effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a vascular endothelial growth factor receptor 2 (VEGFR-2) antagonist, the method comprising administering an effective amount of an anti-diarrheal agent prophylactically.

[0021] Also provided herein are methods of reducing the likelihood of a subject developing diarrhea, wherein the subject has a HER2 positive cancer and is being treated with an effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a vascular endothelial growth factor receptor 2 (VEGFR-2) antagonist the method comprising administering an effective amount of an anti-diarrheal agent prophylactically.

[0022] In some embodiments, the combination therapy and the anti-diarrheal agent are administered concurrently. In some embodiments, the anti-diarrheal agent is administered prior to administration of the combination therapy. In some embodiments, the subject is exhibiting symptoms of diarrhea. In some embodiments, the subject is not exhibiting symptoms of diarrhea.

DESCRIPTION OF THE DRAWINGS

[0023] FIG. 1 Shows a study schema including study visits, and the overall design of phase 2, and phase 3 associated with a phase 2/3 study of tucatinib in combination with trastuzumab, a taxane, and a VEGFR-2 antagonist for HER2+ positive cancers described herein in connection with Example 1.

[0024] FIG. 2 Shows a phase 2 study design associated with a phase 2/3 study of tucatinib in combination with trastuzumab, a taxane, and a VEGFR-2 antagonist for HER2+ positive cancers described herein in connection with Example 1.

[0025] FIG. 3 Shows a phase 3 study design associated with a phase 2/3 study of tucatinib in combination with trastuzumab, a taxane, and a VEGFR-2 antagonist for HER2+ positive cancers described herein in connection with Example 1.

[0026] FIG. 4 provides the amino acid sequence of the heavy (SEQ. ID NO. 1) and light chains (SEQ. ID NO. 2) of trastuzumab and the light chain variable domain (SEQ. ID NO. 3) and the heavy chain variable domain (SEQ. ID NO. 4).

[0027] FIGS. 5A-5C show that a combination of tucatinib and trastuzumab was active in HER2 amplified colorectal cancer (CRC) patient-derived xenograft (PDX) models. Data are shown as group mean $\pm$ S.E.M. FIG. 5A shows the effects of tucatinib and trastuzumab, alone and in combination, on tumor growth in a CTG-0121 CRC PDX model. FIG. 5B shows the effects of tucatinib and trastuzumab, alone and in combination, on tumor growth in a CTG-0784 CRC PDX model. FIG. 5C shows the effects of tucatinib and trastuzumab, alone and in combination, on tumor growth in a CTG-0383 CRC PDX model.

[0028] FIGS. 6A and 6B show that a combination of tucatinib and trastuzumab was active in HER2 amplified esophageal cancer patient-derived xenograft (PDX) models. Data are shown as group mean $\pm$ S.E.M. FIG. 6A shows the effects of tucatinib and trastuzumab, alone and in combination, on tumor growth in a CTG-0137 esophageal cancer PDX model. FIG. 6B shows the effects of tucatinib and trastuzumab, alone and in combination, on tumor growth in a CTG-0138 esophageal cancer PDX model.

[0029] FIGS. 7A-7C show that a combination of tucatinib and trastuzumab was active in HER2 positive gastric cancer patient-derived xenograft (PDX) models. Data are shown as group mean $\pm$ S.D. FIG. 7A shows the effects of tucatinib and trastuzumab, alone and in combination, on tumor growth in a GXA 3038 gastric cancer PDX model. FIG. 7B shows the effects of tucatinib and trastuzumab, alone and in combination, on tumor growth in a GXA 3039 gastric cancer PDX model. FIG. 7C shows the effects of tucatinib and trastuzumab, alone and in combination, on tumor growth in a GXA 3054 gastric cancer PDX model.

[0030] FIG. 8 shows that a combination of tucatinib and trastuzumab was active in a CTG-0927 HER2 positive cholangiocarcinoma patient-derived xenograft (PDX) model. Data are shown as mean $\pm$ S.E.M.

[0031] FIGS. 9A and 9B show that a combination of tucatinib and trastuzumab was active in HER2 positive non-small cell lung cancer (NSCLC) models. Data are shown as group mean $\pm$ S.E.M. FIG. 9A shows the effects of tucatinib and trastuzumab, alone and in combination, on tumor growth in a Calu-3 NSCLC xenograft model. FIG. 9B shows the effects of tucatinib and trastuzumab, alone and in combination, on tumor growth in an NCI-H2170 NSCLC xenograft model.

DETAILED DESCRIPTION

I. Definitions

[0032] In order that the present disclosure can be more readily understood, certain terms are first defined. As used in this application, except as otherwise expressly provided herein, each of the following terms shall have the meaning set forth below. Additional definitions are set forth throughout the application.

[0033] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure is related. For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular Biology, 3rd ed., 1999, Academic Press; and the Oxford Dictionary Of Biochemistry And Molecular Biology, Revised, 2000, Oxford University Press, provide one of skill with a general dictionary of many of the terms used in this disclosure. For purposes of the present disclosure, the following terms are defined.

[0034] Units, prefixes, and symbols are denoted in their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. The headings provided herein are not limitations of the various aspects of the disclosure, which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification in its entirety.

[0035] The terms “a,” “an,” or “the” as used herein not only include aspects with one member, but also include aspects with more than one member. For instance, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a cell” includes a plurality of such cells and reference to “the agent” includes reference to one or more agents known to those skilled in the art, and so forth.

[0036] The term “and/or” where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. Thus, the term “and/or” as used in a phrase such as “A and/or B” herein is intended to include “A and B,” “A or B,” “A” (alone), and “B” (alone). Likewise, the term “and/or” as used in a phrase such as “A, B, and/or C” is intended to encompass each of the following aspects: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

[0037] It is understood that aspects and embodiments of the disclosure described herein include “comprising,” “consisting,” and “consisting essentially of” aspects and embodiments. It is understood that aspects and variations of the embodiments described herein include “consisting of” and/or “consisting essentially of” aspects and variations. In some embodiments, methods consisting essentially of an administration step as disclosed herein include methods wherein a patient has failed a prior therapy (administered to the patient before the period of time) or has been refractory to such prior therapy, and/or wherein the cancer has metastasized or recurred. In some embodiments, methods consisting essentially of an administration step as disclosed herein include methods wherein a patient undergoes surgery, radiation, and/or other regimens prior to, substantially at the same time as, or following such an administration step as disclosed herein, and/or where the patient is administered other chemical and/or biological therapeutic agents following such an administration step as disclosed herein.

[0038] The terms “about” and “approximately” as used herein shall generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Typical, exemplary degrees of error are within 20 percent (%), preferably within 10%, and more preferably within 5% of a given value or range of values. Any reference to “about X” specifically indicates at least the values X, 0.95X, 0.96X, 0.97X, 0.98X, 0.99X, 1.01X, 1.02X, 1.03X, 1.04X, and 1.05X. Thus, “about X” is intended to teach and provide written description support for a claim

limitation of, e.g., “0.98X.” The terms “about” and “approximately,” particularly in reference to a given quantity, encompass and describe the given quantity itself.

[0039] Alternatively, in biological systems, the terms “about” and “approximately” may mean values that are within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold of a given value. Numerical quantities given herein are approximate unless stated otherwise, meaning that the term “about” or “approximately” can be inferred when not expressly stated.

[0040] As used herein, the term “co-administering” includes sequential or simultaneous administration of tucatinib, trastuzumab, a taxane, and a vascular endothelial growth factor receptor 2 (VEGFR-2) antagonist (e.g., tucatinib, trastuzumab, paclitaxel, and ramucirumab). For example, the co-administered compounds can be administered by the same route. In other instances, the co-administered compounds are administered via different routes. For example, one or two compounds can be administered orally, and the other compound(s) can be administered, e.g., sequentially or simultaneously, via intravenous, intramuscular, subcutaneous, or intraperitoneal injection. The simultaneously or sequentially administered compounds or compositions can be administered such that tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist (e.g., tucatinib, trastuzumab, paclitaxel, and ramucirumab) are simultaneously present in a subject or in a cell at an effective concentration.

[0041] As used herein, the term “combination”, “therapeutic combination”, “combination therapy”, or “pharmaceutical combination”, as used herein, defines either a fixed combination in one dosage unit form or a kit of parts or instructions for the combined administration where the tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist (e.g., tucatinib, trastuzumab, paclitaxel, and ramucirumab) may be administered independently at the same time or separately within time intervals that allow that the combination partners show a cooperative, e.g., synergistic, effect.

[0042] A “cancer” refers to a broad group of various diseases characterized by the uncontrolled growth of abnormal cells in the body. A “cancer” or “cancer tissue” can include a tumor.

[0043] In the context of cancer, the term “stage” refers to a classification of the extent of cancer. Factors that are considered when staging a cancer include but are not limited to tumor size, tumor invasion of nearby tissues, and whether the tumor has metastasized to other sites. The specific

criteria and parameters for differentiating one stage from another can vary depending on the type of cancer. Cancer staging can be used, for example, to assist in determining a prognosis or identifying the most appropriate treatment option(s).

[0044] One non-limiting example of a cancer staging system is referred to as the “TNM” system. In the TNM system, “T” refers to the size and extent of the main tumor, “N” refers to the number of nearby lymph nodes to which the cancer has spread, and “M” refers to whether the cancer has metastasized. “TX” denotes that the main tumor cannot be measured, “T0” denotes that the main tumor cannot be found, and “T1,” “T2,” “T3,” and “T4” denote the size or extent of the main tumor, wherein a larger number corresponds to a larger tumor or a tumor that has grown into nearby tissues. “NX” denotes that cancer in nearby lymph nodes cannot be measured, “N0” denotes that there is no cancer in nearby lymph nodes, and “N1,” “N2,” “N3,” and “N4” denote the number and location of lymph nodes to which the cancer has spread, wherein a larger number corresponds to a greater number of lymph nodes containing the cancer. “MX” denotes that metastasis cannot be measured, “M0” denotes that no metastasis has occurred, and “M1” denotes that the cancer has metastasized to other parts of the body.

[0045] As another non-limiting example of a cancer staging system, cancers are classified or graded as having one of five stages: “Stage 0,” “Stage I,” “Stage II,” “Stage III,” or “Stage IV.” Stage 0 denotes that abnormal cells are present, but have not spread to nearby tissue. This is also commonly called carcinoma *in situ* (CIS). CIS is not cancer, but may subsequently develop into cancer. Stages I, II, and III denote that cancer is present. Higher numbers correspond to larger tumor sizes or tumors that have spread to nearby tissues. Stage IV denotes that the cancer has metastasized. One of skill in the art will be familiar with the different cancer staging systems and readily be able to apply or interpret them.

[0046] The term “HER2” (also known as also known as HER2/neu, ERBB2, CD340, receptor tyrosine-protein kinase erbB-2, proto-oncogene Neu, and human epidermal growth factor receptor 2) refers to a member of the human epidermal growth factor receptor (HER/EGFR/ERBB) family of receptor tyrosine kinases. Amplification or overexpression of HER2 plays a significant role in the development and progression of certain aggressive types of cancer, including colorectal cancer, gastric cancer, gastric adenocarcinoma, gastroesophageal junction (GEJ) adenocarcinoma, lung cancer (e.g., non-small cell lung cancer (NSCLC)), biliary cancers (e.g., cholangiocarcinoma,

gallbladder cancer), bladder cancer, esophageal cancer, melanoma, ovarian cancer, liver cancer, prostate cancer, pancreatic cancer, small intestine cancer, head and neck cancer, uterine cancer, cervical cancer, and breast cancer. Non-limiting examples of HER2 nucleotide sequences are set forth in GenBank reference numbers NP_001005862, NP_001289936, NP_001289937, NP_001289938, and NP_004448. Non-limiting examples of HER2 peptide sequences are set forth in GenBank reference numbers NP_001005862, NP_001276865, NP_001276866, NP_001276867, and NP_004439. Each of these sequences are hereby incorporated by reference in their entireties.

[0047] When HER2 is amplified or overexpressed in or on a cell, the cell is referred to as being “HER2 positive.” The level of HER2 amplification or overexpression in HER2 positive cells is commonly expressed as a score ranging from 0 to 3 (*i.e.*, HER2 0, HER2 1+, HER2 2+, or HER2 3+), with higher scores corresponding to greater degrees of expression.

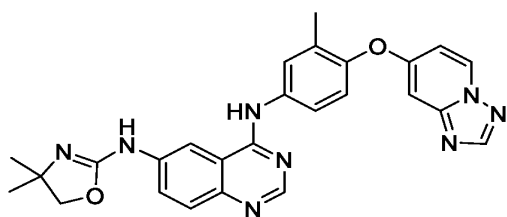
[0048] The term “HER2 positive-associated” with respect to a disease or disorder, as used herein refers to diseases or disorders associated with amplification or overexpression of HER2. Non-limiting examples of HER2 positive-associated diseases or disorders can include, for example, HER2 positive breast cancer (e.g., “HER2 positive breast cancer-associated”).

[0049] The term “metastasis” is an art known term that refers to the spread of cancer cells from the place where they first formed (the primary site) to one or more other sites in a subject (one or more secondary sites). In metastasis, cancer cells break away from the original (primary) tumor, travel through the blood or lymph system, and form a new tumor (a metastatic tumor) in other organs or tissues of the body. The new, metastatic tumor includes the same or similar cancer cells as the primary tumor. At the secondary site, the tumor cell may proliferate and begin the growth or colonization of a secondary tumor at this distant site.

[0050] The term “metastatic cancer” (also known as “secondary cancer”) as used herein refers to a type of cancer that originates in one tissue type, but then spreads to one or more tissues outside of the (primary) cancer’s origin. Following metastasis, the distal tumors can be said to be “derived from” the pre-metastasis tumor. For example, a “tumor derived from” a breast cancer refers to a tumor that can be the result of a metastasized breast cancer. Metastatic brain cancer refers to cancer

in the brain, i.e., cancer which originated in a tissue other than the brain and has metastasized to the brain.

[0051] The term “tucatinib,” also known as ONT-380, ARRY-380, and TUKYSA™, refers to the small molecule tyrosine kinase inhibitor that suppresses or blocks HER2 activation. Tucatinib has the following structure:



. In some instances, tucatinib can be in the form of a pharmaceutically acceptable salt.

[0052] Non-limiting examples of a taxane include paclitaxel, docetaxel, cabazitaxel, larotaxel, BMS-184476, BMS-188797, BMS-275183, milataxel, ortaxel, TL-310, docosaheptaenoic acid-paclitaxel (DHA-paclitaxel), *nab* paclitaxel, EndoTAG+paclitaxel, XRP9881, polymeric-micellar paclitaxel, RPR-109881A, a pharmaceutically acceptable salt or solvate thereof, and a combination thereof.

[0053] The term “vascular endothelial growth factor receptor 2 (VEGFR-2) antagonist”, as used herein refers to a receptor ligand or drug that can reduce, decrease, or otherwise mitigate a biological response by binding and/or blocking a vascular endothelial growth factor receptor 2. Non-limiting examples of a VEGFR-2 antagonist include: bevacizumab, ramucirumab, aflibercept, cetuximab, panitumumab, regorafenib, sunitinib, sorafenib, pazopanib, vandetanib, axitinib, cediranib, vatalanib, and motesanib.

[0054] The term “anti-HER2 antibody-drug conjugate” refers to an anti-HER2 antibody conjugated to a therapeutic agent (i.e., a drug) optionally via a linker.

[0055] An “anti-HER2 antibody”, as used herein, refers to an antibody that binds to the HER2 protein. Anti-HER2 antibodies used for the treatment of cancer are typically monoclonal, although polyclonal antibodies are not excluded by the term. Anti-HER2 antibodies inhibit HER2 activation or downstream signaling by various mechanisms. As non-limiting examples, anti-HER2 antibodies can prevent ligand binding, receptor activation or receptor signal propagation, result in reduced

HER2 expression or localization to the cell surface, inhibit HER2 cleavage, or induce antibody-mediated cytotoxicity. Non-limiting examples of anti-HER2 antibodies that are suitable for use in the methods and compositions of the present invention include trastuzumab, pertuzumab, margetuximab, and combinations thereof.

[0056] The term “ado-trastuzumab emtansine”, also known as T-DM1, refers to an antibody-drug conjugate composed of trastuzumab, a thioether linker, and a derivative of the antimitotic agent maytansine (also known as DM1). Ado-trastuzumab emtansine is sold in the U.S. under the trade name KADCYCLA®. As used herein, “ado-trastuzumab emtansine” also includes biosimilars of trastuzumab, for example, Kanjinti (trastuzumab-anns).

[0057] A “biosimilar” as used herein refers to an antibody or antigen-binding fragment that has the same primary amino acid sequence as compared to a reference antibody (e.g., trastuzumab) and optionally, may have detectable differences in post-translation modifications (e.g., glycosylation and/or phosphorylation) as compared to the reference antibody (e.g., a different glycoform). As a reference, the amino acid sequence of the heavy chain of trastuzumab is provided as SEQ. ID NO. 1, the light chain of trastuzumab is provided as SEQ. ID NO. 2, the light chain variable domain (SEQ. ID NO. 3), and the heavy chain variable domain (SEQ. ID NO. 4) (see also FIG. 4 and U.S. Patent No. 5,821,337, which is incorporated herein in its entirety).

[0058] In some embodiments, a biosimilar is an antibody or antigen-binding fragment thereof that has a light chain that has the same primary amino acid sequence as compared to a reference antibody (e.g., trastuzumab) and a heavy chain that has the same primary amino acid sequence as compared to the reference antibody. In some examples, a biosimilar is an antibody or antigen-binding fragment thereof that has a light chain that includes the same light chain variable domain sequence as a reference antibody (e.g., trastuzumab) and a heavy chain that includes the same heavy chain variable domain sequence as a reference antibody. In some embodiments, a biosimilar can have a similar glycosylation pattern as compared to the reference antibody (e.g., trastuzumab). In other embodiments, a biosimilar can have a different glycosylation pattern as compared to the reference antibody (e.g., trastuzumab).

[0059] The term “tumor growth inhibition (TGI) index” refers to a value used to represent the degree to which an agent (e.g., tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist or a

combination thereof) inhibits the growth of a tumor when compared to an untreated control. The TGI index is calculated for a particular time point (e.g., a specific number of days into an experiment or clinical trial) according to the following formula:

$$TGI = 1 - \left(\frac{Volume_{treated}(Tx\ Day\ X) - Volume_{treated}(Tx\ Day\ 0)}{Volume_{control}(Tx\ Day\ X) - Volume_{control}(Tx\ Day\ 0)} \right) \times 100\%,$$

where “Tx Day 0” denotes the first day that treatment is administered (i.e., the first day that an experimental therapy or a control therapy (e.g., vehicle only) is administered) and “Tx Day X” denotes X number of days after Day 0. Typically, mean volumes for treated and control groups are used. As a non-limiting example, in an experiment where study day 0 corresponds to “Tx Day 0” and the TGI index is calculated on study day 28 (i.e., “Tx Day 28”), if the mean tumor volume in both groups on study day 0 is 250 mm³ and the mean tumor volumes in the experimental and control groups are 125 mm³ and 750 mm³, respectively, then the TGI index on day 28 is 125%.

[0060] As used herein, the term “synergistic” or “synergy” refers to a result that is observed when administering a combination of components or agents (e.g., a combination of tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist) produces an effect (e.g., inhibition of tumor growth, prolongation of survival time) that is greater than the effect that would be expected based on the additive properties or effects of the individual components. In some embodiments, synergism is determined by performing a Bliss analysis (*see, e.g., Fouquier et al. Pharmacol. Res. Perspect.* (2015) 3(3):e00149; hereby incorporated by reference in its entirety for all purposes). The Bliss Independence model assumes that drug effects are outcomes of probabilistic processes, and assumes that the drugs act completely independently (i.e., the drugs do not interfere with one another (e.g., the drugs have different sites of action) but each contributes to a common result).

[0061] The observed effect of a combination of drugs can be based on, for example, the TGI index, tumor size (e.g., volume, mass), an absolute change in tumor size (e.g., volume, mass) between two or more time points (e.g., between the first day a treatment is administered and a particular number of days after treatment is first administered), the rate of change of tumor size (e.g., volume, mass) between two or more time points (e.g., between the first day a treatment is administered and a particular number of days after treatment is first administered), or the survival time of a subject

or a population of subjects. When the TGI index is taken as a measure of the observed effect of a combination of drugs, the TGI index can be determined at one or more time points. When the TGI index is determined at two or more time points, in some instances the mean or median value of the multiple TGI indices can be used as a measure of the observed effect. Furthermore, the TGI index can be determined in a single subject or a population of subjects. When the TGI index is determined in a population, the mean or median TGI index in the population (e.g., at one or more time points) can be used as a measure of the observed effect. When tumor size or the rate of tumor growth is used as a measure of the observed effect, the tumor size or rate of tumor growth can be measured in a subject or a population of subjects. In some instances, the mean or median tumor size or rate of tumor growth is determined for a subject at two or more time points, or among a population of subjects at one or more time points. When survival time is measured in a population, the mean or median survival time can be used as a measure of the observed effect.

[0062] When TGI indices are taken as a measure of the observed effects, the TGI indices can be determined at one or more time points. When TGI indices are determined at two or more time points, in some instances the mean or median values can be used as measures of the observed effects. Furthermore, the TGI indices can be determined in a single subject or a population of subjects in each treatment group. When the TGI indices are determined in populations of subjects, the mean or median TGI indices in each population (e.g., at one or more time points) can be used as measures of the observed effects. When tumor sizes or the rates of tumor growth are used as measures of the observed effects, the tumor sizes or rates of tumor growth can be measured in a subject or a population of subjects in each treatment group. In some instances, the mean or median tumor sizes or rates of tumor growth are determined for subjects at two or more time points, or among populations of subjects at one or more time points. When survival time is measured in a population, mean or median survival times can be used as measures of the observed effects.

[0063] In some embodiments, a combination of tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist is considered to be synergistic when the combination produces an observed TGI index that is greater than the predicted TGI index for the combination of drugs (e.g., when the predicted TGI index is based upon the assumption that the drugs produced a combined effect that is additive). In some instances, the combination is considered to be synergistic when the observed TGI index is at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%,

17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, or 80% greater than the predicted TGI index for the combination of drugs.

[0064] In some embodiments, the rate of tumor growth (e.g., the rate of change of the size (e.g., volume, mass) of the tumor) is used to determine whether a combination of drugs is synergistic (e.g., the combination of drugs is synergistic when the rate of tumor growth is slower than would be expected if the combination of drugs produced an additive effect). In other embodiments, survival time is used to determine whether a combination of drugs is synergistic (e.g., a combination of drugs is synergistic when the survival time of a subject or population of subjects is longer than would be expected if the combination of drugs produced an additive effect).

[0065] "Treatment" or "therapy" of a subject refers to any type of intervention or process performed on, or the administration of an active agent to, the subject with the objective of reversing, alleviating, ameliorating, inhibiting, slowing down, or preventing the onset, progression, development, severity, or recurrence of a symptom, complication, condition, or biochemical indicia associated with a disease. In some embodiments, the disease is cancer. As used herein, the terms "treatment" and "treating" when referring, e.g., to the treatment of a cancer, are not intended to be absolute terms. For example, "treatment of cancer" and "treating cancer", as used in a clinical setting, is intended to include obtaining beneficial or desired clinical results and can include an improvement in the condition of a subject having cancer. Beneficial or desired clinical results include, but are not limited to, one or more of the following: reducing the proliferation of (or destroying) neoplastic or cancerous cells, inhibiting metastasis of neoplastic cells, a decrease in metastasis in a subject, shrinking or decreasing the size of a tumor, change in the growth rate of one or more tumor(s) in a subject, an increase in the period of remission for a subject (e.g., as compared to the one or more metric(s) in a subject having a similar cancer receiving no treatment or a different treatment, or as compared to the one or more metric(s) in the same subject prior to treatment), decreasing symptoms resulting from a disease, increasing the quality of life of those suffering from a disease (e.g., assessed using FACT-G or EORTC-QLQC30), decreasing the dose of other medications required to treat a disease, delaying the progression of a disease, and/or prolonging survival of subjects having a disease.

[0066] The term "prophylactic" or "prophylactically" refers to any type of intervention or process performed on, or the administration of an active agent to, the subject with the objective of

protecting or preventing a disease or condition from developing or at least not developing fully (e.g., to reduce the symptoms or severity of the disease or condition) such as in the development of a side effect (e.g., diarrhea).

[0067] A "subject" includes any human or non-human animal. The term "non-human animal" includes, but is not limited to, vertebrates such as non-human primates, sheep, dogs, and rodents such as mice, rats, and guinea pigs. In some embodiments, the subject is a human. The terms "subject" and "patient" and "individual" are used interchangeably herein.

[0068] An "effective amount" or "therapeutically effective amount" or "therapeutically effective dosage" of a drug or therapeutic agent is any amount of the drug that, when used alone or in combination with another therapeutic agent, protects a subject against the onset of a disease or promotes disease regression evidenced by a decrease in severity of disease symptoms, an increase in frequency and duration of disease symptom-free periods, or a prevention of impairment or disability due to the disease affliction. The ability of a therapeutic agent to promote disease regression can be evaluated using a variety of methods known to the skilled practitioner, such as in human subjects during clinical trials, in animal model systems predictive of efficacy in humans, or by assaying the activity of the agent in *in vitro* assays.

[0069] By way of example for the treatment of tumors, a therapeutically effective amount of an anti-cancer agent inhibits cell growth or tumor growth by at least about 10%, by at least about 20%, by at least about 30%, by at least about 40%, by at least about 50%, by at least about 60%, by at least about 70%, or by at least about 80%, by at least about 90%, by at least about 95%, by at least about 96%, by at least about 97%, by at least about 98%, or by at least about 99% in a treated subject(s) (e.g., one or more treated subjects) relative to an untreated subject(s) (e.g., one or more untreated subjects). In some embodiments, a therapeutically effective amount of an anti-cancer agent inhibits cell growth or tumor growth by 100% in a treated subject(s) (e.g., one or more treated subjects) relative to an untreated subject(s) (e.g., one or more untreated subjects).

[0070] In other embodiments of the disclosure, tumor regression (e.g., brain metastasis regression) can be observed and continue for a period of at least about 20 days, at least about 30 days, at least about 40 days, at least about 50 days, or at least about 60 days.

[0071] As used herein, "subtherapeutic dose" means a dose of a therapeutic compound (e.g., tucatinib) that is lower than the usual or typical dose of the therapeutic compound when administered alone for the treatment of a hyperproliferative disease (e.g., cancer).

[0072] "Simultaneous administration," as used herein, means that the two or more therapies (e.g., in a combination therapy) are administered with a time separation of no more than about 15 minutes, such as no more than about any of 10, 5, or 1 minutes. When the two or more therapies are administered simultaneously, the two or more therapies can be contained in the same composition (e.g., a composition comprising both a first and second therapy) or in separate compositions (e.g., a first therapy in one composition and a second therapy is contained in another composition).

[0073] As used herein, the term "sequential administration" means that the two or more therapies (e.g., in a combination therapy) are administered with a time separation of more than about 15 minutes, such as more than about any of 20, 30, 40, 50, 60, or more minutes. Any of the two or more therapies may be administered first. The two or more therapies are contained in separate compositions, which may be contained in the same or different packages or kits.

[0074] As used herein, the term "concurrent administration" means that the administration of two or more therapies (e.g., in a combination therapy) overlap with each other. For example, the two or more therapies can be administered in the same day, or with a time separation of within one day, within two days, within three days, within four days, within five days, within six days, within seven days, within ten days, within fourteen days, or within twenty-one days.

[0075] By way of example, an "anti-cancer agent" promotes cancer regression in a subject. In some embodiments, a therapeutically effective amount of the drug promotes cancer regression to the point of eliminating the cancer. "Promoting cancer regression" means that administering an effective amount of the drug, alone or in combination with an anti-cancer agent, results in a reduction in tumor growth or size, necrosis of the tumor, a decrease in severity of at least one disease symptom, an increase in frequency and duration of disease symptom-free periods, or a prevention of impairment or disability due to the disease affliction. In addition, the terms "effective" and "effectiveness" with regard to a treatment includes both pharmacological effectiveness and physiological safety. Pharmacological effectiveness refers to the ability of the

drug to promote cancer regression in the patient. Physiological safety refers to the level of toxicity or other adverse physiological effects at the cellular, organ and/or organism level (adverse effects) resulting from administration of the drug.

[0076] "Sustained response" refers to the sustained effect on reducing tumor growth after cessation of a treatment. For example, the tumor size can remain to be the same or smaller as compared to the size at the beginning of the administration phase. In some embodiments, the sustained response has a duration that is at least the same as the treatment duration, or at least 1.5, 2.0, 2.5, or 3 times longer than the treatment duration.

[0077] As used herein, "complete response" or "CR" refers to disappearance of all target lesions; "partial response" or "PR" refers to at least a 30% decrease in the sum of the longest diameters (SLD) of target lesions, taking as reference the baseline SLD; and "stable disease" or "SD" refers to neither sufficient shrinkage of target lesions to qualify for PR, nor sufficient increase to qualify for PD, taking as reference the smallest SLD since the treatment started.

[0078] As used herein, "progression free survival" or "PFS" refers to the length of time during and after treatment during which the disease being treated (e.g., breast cancer) does not get worse. Progression-free survival may include the amount of time patients have experienced a complete response or a partial response, as well as the amount of time patients have experienced stable disease.

[0079] As used herein, "overall response rate" or "ORR" refers to the sum of complete response (CR) rate and partial response (PR) rate.

[0080] As used herein, "overall survival" or "OS" refers to the percentage of individuals in a group who are likely to be alive after a particular duration of time.

[0081] The term "weight-based dose", as referred to herein, means that a dose administered to a subject is calculated based on the weight of the subject. For example, when a subject with 60 kg body weight requires 3.6 mg/kg of an agent, such as tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist one can calculate and use the appropriate amount of the agent (i.e., 216 mg) for administration to said subject.

[0082] The use of the term "fixed dose" with regard to a method of the disclosure means that two or more different agents (e.g., tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist) are administered to a subject in particular (fixed) ratios with each other. In some embodiments, the fixed dose is based on the amount (e.g., mg) of the agents. In certain embodiments, the fixed dose is based on the concentration (e.g., mg/ml) of the agents.

[0083] The use of the term "flat dose" with regard to the methods and dosages of the disclosure means a dose that is administered to a subject without regard for the weight or body surface area (BSA) of the subject. The flat dose is therefore not provided as a mg/kg dose, but rather as an absolute amount of the agent (e.g., tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist). For example, a subject with 60 kg body weight and a subject with 100 kg body weight can receive the same dose of tucatinib (e.g., 300 mg).

[0084] The phrase "pharmaceutically acceptable" indicates that the substance or composition must be compatible chemically and/or toxicologically, with the other ingredients comprising a formulation, and/or the mammal being treated therewith.

[0085] As used herein, the term "pharmaceutically acceptable carrier" refers to a substance that aids the administration of an active agent to a cell, an organism, or a subject. "Pharmaceutically acceptable carrier" refers to a carrier or excipient that can be included in the compositions of the disclosure and that causes no significant adverse toxicological effect on the subject. Non-limiting examples of pharmaceutically acceptable carriers include water, NaCl, normal saline solutions, lactated Ringer's, normal sucrose, normal glucose, binders, fillers, disintegrants, lubricants, coatings, sweeteners, flavors and colors, liposomes, dispersion media, microcapsules, cationic lipid carriers, isotonic and absorption delaying agents, and the like. The carrier may also be substances for providing the formulation with stability, sterility and isotonicity (e.g., antimicrobial preservatives, antioxidants, chelating agents and buffers), for preventing the action of microorganisms (e.g. antimicrobial and antifungal agents, such as parabens, chlorobutanol, phenol, sorbic acid and the like) or for providing the formulation with an edible flavor *etc.* In some instances, the carrier is an agent that facilitates the delivery of a small molecule drug or antibody to a target cell or tissue. One of skill in the art will recognize that other pharmaceutical carriers are useful in the present disclosure.

[0086] The phrase "pharmaceutically acceptable salt" as used herein, refers to pharmaceutically acceptable organic or inorganic salts of a compound of the disclosure. Exemplary salts include, but are not limited, to sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucuronate, saccharate, formate, benzoate, glutamate, methanesulfonate (mesylate), ethanesulfonate, benzenesulfonate, p-toluenesulfonate, pamoate (i.e., 4,4'-methylene-bis -(2-hydroxy-3-naphthoate)) salts, alkali metal (e.g., sodium and potassium) salts, alkaline earth metal (e.g., magnesium) salts, and ammonium salts. A pharmaceutically acceptable salt may involve the inclusion of another molecule such as an acetate ion, a succinate ion or other counter ion. The counter ion may be any organic or inorganic moiety that stabilizes the charge on the parent compound. Furthermore, a pharmaceutically acceptable salt may have more than one charged atom in its structure. Instances where multiple charged atoms are part of the pharmaceutically acceptable salt can have multiple counter ions. Hence, a pharmaceutically acceptable salt can have one or more charged atoms and/or one or more counter ion.

[0087] As used herein, the term "solid dispersion" means a system in a solid state comprising at least two components, wherein one component is dispersed throughout the other component. For example, a solid dispersion as described herein can include one component of tucatinib dispersed throughout another component, such as a dispersion polymer.

[0088] As used herein, the term "amorphous" means a solid in a solid state that is a non-crystalline state. Amorphous solids generally possess crystal-like short range molecular arrangement, but no long range order of molecular packing as found in crystalline solids. The solid state form of a solid may be determined by polarized light microscopy, X-ray powder diffraction ("XRPD"), differential scanning calorimetry ("DSC"), or other standard techniques known to those of skill in the art.

[0089] As used herein, the term "amorphous solid dispersion" means a solid comprising a drug substance and a dispersion polymer. The amorphous solid dispersion discussed herein comprises amorphous tucatinib and a dispersion polymer, wherein the amorphous solid dispersion contains tucatinib in a substantially amorphous solid state form. In certain embodiments, the substantially amorphous solid state form means that the tucatinib component in the amorphous solid dispersion

is at least 80% amorphous tucatinib. In certain embodiments, the substantially amorphous solid state form means that the tucatinib component in the amorphous solid dispersion is at least 85% amorphous tucatinib. In certain embodiments, the substantially amorphous solid state form means that the tucatinib component in the amorphous solid dispersion is at least 90% tucatinib. In certain embodiments, the substantially amorphous solid state form means that the tucatinib component in the amorphous solid dispersion is at least 95% amorphous tucatinib.

[0090] As used herein, the term “dispersion polymer” means a polymer that allows for tucatinib to be dispersed throughout such that a solid dispersion may form. The dispersion polymer is preferably neutral or basic. The dispersion polymer may contain a mixture of two or more polymers. Examples of dispersion polymers include, but are not limited to, vinyl polymers and copolymers, vinylpyrrolidone vinylacetate copolymer (“PVP-VA”), polyvinyl alcohols, polyvinyl alcohol polyvinyl acetate copolymers, polyvinyl pyrrolidone (“PVP”), acrylate and methacrylate copolymers, methylacrylic acid methyl methacrylate copolymer (such as Eudragit®), polyethylene polyvinyl alcohol copolymers, polyoxyethylene-polyoxypropylene block copolymers (also referred to as poloxamers), graft copolymer comprised of polyethylene glycol, polyvinyl caprolactam and polyvinyl acetate (such as Soluplus®), cellulosic polymers, such as hydroxypropyl methyl cellulose acetate (“HPMCA”), hydroxypropyl methyl cellulose (“HPMC”), hydroxypropyl cellulose (“HPC”), methyl cellulose, hydroxyethyl methyl cellulose, hydroxyethyl cellulose, hydroxyethyl cellulose acetate, and hydroxyethyl ethyl cellulose, hydroxypropyl methyl cellulose acetate succinate (“HPMCAS”), hydroxypropyl methyl cellulose phthalate (“HPMCP”), carboxymethylethyl cellulose (“CMEC”), cellulose acetate phthalate (“CAP”), cellulose acetate succinate (“CAS”), hydroxypropyl methyl cellulose acetate phthalate (“HPMCAP”), cellulose acetate trimellitate (“CAT”), hydroxypropyl methyl cellulose acetate trimellitate (“HPMCAT”), and carboxymethylcellulose acetate butyrate (“CMCAB”), and the like.

[0091] As used herein, the term “spray drying” means processes involved in breaking up liquid mixtures into small droplets (atomization) and rapidly removing solvent from the mixture in a spray drying apparatus where there is a strong driving force for evaporation of solvent from the droplets. The phrase spray drying is used conventionally and broadly. Spray drying processes and spray drying equipment are described generally in Perry, Robert H., and Don W. Green (eds.). *Perry's Chemical Engineers' Handbook*. New York: McGraw-Hill, 2007 (8th edition).

[0092] As used herein, “polymorphs” refer to distinct solids sharing the same molecular formula, yet each polymorph may have distinct solid state physical properties. A single compound may give rise to a variety of polymorphic forms where each form has different and distinct solid state physical properties, such as different solubility profiles, melting point temperatures, flowability, dissolution rates and/or different X-ray diffraction peaks. These practical physical characteristics are influenced by the conformation and orientation of molecules in the unit cell, which defines a particular polymorphic form of a substance. Polymorphic forms of a compound can be distinguished in a laboratory by X-ray diffraction spectroscopy, such as X-ray powder diffraction (“XRPD”), and by other methods, such as infrared spectrometry. Additionally, polymorphic forms of the same drug substance or active pharmaceutical ingredient can be administered by itself or formulated as a drug product (pharmaceutical composition) and are well known in the pharmaceutical art to affect, for example, the solubility, stability, flowability, tractability and compressibility of drug substances and the safety and efficacy of drug products. For more, see Hilfiker, Rolf (ed.), *Polymorphism in the Pharmaceutical Industry*. Weinheim, Germany: Wiley-VCH 2006.

[0093] "Administering" or “administration” refer to the physical introduction of a therapeutic agent to a subject, using any of the various methods and delivery systems known to those skilled in the art. Exemplary routes of administration include oral, intravenous, intramuscular, subcutaneous, intraperitoneal, spinal or other parenteral routes of administration, for example by injection or infusion (e.g., intravenous infusion). The phrase "parenteral administration" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intralymphatic, intralesional, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion, as well as *in vivo* electroporation. A therapeutic agent can be administered via a non-parenteral route, or orally. Other non-parenteral routes include a topical, epidermal or mucosal route of administration, for example, intranasally, vaginally, rectally, sublingually or topically. Administration can also be performed, for example, once, a plurality of times, and/or over one or more extended periods.

[0094] The terms "baseline" or "baseline value" used interchangeably herein can refer to a measurement or characterization of a symptom before the administration of the therapy or at the beginning of administration of the therapy. The baseline value can be compared to a reference value in order to determine the reduction or improvement of a symptom of a disease contemplated herein (e.g., breast cancer). The terms "reference" or "reference value" used interchangeably herein can refer to a measurement or characterization of a symptom after administration of the therapy. The reference value can be measured one or more times during a dosage regimen or treatment cycle or at the completion of the dosage regimen or treatment cycle. A "reference value" can be an absolute value; a relative value; a value that has an upper and/or lower limit; a range of values; an average value; a median value; a mean value; or a value as compared to a baseline value.

[0095] Similarly, a "baseline value" can be an absolute value; a relative value; a value that has an upper and/or lower limit; a range of values; an average value; a median value; a mean value; or a value as compared to a reference value. The reference value and/or baseline value can be obtained from one individual, from two different individuals or from a group of individuals (e.g., a group of two, three, four, five or more individuals).

[0096] An "adverse event" (AE) as used herein is any unfavorable and generally unintended or undesirable sign (including an abnormal laboratory finding), symptom, or disease associated with the use of a medical treatment. A medical treatment can have one or more associated AEs and each AE can have the same or different level of severity. Reference to methods capable of "altering adverse events" means a treatment regime that decreases the incidence and/or severity of one or more AEs associated with the use of a different treatment regime.

[0097] A "serious adverse event" or "SAE" as used herein is an adverse event that meets one of the following criteria:

- Is fatal or life-threatening (as used in the definition of a serious adverse event, "life-threatening" refers to an event in which the patient was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it was more severe.
- Results in persistent or significant disability/incapacity
- Constitutes a congenital anomaly/birth defect

- Is medically significant, i.e., defined as an event that jeopardizes the patient or may require medical or surgical intervention to prevent one of the outcomes listed above. Medical and scientific judgment must be exercised in deciding whether an AE is “medically significant”

- Requires inpatient hospitalization or prolongation of existing hospitalization, excluding the following: 1) routine treatment or monitoring of the underlying disease, not associated with any deterioration in condition; 2) elective or pre-planned treatment for a pre-existing condition that is unrelated to the indication under study and has not worsened since signing the informed consent; and 3) social reasons and respite care in the absence of any deterioration in the patient’s general condition.

[0098] The terms "once about every week," "once about every two weeks," or any other similar dosing interval terms as used herein mean approximate numbers. "Once about every week" can include every seven days $\pm$ one day, i.e., every six days to every eight days. "Once about every two weeks" can include every fourteen days $\pm$ two days, i.e., every twelve days to every sixteen days. "Once about every three weeks" can include every twenty-one days $\pm$ three days, i.e., every eighteen days to every twenty-four days. Similar approximations apply, for example, to once about every four weeks, once about every five weeks, once about every six weeks, and once about every twelve weeks. In some embodiments, a dosing interval of once about every six weeks or once about every twelve weeks means that the first dose can be administered any day in the first week, and then the next dose can be administered any day in the sixth or twelfth week, respectively. In other embodiments, a dosing interval of once about every six weeks or once about every twelve weeks means that the first dose is administered on a particular day of the first week (e.g., Monday) and then the next dose is administered on the same day of the sixth or twelfth weeks (i.e., Monday), respectively.

[0099] As described herein, any concentration range, percentage range, ratio range, or integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated.

[0100] Various aspects of the disclosure are described in further detail in the following herein.

II. Description of the Embodiments

A. Methods for Treating Cancer with Tucatinib in Combination with Trastuzumab, a Taxane, and a VEGFR-2 antagonist

[0101] HER2 is a validated target in multiple solid tumors, with anti-HER2 biologics and small-molecule drugs approved for patients with HER2+ breast and gastric cancers. Amplification of the HER2-gene or overexpression of its protein occurs in approximately 15% to 20% of breast cancers and 6% to 30% of gastric and esophageal cancers. Recently, interest has grown in HER2-targeting strategies for patients with refractory metastatic colorectal carcinoma (CRC), where overexpression of HER2 has been found to occur in approximately 3% to 5% of patients. HER2 can also be overexpressed in other gastrointestinal cancers, such as cholangiocarcinoma and gallbladder carcinoma, where studies suggest ERBB2 amplification ranges from 1% to 6%.

[0102] The current standard of care for patients with HER2+ metastatic disease consists of treatment with pertuzumab plus trastuzumab and a taxane as first-line treatment for metastatic disease, followed by T-DM1 in second line. Treatment options for patients who progress after treatment with both pertuzumab and T-DM1 remain relatively limited. Patients are generally treated with a continuation of anti-HER2 therapy (in the form of trastuzumab or lapatinib) in combination with cytotoxic chemotherapy, such as capecitabine. Combined HER2 therapy with trastuzumab and lapatinib can also be considered. In some HER2 positive gastrointestinal cancers, the standard of care of treatment with trastuzumab and chemotherapy, while treatment with lapatinib has been relatively ineffective against gastrointestinal cancers. In other HER2 positive cancers such as gastroesophageal, colorectal, biliary tract, and gallbladder cancers, the standard of care is an oxaliplatin-based chemotherapy comprising the combination of oxaliplatin, fluorouracil, and leucovorin (e.g., FOLFOX and/or a modified FOLFOX regimen). However, better options for these patients are needed. The treatment and prevention of HER2 positive cancers represents an unmet need.

[0103] In some aspects, the disclosure provides a method of treating a HER2 positive cancer in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a

vascular endothelial growth factor receptor 2 (VEGFR-2) antagonist(e.g., tucatinib, trastuzumab, paclitaxel, and ramucirumab).

[0104] In some aspects, the disclosure provides a method of treating a HER2 positive cancer in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a combination therapy comprising tucatinib, trastuzumab, paclitaxel, and ramucirumab.

[0105] In some aspects, the disclosure provides a method of treating cancer in a subject in need thereof comprising, identifying the subject as having a HER2 positive cancer. For example, the subject can have a HER2 positive cancer that is histologically or cytologically confirmed. In some embodiments, the HER2 positive cancer is selected from the group consisting of gastric adenocarcinoma, gastroesophageal junction (GEJ) adenocarcinoma, esophageal adenocarcinoma, colorectal carcinoma (CRC), cholangiocarcinoma, gallbladder carcinoma, gastric cancer, lung cancer, biliary cancers, bladder cancer, esophageal cancer, melanoma, ovarian cancer, liver cancer, prostate cancer, pancreatic cancer, small intestine cancer, non-small cell lung cancer, head and neck cancer, uterine cancer, cervical cancer, brain cancer, and breast cancer. In some embodiments, the HER2 positive cancer is selected from the group consisting of gastric adenocarcinoma, gastroesophageal junction (GEJ) adenocarcinoma, esophageal adenocarcinoma, colorectal carcinoma (CRC), cholangiocarcinoma, gallbladder carcinoma, gastric cancer, lung cancer, biliary cancers, bladder cancer, esophageal cancer, melanoma, ovarian cancer, liver cancer, prostate cancer, pancreatic cancer, small intestine cancer, non-small cell lung cancer, head and neck cancer, uterine cancer, cervical cancer, and brain cancer. In some embodiments, the HER2 positive cancer is unresectable or metastatic. In some embodiments, the method can further include administering to the subject a therapeutically effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist (e.g., tucatinib, trastuzumab, paclitaxel, and ramucirumab).

[0106] In some embodiments, method can include administering to the subject a therapeutically effective amount of a combination therapy comprising a biosimilar. In such embodiments, a trastuzumab biosimilar can be used in the combination therapy such that the combination therapy comprises tucatinib, a trastuzumab biosimilar, a taxane, and a VEGFR-2 antagonist.

[0107] In some embodiments, the taxane is selected from the group consisting of paclitaxel, docetaxel, cabazitaxel, larotaxel, BMS-184476, BMS-188797, BMS-275183, milataxel, ortaxel, TL-310, docosahexaenoic acid-paclitaxel (DHA-paclitaxel), *nab* paclitaxel, EndoTAG+paclitaxel, XRP9881, polymeric-micellar paclitaxel, RPR-109881A, a pharmaceutically acceptable salt or solvate thereof, and a combination thereof. In some embodiments, the taxane is selected from the group consisting of paclitaxel, docetaxel, and cabazitaxel. In some embodiments, the taxane is paclitaxel.

[0108] In some embodiments, the VEGFR-2 antagonist is selected from the group consisting of bevacizumab, ramucirumab, aflibercept, cetuximab, panitumumab, regorafenib, sunitinib, sorafenib, pazopanib, vandetanib, axitinib, cediranib, vatalanib, motesanib, lucatinib, intedanib, semaxanib, apatinib, lenvatinib, carbozantinib, and a combination thereof. In some embodiments, the VEGFR-2 is a monoclonal antibody. In some embodiments, the VEGFR-2 antagonist is a monoclonal antibody selected from the group consisting of bevacizumab, ramucirumab, aflibercept, cetuximab, panitumumab, and combinations thereof. In some embodiments, the monoclonal antibody is ramucirumab.

[0109] In some embodiments, the subject was previously treated with at least one anticancer therapy. In such embodiments, the subject may have been treated with at least one anticancer therapy selected from the group consisting of trastuzumab (or a trastuzumab biosimilar), lapatinib, trastuzumab and a taxane, pertuzumab, and combinations thereof. In some embodiments, the subject has been previously treated with one or more additional therapeutic agents for the cancer and did not respond to the treatment. In some embodiments, the subject has been previously treated with one or more additional therapeutic agents for the cancer and relapsed after the treatment. In some embodiments, the subject can be refractory or develop a brain metastasis during the previous anticancer therapy.

[0110] In some embodiments, the subject has been previously treated with a HER2-directed antibody. In some such embodiments, the subject may have locally-advanced unresectable or metastatic HER2+ GEC who received prior treatment with a HER2-directed antibody; further, the subject may have received at least one prior line of therapy in the advance disease setting.

[0111] In some embodiments, the subject has not been previously treated. In some embodiments, the subject has not been previously treated with an anti-HER2 and/or an anti-EGFR tyrosine kinase inhibitor. In some embodiments, the subject has not been treated with the anti-HER2/EGFR tyrosine kinase inhibitor selected from the group consisting of tucatinib, lapatinib, neratinib, or afatinib.

[0112] In some embodiments, the subject has not been previously treated with a HER2-directed antibody-drug conjugate. In some embodiments, the subject was not treated with an antibody-drug conjugate is selected from the group consisting of ado-trastuzumab (T-DM1) or trastuzumab deruxtecan (DS8201a).

[0113] In some embodiments, the subject may have not been previously treated with tucatinib. In some embodiments, the subject has not been previously treated with an anthracycline. In some embodiments, the subject was not previously treated with anthracycline selected from the group consisting of doxorubicin, epirubicin, mitoxantrone, idarubicin, liposomal doxorubicin, and combinations thereof.

[0114] In some embodiments, the combination therapy is administered in a 28-day cycle. In some embodiments, tucatinib is dosed at approximately the same time as the taxane. In some such embodiments, tucatinib is dosed at approximately the same time as the start of the taxane infusion. For example, the tucatinib can be administered with the taxane on cycle 1 day 8. In some embodiments, the taxane is administered first, followed by trastuzumab (or a trastuzumab biosimilar), and ramucirumab. In some embodiments, the combination therapy is administered by IV.

[0115] In some embodiments, the combination therapy is administered in a 28-day cycle. In some embodiments, tucatinib is dosed at approximately the same time as paclitaxel. In some such embodiments, tucatinib is dosed at approximately the same time as the start of the paclitaxel infusion. For example, the tucatinib can be administered with the paclitaxel on cycle 1 day 8. In some embodiments, the paclitaxel is administered first, followed by trastuzumab (or a trastuzumab biosimilar), and ramucirumab. In some embodiments, the combination therapy is administered by IV.

[0116] In some embodiments, the tucatinib is administered to the subject at a dose of about 150 mg to about 650 mg. In some embodiments, the tucatinib is administered to the subject at a dose of about 300 mg. In some embodiments, the tucatinib is administered twice daily. In some embodiments, the tucatinib is administered to the subject orally. In some embodiments, the tucatinib is administered to the subject orally, twice daily, beginning on cycle 1 day 1 and onward.

[0117] In some embodiments, trastuzumab is dosed at 6 mg/kg. In some embodiments, the trastuzumab is administered to the subject via IV. In some embodiments, trastuzumab is administered at 6 mg/kg as a loading dose and is administered on cycle 1 day 1. In some embodiments, following the loading dose, the trastuzumab is administered at 4 mg/kg. In some embodiments, following the loading dose, the trastuzumab is administered via IV at 4 mg/kg on cycle 1 day 15 and then days 1 and 15 of each cycle thereafter. In some embodiments, the trastuzumab is a trastuzumab biosimilar.

[0118] In some embodiments, a VEGFR-2 antagonist is administered on days 1 and 15 of each cycle. In some embodiments, the VEGFR-2 antagonist is administered via IV. In some embodiments, the VEGFR-2 antagonist is ramucirumab. In some embodiments, ramucirumab is administered on Days 1 and 15 of each cycle. In some embodiments, the ramucirumab is administered at a dose of about 4 mg/kg to about 12 mg/kg. In some embodiments, ramucirumab is administered at a dose of about 8 mg/kg. In some embodiments, ramucirumab is administered by IV.

[0119] In some embodiments, a taxane is administered on Days 1, 8, and 15 of each cycle. In some embodiments, the taxane is selected from the group consisting of paclitaxel, docetaxel, and cabazitaxel. In some embodiments, the taxane is administered by IV. In some embodiments, the taxane is paclitaxel. In some embodiments, paclitaxel is administered on Days 1, 8, and 15 of each cycle. In some embodiments, the paclitaxel is administered to the subject at a dose of about 50 mg/m² to about 100 mg/m². In some embodiments, the paclitaxel is administered to the subject at a dose of about 80 mg/m². In some embodiments, paclitaxel is administered by IV.

[0120] In some embodiments, the subject has been previously treated with one or more additional therapeutic agents for the cancer and experienced disease progression during the treatment. In some embodiments, the one or more additional therapeutic agents is an HER2 directed antibody.

In some embodiments, the subject has been previously treated with trastuzumab, or pertuzumab. In some embodiments, the subject has been previously treated with trastuzumab. In some embodiments, the subject has been previously treated with pertuzumab. In some embodiments, the subject has been previously treated with trastuzumab and pertuzumab.

[0121] In some embodiments, the subject has not been previously treated with another anticancer therapy for the cancer within the past 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 2 weeks, 3 weeks, 4 weeks, 6 weeks, 2 months, 3 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, 15 months, 18 months, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 9 years or 10 years prior to being administered the therapeutically effective amount of tucatinib, or salt or solvate thereof. In some embodiments, the subject has not been previously treated with another therapeutic agent for the cancer within the past 12 months prior to being administered the therapeutically effective amount of tucatinib, or salt or solvate thereof. In some embodiments, the subject has not been previously treated with another therapeutic agent for the cancer. In some embodiments, the subject has not been treated with another anticancer therapy within the past three weeks. In some embodiments, the subject has not been previously treated with lapatinib, neratinib, afatinib, or capecitabine. In some embodiments, the subject has not been previously treated with lapatinib. In some embodiments, the subject has not been previously treated with neratinib. In some embodiments, the subject has not been previously treated with afatinib. In some embodiments, the subject has not been previously treated with capecitabine. In some embodiments, the subject has not been previously treated with an anti-HER2 antibody-drug conjugate (e.g., ado-trastuzumab emtansine).

[0122] In some embodiments, the HER2 status of a sample cell is determined. The determination can be made before treatment (i.e., administration of a combination of tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist) begins, during treatment, or after treatment has been completed. In some instances, determination of the HER2 status results in a decision to change therapy (e.g., adding, changing, or discontinuing the use of the combination of tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist (e.g., tucatinib, trastuzumab, paclitaxel, and ramucirumab), discontinuing therapy altogether, or switching from another treatment method to a method of the present disclosure).

[0123] In some embodiments, the sample cell is determined to be overexpressing or not overexpressing HER2. In particular embodiments, the cell is determined to be HER2 3+, HER2 2+, HER2 1+, or HER2 0 (i.e., HER is not overexpressed).

[0124] In some embodiments, the sample cell is a cancer cell. In some instances, the sample cell is obtained from a subject who has cancer. The sample cell can be obtained as a biopsy specimen, by surgical resection, or as a fine needle aspirate (FNA). In some embodiments, the sample cell is a circulating tumor cell (CTC).

[0125] HER2 expression can be compared to a reference cell. In some embodiments, the reference cell is a non-cancer cell obtained from the same subject as the sample cell. In other embodiments, the reference cell is a non-cancer cell obtained from a different subject or a population of subjects. In some embodiments, measuring expression of HER2 comprises, for example, determining *HER2* gene copy number or amplification, nucleic acid sequencing (e.g., sequencing of genomic DNA or cDNA), measuring mRNA expression, measuring protein abundance, or a combination thereof. HER2 testing methods include immunohistochemistry (IHC), *in situ* hybridization, fluorescence *in situ* hybridization (FISH), chromogenic *in situ* hybridization (CISH), ELISAs, and RNA quantification (e.g., of *HER2* expression) using techniques such as RT-PCR and microarray analysis.

[0126] In some embodiments, the sample cell is determined to be HER2 positive when HER2 is expressed at a higher level in the sample cell compared to a reference cell. In some embodiments, the cell is determined to be HER2 positive when HER2 is overexpressed at least about 1.5-fold (e.g., about 1.5-fold, 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 4.5-fold, 5-fold, 5.5-fold, 6-fold, 6.5-fold, 7-fold, 7.5-fold, 8-fold, 8.5-fold, 9-fold, 9.5-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold, 20-fold, 25-fold, 30-fold, 35-fold, 40-fold, 45-fold, 50-fold, 55-fold, 60-fold, 65-fold, 70-fold, 75-fold, 80-fold, 85-fold, 90-fold, 95-fold, 100-fold, or more) compared to a reference cell. In particular embodiments, the cell is determined to be HER2 positive when HER2 is overexpressed at least about 1.5-fold compared to the reference cell.

[0127] In some embodiments, the sample cell is determined to be HER2 positive when the FISH or CISH signal ratio is greater than 2. In other embodiments, the sample cell is determined to be HER2 positive when the *HER2* gene copy number is greater than 6.

[0128] In one aspect, provided herein are methods for treating or ameliorating a HER2 positive cancer in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist (e.g., tucatinib, trastuzumab, paclitaxel, and ramucirumab).

[0129] In one aspect provided herein are methods for treating a HER2 positive cancer in a subject that has exhibited an adverse event after starting treatment with a combination therapy comprising tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist at an initial dosage level, comprising administering to the subject at least one of the combination therapy at a reduced dosage level. For example, one, two, or all of the components included in the combination therapy can be reduced. In such embodiments, an individual component (i.e., one of tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist) of the combination therapy can be reduced following an adverse reaction experienced by the subject while the other components of the compound therapy remain at their initial dosage levels. In another such embodiment, two of the components (i.e., two of tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist) of the combination therapy can be reduced following an adverse reaction experienced by the subject while the remaining component of the compound therapy remains at its initial dosage level. In another such embodiment, all of the components (i.e., tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist) of the combination therapy can be reduced following an adverse reaction experienced by the subject and none of the components that comprise the compound therapy remain at the initial dosage level. For example, the methods can include methods for treating the HER2 positive cancer in a subject that has exhibited an adverse event after starting treatment with at least one of the combination therapy comprising tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist.

[0130] In some aspects, provided herein are methods for treating a HER2 positive cancer in a subject that has exhibited an adverse event after starting treatment with a combination therapy comprising tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist at an initial dosage level, comprising administering to the subject at least one component of the combination therapy at a reduced dosage level. In some embodiments, the taxane is paclitaxel. In some embodiments, the VEGFR-2 is ramucirumab.

[0131] In such embodiments, the paclitaxel is administered to the subject at an initial dose of about 50 mg/m² to about 100 mg/m². In some embodiments, the paclitaxel is administered to the

subject at an initial dose of about 80 mg/m². In some embodiments, the paclitaxel is administered to the subject at a reduced dose of about 50 mg/m² to about 75 mg/m². In some embodiments, the paclitaxel is administered to the subject at a reduced dose of about 70 mg/m². In some embodiments, the paclitaxel is administered to the subject at a reduced dose of about 60 mg/m². In some embodiments, the tucatinib is administered to the subject at an initial dose of about 150 mg to about 650 mg. In such embodiments, the tucatinib is administered to the subject at an initial dose of about 300 mg. In some embodiments, the tucatinib is administered to the subject at a reduced dose of about 125 mg to about 275 mg. In some embodiments, wherein the ramucirumab is administered to the subject at an initial dose of about 8 mg/kg. In some embodiments, the ramucirumab is administered to the subject at a reduced dose of about 6 mg/kg. In some embodiments, the ramucirumab is administered to the subject at a reduced dose of about 5 mg/kg.

[0132] In some embodiments of any of the methods described herein, the method can further comprise treating a HER2 positive cancer in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist, and administering an effective amount of an anti-diarrheal agent.

[0133] In some embodiments, the anti-diarrheal agent is administered prophylactically. In some embodiments of any of the methods described herein, the method can include reducing the severity or incidents of diarrhea, or preventing diarrhea in a subject having a HER2 positive cancer and being treated with an effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist, the method comprising administering an effective amount of an anti-diarrheal agent prophylactically.

[0134] In another embodiment of any of the methods described herein, the method can include a method of reducing the likelihood of a subject developing diarrhea, wherein the subject has a HER2 positive cancer and is being treated with an effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist the method comprising administering an effective amount of an anti-diarrheal agent prophylactically.

[0135] In some embodiments, the combination therapy and the anti-diarrheal agent are administered sequentially. In some embodiments, the combination therapy and the anti-diarrheal

agent are administered concurrently. In some embodiments, the anti-diarrheal agent is administered prior to administration of the combination therapy. For example, one hour before, two hours before, four hours before, six hours before, twelve hours before, one day before, two days before, three days before, four days before, five days before, or one week before. In some cases, the subject is exhibiting symptoms of diarrhea prior to administration of the anti-diarrheal agent. In other cases, the subject is not exhibiting symptoms of diarrhea prior to administration of the anti-diarrheal agent.

[0136] Non-limiting examples of anti-diarrheal agents include loperamide, budesonide (e.g., in combination with loperamide), prophylactic antibiotics (e.g., doxycycline), probiotics, electrolyte replacement solutions, colestipol, colestipol in combination with loperamide, octreotide, crofelemer, TJ14, *Bacillus Cereus*, calcium aluminosilicate, sulfasalazine, cefpodoxime, elsiglutide, glutamine, codeine, diphenoxylate, atropine, bismuth subsalicylate, diphenoxylate, atropine, attapulgit, activated charcoal, bentonite, *saccharomyces boulardii* lyo, rifaximin, neomycin, alosetron, octreotide, crofelemer, opium, cholestyramine, and colesevelam.

[0137] In some embodiments, the combination therapy and the anti-diarrheal agent are administered sequentially. In some embodiments, the combination therapy and the anti-diarrheal agent are administered concurrently. In some embodiments, the anti-diarrheal agent is administered prior to administration of the combination therapy. For example, one hour before, two hours before, four hours before, six hours before, twelve hours before, one day before, two days before, three days before, four days before, five days before, or one week before. In some cases, the subject is exhibiting symptoms of diarrhea prior to administration of the antiemetic agent. In other cases, the subject is not exhibiting symptoms of diarrhea prior to administration of the antiemetic agent.

B. Tucatinib Dose and Administration

[0138] In some embodiments, a dose of tucatinib is between about 0.1 mg and 10 mg per kg of the subject's body weight (e.g., about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, or 10 mg per kg of the subject's body weight). In other embodiments, a dose of tucatinib is between about 10 mg and 100 mg per kg of the subject's body weight (e.g., about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30,

31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 mg per kg of the subject's body weight). In some embodiments, a dose of tucatinib is at least about 100 mg to 500 mg per kg of the subject's body weight (e.g., at least about 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 mg per kg of the subject's body weight). In particular embodiments, a dose of tucatinib is between about 1 mg and 50 mg per kg of the subject's body weight (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 mg per kg of the subject's body weight). In some instances, a dose of tucatinib is about 50 mg per kg of the subject's body weight.

[0139] In some embodiments, a dose of tucatinib comprises between about 1 mg and 100 mg (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 mg) of tucatinib. In other embodiments, a dose of tucatinib comprises between about 100 mg and 1,000 mg (e.g., about 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, or 1,000 mg) of tucatinib. In particular embodiments, a dose of tucatinib is about 300 mg (e.g., when administered twice per day). In certain of these embodiments, a dose of tucatinib is 300 mg (e.g., 6 × 50 mg tablets; or 2 × 150 mg tablets), administered twice per day.

[0140] In some embodiments, a dose of tucatinib comprises at least about 1,000 mg to 10,000 mg (e.g., at least about 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,100, 2,200, 2,300, 2,400, 2,500, 2,600, 2,700, 2,800, 2,900, 3,000, 3,100, 3,200, 3,300, 3,400, 3,500, 3,600, 3,700, 3,800, 3,900, 4,000, 4,100, 4,200, 4,300, 4,400, 4,500, 4,600, 4,700, 4,800, 4,900, 5,000, 5,100, 5,200, 5,300, 5,400, 5,500, 5,600, 5,700, 5,800, 5,900, 6,000, 6,100, 6,200, 6,300, 6,400, 6,500, 6,600, 6,700, 6,800, 6,900, 7,000, 7,100, 7,200, 7,300, 7,400, 7,500, 7,600, 7,700, 7,800, 7,900, 8,000, 8,100, 8,200, 8,300, 8,400, 8,500, 8,600, 8,700, 8,800, 8,900, 9,000, 9,100, 9,200, 9,300, 9,400, 9,500, 9,600, 9,700, 9,800, 9,900, 10,000 or more mg) of tucatinib.

[0141] In some embodiments, a dose of tucatinib, or salt or solvate thereof, contains a therapeutically effective amount of tucatinib, or salt or solvate thereof. In other embodiments, a dose of tucatinib, or salt or solvate thereof, contains less than a therapeutically effective amount

of tucatinib, or salt or solvate thereof, (e.g., when multiple doses are given in order to achieve the desired clinical or therapeutic effect).

[0142] Tucatinib, or salt or solvate thereof, can be administered by any suitable route and mode. Suitable routes of administering combination therapies of the present disclosure are well known in the art and may be selected by those of ordinary skill in the art. In one embodiment, tucatinib administered parenterally. Parenteral administration refers to modes of administration other than enteral and topical administration, usually by injection, and include epidermal, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, intratendinous, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, intracranial, intrathoracic, epidural and intrasternal injection and infusion. In some embodiments, the route of administration of tucatinib is intravenous injection or infusion. In some embodiments, the route of administration of tucatinib is intravenous infusion. In some embodiments, the route of administration of tucatinib is intravenous injection or infusion. In some embodiments, the tucatinib is intravenous infusion. In some embodiments, the route of administration of tucatinib is oral.

[0143] In one embodiment of the methods or uses or product for uses provided herein, tucatinib is administered to the subject at a dose of about 150 mg to about 650 mg.

[0144] In one embodiment of the methods or uses or product for uses provided herein, tucatinib is administered to the subject daily, twice daily, three times daily or four times daily. In some embodiments, tucatinib is administered to the subject every other day, once about every week or once about every three weeks. In some embodiments, tucatinib is administered to the subject once per day. In some embodiments, tucatinib is administered to the subject twice per day. In some embodiments, tucatinib is administered to the subject at a dose of about 300 mg twice per day. In some embodiments, tucatinib is administered to the subject at a dose of 300 mg twice per day. In some embodiments, tucatinib is administered to the subject at a dose of about 600 mg once per day. In some embodiments, tucatinib is administered to the subject at a dose of 600 mg once per day. In some embodiments, tucatinib is administered to the subject twice per day on each day of a 21 day treatment cycle. In some embodiments, the tucatinib is administered to the subject orally.

C. Trastuzumab Dose and Administration

[0145] In some embodiments, trastuzumab (or a biosimilar thereof) is administered on Day 1 and 15 of every 28-day cycle. In some embodiments, trastuzumab is administered as a loading dose. In some embodiments, a loading dose of about 6 mg/kg is administered. In some embodiments, the loading dose is administered via IV. In some embodiments, the loading dose is administered on cycle 1 day 1. In some embodiments, following the loading dose, the trastuzumab is dosed at 4 mg/kg with each subsequent dose. In some embodiments, the trastuzumab is administered on a weekly basis. In some embodiments, the trastuzumab is administered at 2 mg/kg via IV once weekly.

D. VEGFR-2 antagonist Dose and Administration

[0146] In some embodiments, a VEGFR-2 antagonist (e.g., ramucirumab) is administered on Days 1 and 15 of each 28 day cycle. In some embodiments, the VEGFR-2 antagonist is ramucirumab. In some embodiments, ramucirumab is administered on Days 1 and 15 of each 28 day cycle. In some embodiments, ramucirumab is administered at a dose of about 8 mg/kg on Days 1 and 15 of each 28-day cycle. In some embodiments, the VEGFR-2 antagonist is administered via IV. In some embodiments, ramucirumab is administered via IV.

E. Taxane Dose and Administration

[0147] In some embodiments, a taxane (e.g., paclitaxel) is administered on Days 1, 8, and 15 of each cycle. In some embodiments, the taxane is selected from the group consisting of paclitaxel, docetaxel, and cabazitaxel. In some embodiments, the taxane is paclitaxel. In some embodiments, paclitaxel is administered at a dose of about 80 mg/m². In some embodiments, the paclitaxel is administered on Days 1, 8, and 15 of each 28-day cycle. In some embodiments, a taxane is administered via IV. In some embodiments, paclitaxel is administered via IV. In some embodiments, paclitaxel is administered at a dose of about 80 mg/m² is on Days 1, 8, and 15 of each 28-day cycle.

F. Combination Therapy

[0148] Provided herein are methods of treatment comprising administering to the subject a combination therapy comprising tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist (e.g.,

tucatinib, trastuzumab, paclitaxel, and ramucirumab). In some embodiments, the combination therapy consists essentially of tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist (e.g., tucatinib, trastuzumab, paclitaxel, and ramucirumab).

[0149] In some embodiments, the tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist are administered to the subject on a treatment cycle. In some embodiments, the tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist are administered to a subject on a 28 day treatment cycle. In some embodiments, the subject will be treated with tucatinib, trastuzumab, a taxane selected from paclitaxel, docetaxel, cabazitaxel, larotaxel, BMS-184476, BMS-188797, BMS-275183, milataxel, ortaxel, TL-310, docosahexaenoic acid-paclitaxel (DHA-paclitaxel), *nab* paclitaxel, EndoTAG+paclitaxel, XRP9881, polymeric-micellar paclitaxel, RPR-109881A, a pharmaceutically acceptable salt or solvate thereof, and a combination thereof, and a VEGFR-2 antagonist that is selected from the group consisting of bevacizumab, ramucirumab, aflibercept, cetuximab, panitumumab, regorafenib, sunitinib, sorafenib, pazopanib, vandetanib, axitinib, cediranib, vatalanib, motesanib given in 28-day cycles. For instance, the subject can be treated with tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist given in 28-day cycles. An eligible subject may have received prior treatment of a HER2-directed antibody prior to receiving the combination treatment. The starting dose of tucatinib is 300 mg orally (PO) BID, and the first dose will be administered on cycle 1 day 8 and continuously thereafter.

G. Compositions

[0150] In another aspect, the present disclosure provides a pharmaceutical composition comprising tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist (e.g., tucatinib, trastuzumab, paclitaxel, and ramucirumab), and a pharmaceutically acceptable carrier. In another aspect, the present disclosure provides a pharmaceutical combination comprising tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist, and a pharmaceutically acceptable carrier. In some examples, the tucatinib can be administered separately from the trastuzumab, the taxane and the VEGFR-2 antagonist. In other examples, the tucatinib can be administered with one, two, or all of the trastuzumab, taxane, and/or the VEGFR-2 antagonist.

[0151] The pharmaceutical compositions of the present disclosure may be prepared by any of the methods well-known in the art of pharmacy. Pharmaceutically acceptable carriers suitable for use

with the present disclosure include any of the standard pharmaceutical carriers, buffers and excipients, including phosphate-buffered saline solution, water, and emulsions (such as an oil/water or water/oil emulsion), and various types of wetting agents or adjuvants. Suitable pharmaceutical carriers and their formulations are described in Remington's Pharmaceutical Sciences (Mack Publishing Co., Easton, 19th ed. 1995). Preferred pharmaceutical carriers depend upon the intended mode of administration of the active agent.

[0152] The pharmaceutical compositions of the present disclosure can include a combination of drugs (e.g., tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist), or any pharmaceutically acceptable salts thereof, as active ingredients and a pharmaceutically acceptable carrier or excipient or diluent. A pharmaceutical composition may optionally contain other therapeutic ingredients.

[0153] The compositions (e.g., tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist) can be combined as the active ingredients in intimate admixture with a suitable pharmaceutical carrier or excipient according to conventional pharmaceutical compounding techniques. Any carrier or excipient suitable for the form of preparation desired for administration is contemplated for use with the compounds disclosed herein.

[0154] The pharmaceutical compositions include those suitable for oral, topical, parenteral, pulmonary, nasal, or rectal administration. The most suitable route of administration in any given case will depend in part on the nature and severity of the cancer condition and also optionally the HER2 status or stage of the cancer.

[0155] Other pharmaceutical compositions include those suitable for systemic (e.g., enteral or parenteral) administration. Systemic administration includes oral, rectal, sublingual, or sublabial administration. Parenteral administration includes, e.g., intravenous, intramuscular, intra-arteriole, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial. Other modes of delivery include, but are not limited to, the use of liposomal formulations, intravenous infusion, transdermal patches, etc. In particular embodiments, pharmaceutical compositions of the present disclosure may be administered intratumorally.

[0156] Compositions for systemic administration include, but are not limited to, dry powder compositions consisting of the composition as set forth herein (e.g., tucatinib, trastuzumab, a

taxane, and a VEGFR-2 antagonist) and the powder of a suitable carrier or excipient. The compositions for systemic administration can be represented by, but not limited to, tablets, capsules, pills, syrups, solutions, and suspensions.

[0157] In some embodiments, the compositions (e.g., tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist) further include a pharmaceutical surfactant. In other embodiments, the compositions further include a cryoprotectant. In some embodiments, the cryoprotectant is selected from the group consisting of glucose, sucrose, trehalose, lactose, sodium glutamate, PVP, HP β CD, CD, glycerol, maltose, mannitol, and saccharose.

[0158] Pharmaceutical compositions or medicaments for use in the present disclosure can be formulated by standard techniques using one or more physiologically acceptable carriers or excipients. Suitable pharmaceutical carriers are described herein and in Remington: The Science and Practice of Pharmacy, 21st Ed., University of the Sciences in Philadelphia, Lippencott Williams & Wilkins (2005).

Pharmaceutical Compositions of Tucatinib

[0159] In some embodiments, a pharmaceutical composition comprising tucatinib and a pharmaceutically acceptable carrier is provided herein, wherein the pharmaceutical composition comprises a solid dispersion of tucatinib.

[0160] The solid dispersions are generally prepared by dissolving the drug substance and the dispersion polymer in a suitable solvent to form a feed solution, and then the feed solution may be spray dried to form the solid dispersion (and remove the solvent). Spray drying is a known process. Spray drying is generally performed by dissolving tucatinib and the dispersion polymer in a suitable solvent to prepare a feed solution. The feed solution may be pumped through an atomizer into a drying chamber. The feed solution can be atomized by conventional means known in the art, such as a two-fluid sonicating nozzle, a pressure nozzle, a rotating nozzle and a two-fluid non-sonicating nozzle. Then, the solvent is removed in the drying chamber to form the solid dispersion. A typical drying chamber uses hot gases, such as forced air, nitrogen, nitrogen-enriched air, or argon to dry particles. The size of the drying chamber may be adjusted to achieve particle properties or throughput. Although the solid dispersion are preferably prepared by conventional

spray drying techniques, other techniques known in the art may be used, such as melt extrusion, freeze drying, rotary evaporation, drum drying or other solvent removal processes.

[0161] In some embodiments, a process of preparing a solid dispersion is provided, comprising: (a) dissolving tucatinib and a dispersion polymer in a suitable solvent; and (b) evaporating the solvent to form the solid dispersion. In certain embodiments, the evaporation of the solvent in step (b) is performed by spray drying, melt extrusion, freeze drying, rotary evaporation, drum drying or other solvent removal processes.

[0162] In certain embodiments, the dispersion polymer is selected from PVP-VA, methylacrylic acid methyl methacrylate copolymer, HPMCP, CAP, HPMCAS and HPMC and mixtures thereof. In certain embodiments, the dispersion polymer is selected from PVP-VA, methylacrylic acid methyl methacrylate copolymer, HPMCP, CAP, HPMCAS and HPMC. In certain embodiments, the dispersion polymer is selected from PVP-VA, Eudragit® L100, HPMCP H-55, CAP, HPMCAS Grade M, HPMC and mixtures thereof. In certain embodiments, the dispersion polymer is selected from PVP-VA, Eudragit® L100, HPMCP H-55, CAP, HPMCAS Grade M and HPMC.

[0163] In certain embodiments, the dispersion polymer is selected from PVP-VA, methylacrylic acid methyl methacrylate copolymer, HPMCP, CAP and HPMCAS, and mixtures thereof. In certain embodiments, the dispersion polymer is selected from PVP-VA, methylacrylic acid methyl methacrylate copolymer, HPMCP, CAP and HPMCAS. In certain embodiments, the dispersion polymer is selected from PVP-VA, Eudragit® L100, HPMCP H-55, CAP and HPMCAS Grade M, and mixtures thereof. In certain embodiments, the dispersion polymer is selected from PVP-VA, Eudragit® L100, HPMCP H-55, CAP and HPMCAS Grade M.

[0164] In certain embodiments, the dispersion polymer is selected from PVP-VA, methylacrylic acid methyl methacrylate copolymer, HPMCP, CAP and HPMC, and mixtures thereof. In certain embodiments, the dispersion polymer is selected from PVP-VA, methylacrylic acid methyl methacrylate copolymer, HPMCP, CAP and HPMC. In certain embodiments, the dispersion polymer is selected from PVP-VA, Eudragit® L100, HPMCP H-55, CAP and HPMC, and mixtures thereof. In certain embodiments, the dispersion polymer is selected from PVP-VA, Eudragit® L100, HPMCP H-55, CAP and HPMC

[0165] In certain embodiments, the dispersion polymer is selected from PVP-VA, methylacrylic acid methyl methacrylate copolymer, HPMCP and CAP, and mixtures thereof. In certain embodiments, the dispersion polymer is selected from PVP-VA, methylacrylic acid methyl methacrylate copolymer, HPMCP and CAP. In certain embodiments, the dispersion polymer is selected from PVP-VA, Eudragit® L100, HPMCP H-55 and CAP, and mixtures thereof. In certain embodiments, the dispersion polymer is selected from PVP-VA, Eudragit® L100, HPMCP H-55 and CAP. In certain embodiments, the dispersion polymer is PVP-VA.

[0166] In certain embodiments, the dispersion polymer is methylacrylic acid methyl methacrylate copolymer. In certain embodiments, the dispersion polymer is Eudragit®. In certain embodiments, the dispersion polymer is Eudragit® L100.

[0167] In certain embodiments, the dispersion polymer is HPMCP. In certain embodiments, the dispersion polymer is HPMCP H-55.

[0168] In certain embodiments, the dispersion polymer is CAP.

[0169] In certain embodiments, the dispersion polymer is HPMCAS. In certain embodiments, the dispersion polymer is HPMCAS Grade M.

[0170] In certain embodiments, the dispersion polymer is preferably neutral or basic.

[0171] In certain embodiments, the dispersion polymer is selected from PVP-VA and HPMC. In certain embodiments, the dispersion polymer is HPMC.

[0172] Suitable solvents are a solvent or mixture of solvents in which both tucatinib and the dispersion polymer have adequate solubility (solubility greater than 1 mg/mL). A mixture of solvents may be used if each component of the solid dispersion (i.e., tucatinib and dispersion polymer) require different solvents to obtain the desired solubility. The solvent may be volatile with a boiling point of 150° C. or less. In addition, the solvent should have relatively low toxicity and be removed from the dispersion to a level that is acceptable to The International Committee on Harmonization (“ICH”) guidelines. Removal of solvent to this level may require a subsequent processing step, such as tray drying. Examples of suitable solvents include, but are not limited to, alcohols, such as methanol (“MeOH”), ethanol (“EtOH”), n-propanol, isopropanol (“IPA”) and butanol; ketones, such as acetone, methyl ethyl ketone (“MEK”) and methyl isobutyl ketone;

esters, such as ethyl acetate (“EA”) and propyl acetate; and various other solvents, such as tetrahydrofuran (“THF”), acetonitrile (“ACN”), methylene chloride, toluene and 1,1,1-trichloroethane. Lower volatility solvents, such as dimethyl acetate or dimethylsulfoxide (“DMSO”), may be used. Mixtures of solvents with water may also be used, so long as the polymer and tucatinib are sufficiently soluble to make the spray drying process practicable. Generally, due to the hydrophobic nature of low solubility drugs, non-aqueous solvents may be used, meaning the solvent comprises less than about 10 weight % water.

[0173] In certain embodiments, the suitable solvent is selected from MeOH and THF, and mixtures thereof. In certain embodiments, the suitable solvent is MeOH:THF solvent system of about 1:3. In certain embodiments, the suitable solvent is a 1:3 MeOH:THF solvent system.

[0174] In certain embodiments, the suitable solvent is selected from MeOH, THF and water, and mixtures thereof. In certain embodiments, the suitable solvent is selected from MeOH, THF and water. In certain embodiments, the suitable solvent is a THF:MeOH:water solvent system of about 80:10:10. In certain embodiments, the suitable solvent is a 80:10:10 THF:MeOH:water solvent system. In certain embodiments, the suitable solvent is a THF:MeOH:water solvent system of about 82:8:10. In certain embodiments, the suitable solvent is a 82:8:10 THF:MeOH:water solvent system. In certain embodiments, the suitable solvent is a THF:MeOH:water solvent system of about 82.2:8.2:9.6. In certain embodiments, the suitable solvent is a 82.2:8.2:9.6 THF:MeOH:water solvent system.

[0175] In certain embodiments, the amount of tucatinib in the solid dispersion ranges from about 0.1% to about 70% by weight relative to the dispersion polymer. In certain embodiments, the amount of tucatinib in the solid dispersion ranges from 0.1% to 70% by weight relative to the dispersion polymer.

[0176] In certain embodiments, the amount of tucatinib in the solid dispersion ranges from about 1% to about 60% by weight relative to the dispersion polymer. In certain embodiments, the amount of tucatinib in the solid dispersion ranges from 1% to 60% by weight relative to the dispersion polymer.

[0177] In certain embodiments, the amount of tucatinib in the solid dispersion ranges from about 5% to about 60% by weight relative to the dispersion polymer. In certain embodiments, the amount

of tucatinib in the solid dispersion ranges from 5% to 60% by weight relative to the dispersion polymer.

[0178] In certain embodiments, the amount of tucatinib in the solid dispersion ranges from about 55% to about 65% by weight relative to the dispersion polymer. In certain embodiments, the amount of tucatinib in the solid dispersion ranges from 55% to 65% by weight relative to the dispersion polymer. In certain embodiments, the amount of tucatinib in the solid dispersion is about 60% by weight relative to the dispersion polymer. In certain embodiments, the amount of tucatinib in the solid dispersion is 60% by weight relative to the dispersion polymer.

[0179] In certain embodiments, the amount of tucatinib in the solid dispersion ranges from about 25% to about 35% by weight relative to the dispersion polymer. In certain embodiments, the amount of tucatinib in the solid dispersion ranges from 25% to 35% by weight relative to the dispersion polymer. In certain embodiments, the amount of tucatinib in the solid dispersion is about 30% by weight relative to the dispersion polymer. In certain embodiments, the amount of tucatinib in the solid dispersion is 30% by weight relative to the dispersion polymer.

[0180] In certain embodiments, the amount of tucatinib in the solid dispersion ranges from about 45% to about 55% by weight relative to the dispersion polymer. In certain embodiments, the amount of tucatinib in the solid dispersion ranges from 45% to 55% by weight relative to the dispersion polymer. In certain embodiments, the amount of tucatinib in the solid dispersion is about 50% by weight relative to the dispersion polymer. In certain embodiments, the amount of tucatinib in the solid dispersion is 50% by weight relative to the dispersion polymer.

[0181] In certain embodiments, the solid dispersion is an amorphous solid dispersion.

[0182] Another embodiment provides a pharmaceutical composition comprising a solid dispersion of tucatinib and a dispersion polymer, and a carrier or excipient.

[0183] Suitable carriers and excipients are well known to those skilled in the art and are described in detail in, e.g., Ansel, Howard C., et al., *Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems*. Philadelphia: Lippincott, Williams & Wilkins, 2004; Gennaro, Alfonso R., et al. *Remington: The Science and Practice of Pharmacy*. Philadelphia: Lippincott, Williams & Wilkins, 2000; and Rowe, Raymond C. *Handbook of Pharmaceutical Excipients*. Chicago, Pharmaceutical Press, 2005.

[0184] The pharmaceutical compositions may also include one or more additional components, such as buffers, dispersion agents, surfactants, wetting agents, lubricating agents, emulsifiers, suspending agents, preservatives, antioxidants, opaquing agents, glidants, processing aids, colorants, sweeteners, perfuming agents, flavoring agents, diluents and other known additives to provide an elegant presentation of the drug, i.e., a compound described herein or pharmaceutical composition thereof, or aid in the manufacturing of the pharmaceutical product, i.e., medicament (see Ansel; Gennaro; and Rowe above). The components of the pharmaceutical composition should be pharmaceutically acceptable.

[0185] Certain embodiments provide a pharmaceutical composition comprising: (a) about 1 to about 70 weight % of a solid dispersion of tucatinib; (b) about 0.1 to about 20 weight % of a disintegrant; (c) about 0.1 to about 25 weight % of an osmogen; (d) about 0.1 to about 10 weight % of a glidant; (e) about 0.1 to about 10 weight % of a lubricant; and (f) about 0.1 to about 25 weight % of a binder/diluent.

[0186] In certain embodiments, the pharmaceutical composition comprises: (a) 1 to 70 weight % of a solid dispersion of tucatinib; (b) 0.1 to 20 weight % of a disintegrant; (c) 0.1 to 25 weight % of an osmogen; (d) 0.1 to 10 weight % of a glidant; (e) 0.1 to 10 weight % of a lubricant; and (f) 0.1 to 25 weight % of a binder/diluent.

[0187] Certain embodiments provide a pharmaceutical composition comprising: (a) about 25 to about 60 weight % of a solid dispersion of tucatinib; (b) about 5 to about 15 weight % of a disintegrant; (c) about 15 to about 25 weight % of an osmogen; (d) about 0.1 to about 3 weight % of a glidant; (e) about 0.1 to about 3 weight % of a lubricant; and (f) about 10 to about 25 weight % of a binder/diluent.

[0188] In certain embodiments, the pharmaceutical composition comprises: (a) 25 to 60 weight % of a solid dispersion of tucatinib; (b) 5 to 15 weight % of a disintegrant; (c) 15 to 25 weight % of an osmogen; (d) 0.1 to 3 weight % of a glidant; (e) 0.1 to 3 weight % of a lubricant; and (f) 10 to 25 weight % of a binder/diluent.

[0189] Certain embodiments provide a pharmaceutical composition comprising: (a) about 40 to about 60 weight % of a solid dispersion of tucatinib; (b) about 5 to about 15 weight % of a disintegrant; (c) about 15 to about 25 weight % of an osmogen; (d) about 0.1 to about 3 weight %

of a glidant; (e) about 0.1 to about 3 weight % of a lubricant; and (f) about 10 to about 25 weight % of a binder/diluent.

[0190] In certain embodiments, the pharmaceutical composition comprises: (a) 40 to 60 weight % of a solid dispersion of tucatinib; (b) 5 to 15 weight % of a disintegrant; (c) 15 to 25 weight % of an osmogen; (d) 0.1 to 3 weight % of a glidant; (e) 0.1 to 3 weight % of a lubricant; and (f) 10 to 25 weight % of a binder/diluent.

[0191] Certain embodiments provide a pharmaceutical composition comprising: (a) about 1 to about 70 weight % of a solid dispersion of tucatinib; (b) about 0.1 to about 20 weight % of a disintegrant; (c) about 0.1 to about 25 weight % of an osmogen; (d) about 0.1 to about 10 weight % of a glidant; (e) about 0.1 to about 10 weight % of a lubricant; and (f) about 0.1 to about 25 weight % of a filler.

[0192] In certain embodiments, the pharmaceutical composition comprises: (a) 1 to 70 weight % of a solid dispersion of tucatinib; (b) 0.1 to 20 weight % of a disintegrant; (c) 0.1 to 25 weight % of an osmogen; (d) 0.1 to 10 weight % of a glidant; (e) 0.1 to 10 weight % of a lubricant; and (f) 0.1 to 25 weight % of a filler.

[0193] Certain embodiments provide a pharmaceutical composition comprising: (a) about 25 to about 60 weight % of a solid dispersion of tucatinib; (b) about 1 to about 10 weight % of a disintegrant; (c) about 15 to about 25 weight % of an osmogen; (d) about 0.1 to about 3 weight % of a glidant; (e) about 0.1 to about 3 weight % of a lubricant; and (f) about 10 to about 25 weight % of a filler.

[0194] In certain embodiments, the pharmaceutical composition comprises: (a) 25 to 60 weight % of a solid dispersion of tucatinib; (b) 1 to 10 weight % of a disintegrant; (c) 15 to 25 weight % of an osmogen; (d) 0.1 to 3 weight % of a glidant; (e) 0.1 to 3 weight % of a lubricant; and (f) 10 to 25 weight % of a filler.

[0195] Certain embodiments provide a pharmaceutical composition comprising: (a) about 40 to about 60 weight % of a solid dispersion of tucatinib; (b) about 1 to about 10 weight % of a disintegrant; (c) about 15 to about 25 weight % of an osmogen; (d) about 0.1 to about 3 weight % of a glidant; (e) about 0.1 to about 3 weight % of a lubricant; and (f) about 10 to about 25 weight % of a filler.

[0196] In certain embodiments, the pharmaceutical composition comprises: (a) 40 to 60 weight % of a solid dispersion of tucatinib; (b) 1 to 10 weight % of a disintegrant; (c) 15 to 25 weight % of an osmogen; (d) 0.1 to 3 weight % of a glidant; (e) 0.1 to 3 weight % of a lubricant; and (f) 10 to 25 weight % of a filler.

[0197] In certain embodiments, the osmogen is selected from NaCl and KCl, and mixtures thereof.

[0198] In certain embodiments, the lubricant is magnesium stearate.

[0199] In certain embodiments, the glidant is colloidal silicon dioxide.

[0200] In certain embodiments, the binder/diluent is microcrystalline cellulose. In certain embodiments, the binder/diluent acts as both a binder and a diluent.

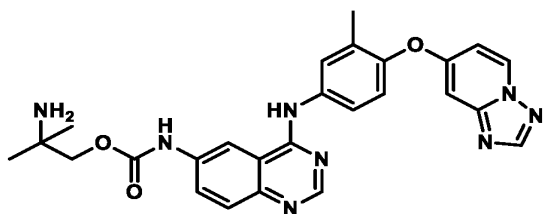
[0201] In certain embodiments, the binder is microcrystalline cellulose.

[0202] In certain embodiments, the diluent is microcrystalline cellulose.

[0203] In certain embodiments, the filler is lactose.

[0204] In certain embodiments, the disintegrant is selected from croscopovidone and sodium bicarbonate (NaHCO_3), and mixtures thereof. In certain embodiments, the disintegrant is selected from croscopovidone and sodium bicarbonate. In certain embodiments, the disintegrant is sodium bicarbonate. In certain embodiments, the disintegrant is croscopovidone.

[0205] In certain embodiments, the composition contains sodium bicarbonate. tucatinib may slowly degrade, through hydrolysis or other means, to a carbamate impurity:



Sodium bicarbonate helps to slow the degradation to the carbamate impurity. Sodium bicarbonate also helps to provide consistent tablet disintegration when the tablets are exposed to different humidities.

[0206] Certain embodiments provide a pharmaceutical composition comprising: (a) tucatinib; and (b) sodium bicarbonate.

[0207] Certain embodiments provide a pharmaceutical composition comprising: (a) about 1 to about 70 weight % of a solid dispersion of tucatinib; and (b) about 0.1 to about 30 weight % sodium bicarbonate.

[0208] In certain embodiments, the pharmaceutical composition comprises: (a) 1 to 70 weight % of a solid dispersion of tucatinib; and (b) 0.1 to 30 weight % sodium bicarbonate.

[0209] Certain embodiments provide a pharmaceutical composition comprising: (a) about 1 to about 70 weight % of a solid dispersion of tucatinib; (b) about 0.1 to about 30 weight % sodium bicarbonate; and (c) the remaining weight is other pharmaceutically acceptable excipients and carriers.

[0210] In certain embodiments, the pharmaceutical composition comprises: (a) 1 to 70 weight % of a solid dispersion of tucatinib; (b) 0.1 to 30 weight % sodium bicarbonate; and (c) the remaining weight is other pharmaceutically acceptable excipients and carriers.

[0211] Certain embodiments provide a pharmaceutical composition comprising: (a) about 25 to about 60 weight % of a solid dispersion of tucatinib; and (b) about 1 to about 15 weight % of sodium bicarbonate.

[0212] In certain embodiments, the pharmaceutical composition comprises: (a) 25 to 60 weight % of a solid dispersion of tucatinib; and (b) 1 to 15 weight % of sodium bicarbonate.

[0213] Certain embodiments provide a pharmaceutical composition comprising: (a) about 25 to about 60 weight % of a solid dispersion of tucatinib; (b) about 1 to about 15 weight % of sodium bicarbonate; and (c) the remaining weight is other pharmaceutically acceptable excipients and carriers.

[0214] In certain embodiments, the pharmaceutical composition comprises: (a) 25 to 60 weight % of a solid dispersion of tucatinib; (b) 1 to 15 weight % of sodium bicarbonate; and (c) the remaining weight is other pharmaceutically acceptable excipients and carriers.

[0215] Certain embodiments provide a pharmaceutical composition comprising: (a) about 40 to about 60 weight % of a solid dispersion of tucatinib; and (b) about 1 to about 15 weight % of sodium bicarbonate.

[0216] In certain embodiments, the pharmaceutical composition comprises: (a) 40 to 60 weight % of a solid dispersion of tucatinib; and (b) 1 to 15 weight % of sodium bicarbonate.

[0217] Certain embodiments provide a pharmaceutical composition comprising: (a) about 40 to about 60 weight % of a solid dispersion of tucatinib; (b) about 1 to about 15 weight % of sodium bicarbonate; (c) the remaining weight is other pharmaceutically acceptable excipients and carriers.

[0218] In certain embodiments, the pharmaceutical composition comprises: (a) 40 to 60 weight % of a solid dispersion of tucatinib; (b) 1 to 15 weight % of sodium bicarbonate; (c) the remaining weight is other pharmaceutically acceptable excipients and carriers.

[0219] Certain embodiments provide a pharmaceutical composition comprising: (a) about 40 to about 60 weight % of a solid dispersion of tucatinib; (b) about 5 to about 15 weight % of a disintegrant which is selected from the group of crospovidone, sodium bicarbonate (NaHCO_3), and mixtures thereof; (c) about 15 to about 25 weight % of an osmogen which is selected from the group consisting of NaCl, KCl, and mixtures thereof; (d) about 0.1 to about 3 weight % of a glidant which is colloidal silicon dioxide; (e) about 0.1 to about 3 weight % of a lubricant which is magnesium stearate; and (f) about 10 to about 25 weight % of a binder/diluent which is microcrystalline cellulose.

[0220] In certain embodiments, the pharmaceutical composition comprises: (a) 40 to 60 weight % of a solid dispersion of tucatinib; (b) 5 to 15 weight % of a disintegrant which is selected from the group of crospovidone, sodium bicarbonate (NaHCO_3), and mixtures thereof; (c) 15 to 25 weight % of an osmogen which is selected from the group consisting of NaCl, KCl, and mixtures thereof; (d) 0.1 to 3 weight % of a glidant which is colloidal silicon dioxide; (e) 0.1 to 3 weight % of a lubricant which is magnesium stearate; and (f) 10 to 25 weight % of a binder/diluent which is microcrystalline cellulose.

[0221] Certain embodiments provide a pharmaceutical composition comprising: (a) about 40 to about 60 weight % of a solid dispersion of tucatinib; (b) about 1 to about 10 weight % of a disintegrant which is selected from the group of crospovidone, sodium bicarbonate (NaHCO_3), and mixtures thereof; (c) about 15 to about 25 weight % of an osmogen which is selected from the group consisting of NaCl, KCl, and mixtures thereof; (d) about 0.1 to about 3 weight % of a glidant

which is colloidal silicon dioxide; (e) about 0.1 to about 3 weight % of a lubricant which is magnesium stearate; and (f) about 10 to about 25 weight % of a filler which is lactose.

[0222] In certain embodiments, the pharmaceutical composition comprises: (a) 40 to 60 weight % of a solid dispersion of tucatinib; (b) 1 to 10 weight % of a disintegrant which is selected from the group of crospovidone, sodium bicarbonate (NaHCO_3), and mixtures thereof; (c) 15 to 25 weight % of an osmogen which is selected from the group consisting of NaCl, KCl, and mixtures thereof; (d) 0.1 to 3 weight % of a glidant which is colloidal silicon dioxide; (e) 0.1 to 3 weight % of a lubricant which is magnesium stearate; and (f) 10 to 25 weight % of a filler which is lactose.

[0223] In certain embodiments, the pharmaceutical composition is selected from the group consisting of:

Function	Ingredient	% of Blend
API	Solid dispersion of tucatinib	about 50
Disintegrant	Crospovidone - Polyplasdone®	about 6
Osmogen	NaCl	about 5
Osmogen	KCl	about 5
Glidant	Colloidal Silicon Dioxide	about 0.5
Lubricant	Magnesium Stearate	about 0.25
Extragranular		
Binder/Diluent	Microcrystalline cellulose - Avicel®	about 19.25
Osmogen	NaCl	about 4.625
Osmogen	KCl	about 4.625
Disintegrant	Polyplasdone	about 4
Glidant	Colloidal Silicon Dioxide	about 0.5
Lubricant	Magnesium Stearate	about 0.25

Function	Ingredient	% of Blend
API	Solid dispersion of tucatinib	about 50
Disintegrant	Crospovidone - Polyplasdone®	about 6
Disintegrant	NaHCO_3	about 3
Osmogen	NaCl	about 5
Osmogen	KCl	about 5
Glidant	Colloidal Silicon Dioxide	about 0.5
Lubricant	Magnesium Stearate	about 0.25

Extragranular		
Binder/Diluent	Microcrystalline cellulose - Avicel®	about 16.25
Osmogen	NaCl	about 4.625
Osmogen	KCl	about 4.625
Disintegrant	Polyplasdone	about 4
Glidant	Colloidal Silicon Dioxide	about 0.5
Lubricant	Magnesium Stearate	about 0.25

Function	Ingredient	% of Blend
API	Solid dispersion of tucatinib	about 50
Disintegrant	Crospovidone - Polyplasdone®	about 6
Osmogen	NaCl	about 10.625
Osmogen	KCl	about 10.625
Filler	Lactose	about 21.25
Glidant	Colloidal Silicon Dioxide	about 0.5
Lubricant	Magnesium Stearate	about 0.25
Extragranular		
Glidant	Colloidal Silicon Dioxide	about 0.5
Lubricant	Magnesium Stearate	about 0.25

[0224] In certain embodiments, the pharmaceutical composition is selected from the group consisting of:

Function	Ingredient	% of Blend
API	Solid dispersion of tucatinib	50
Disintegrant	Crospovidone - Polyplasdone®	6
Osmogen	NaCl	5
Osmogen	KCl	5
Glidant	Colloidal Silicon Dioxide	0.5
Lubricant	Magnesium Stearate	0.25
Extragranular		
Binder/Diluent	Microcrystalline cellulose - Avicel®	19.25
Osmogen	NaCl	4.625
Osmogen	KCl	4.625
Disintegrant	Polyplasdone	4
Glidant	Colloidal Silicon Dioxide	0.5
Lubricant	Magnesium Stearate	0.25

Function	Ingredient	% of Blend
API	Solid dispersion of tucatinib	50
Disintegrant	Crospovidone - Polyplasdone®	6
Disintegrant	NaHCO ₃	3
Osmogen	NaCl	5
Osmogen	KCl	5
Glidant	Colloidal Silicon Dioxide	0.5
Lubricant	Magnesium Stearate	0.25
Extragranular		
Binder/Diluent	Microcrystalline cellulose - Avicel®	16.25
Osmogen	NaCl	4.625
Osmogen	KCl	4.625
Disintegrant	Polyplasdone	4
Glidant	Colloidal Silicon Dioxide	0.5
Lubricant	Magnesium Stearate	0.25

Function	Ingredient	% of Blend
API	Solid dispersion of tucatinib	50
Disintegrant	Crospovidone - Polyplasdone®	6
Osmogen	NaCl	10.625
Osmogen	KCl	10.625
Filler	Lactose	21.25
Glidant	Colloidal Silicon Dioxide	0.5
Lubricant	Magnesium Stearate	0.25
Extragranular		
Glidant	Colloidal Silicon Dioxide	0.5
Lubricant	Magnesium Stearate	0.25

[0225] The pharmaceutical composition preferably contains a therapeutically effective amount of tucatinib. However, in some embodiments, each individual dose contains a portion of a therapeutically effective amount of tucatinib, such that multiple doses of the composition may be required (for example, two or more tablets are required for a therapeutically effective amount). Thus, in this application when it states that the pharmaceutical composition contains a therapeutically effective amount it means that the composition may be one dose (for example, one tablet) or multiple doses (for example, two tablets). In certain embodiments, the pharmaceutical composition contains between 1 and 500 mg of tucatinib.

[0226] In certain embodiments, the pharmaceutical composition contains between about 25 and about 400 mg of tucatinib. In certain embodiments, the pharmaceutical composition contains between 25 and 400 mg of tucatinib.

[0227] In certain embodiments, the pharmaceutical composition contains between about 25 and about 100 mg (e.g., about 25 mg, about 30 mg, about 35 mg, about 40 mg, about 45 mg, about 50 mg, about 55 mg, about 60 mg, about 65 mg, about 70 mg, about 75 mg, about 80 mg, about 85 mg, about 90 mg, about 95 mg, about 100 mg) of tucatinib. In certain embodiments, the pharmaceutical composition contains between 25 and 100 mg (e.g., 25 mg, 30 mg, 35 mg, 40 mg, 45 mg, 50 mg, 55 mg, 60 mg, 65 mg, 70 mg, 75 mg, 80 mg, 85 mg, 90 mg, 95 mg, and 100 mg) of tucatinib. In certain embodiments, the pharmaceutical composition contains between about 25 and about 75 mg of tucatinib. In certain embodiments, the pharmaceutical composition contains between 25 and 75 mg of tucatinib. In certain embodiments, the pharmaceutical composition contains about 50 mg of tucatinib. In certain particular embodiments, the pharmaceutical composition contains 50 mg of tucatinib. In certain of the foregoing embodiments, the pharmaceutical composition is formulated as a tablet. As a non-limiting example, the pharmaceutical composition is formulated as a tablet and contains 50 mg of tucatinib.

[0228] In certain embodiments, the pharmaceutical composition contains between about 100 and about 300 mg (e.g., about 100 mg, about 110 mg, about 120 mg, about 130 mg, about 140 mg, about 150 mg, about 160 mg, about 170 mg, about 180 mg, about 190 mg, about 200 mg, about 210 mg, about 220 mg, about 230 mg, about 240 mg, about 250 mg, about 260 mg, about 270 mg, about 280 mg, about 290 mg, about 300 mg) of tucatinib. In certain embodiments, the pharmaceutical composition contains between 100 and 300 mg (e.g., 100 mg, 110 mg, 120 mg, 130 mg, 140 mg, 150 mg, 160 mg, 170 mg, 180 mg, 190 mg, 200 mg, 210 mg, 220 mg, 230 mg, 240 mg, 250 mg, 260 mg, 270 mg, 280 mg, 290 mg, 300 mg) of tucatinib. In certain embodiments, the pharmaceutical composition contains between about 100 and about 200 mg of tucatinib. In certain embodiments, the pharmaceutical composition contains between 100 and 200 mg of tucatinib. In certain embodiments, the pharmaceutical composition contains between about 125 and about 175 mg of tucatinib. In certain embodiments, the pharmaceutical composition contains between 125 and 175 mg of tucatinib. In certain embodiments, the pharmaceutical composition contains about 150 mg of tucatinib. In certain particular embodiments, the pharmaceutical

composition contains 150 mg of tucatinib. In certain of the foregoing embodiments, the pharmaceutical composition is formulated as a tablet. As a non-limiting example, the pharmaceutical composition is formulated as a tablet and contains 150 mg of tucatinib.

[0229] The pharmaceutical compositions described herein may be administered by any convenient route appropriate to the condition to be treated. Suitable routes include oral, parenteral (including subcutaneous, intramuscular, intravenous, intraarterial, intradermal, intrathecal and epidural), transdermal, rectal, nasal, topical (including buccal and sublingual), ocular, vaginal, intraperitoneal, intrapulmonary and intranasal. If parenteral administration is desired, the compositions will be sterile and in a solution or suspension form suitable for injection or infusion.

[0230] The compounds may be administered in any convenient administrative form, e.g., tablets, powders, capsules, dispersions, suspensions, syrups, sprays, suppositories, gels, emulsions, patches, etc.

[0231] The pharmaceutical compositions described herein are typically administered orally. The pharmaceutical compositions described herein are typically administered as a tablet, caplet, hard or soft gelatin capsule, pill, granules or a suspension.

[0232] Additional examples of pharmaceutical compositions of tucatinib and methods of preparation thereof are described in U.S. Patent No. 9,457,093, which is incorporated by reference herein in its entirety.

[0233] The pharmaceutical compositions described herein may comprise one or more polymorphs of tucatinib. Exemplary polymorphs of tucatinib and methods of preparation thereof are described in U.S. Patent No. 9,168,254, which is incorporated by reference herein in its entirety.

[0234] In some embodiments, the pharmaceutical composition comprises amorphous tucatinib. In certain embodiments, tucatinib in the pharmaceutical composition is substantially amorphous (e.g., at least 80%, at least 85%, at least 90%, or at least 95% amorphous).

[0235] In some embodiments, the pharmaceutical composition comprises a crystalline polymorph of tucatinib. In certain embodiments, tucatinib in the pharmaceutical composition is substantially crystalline (e.g., at least 80%, at least 85%, at least 90%, or at least 95% crystalline).

[0236] In certain embodiments, the pharmaceutical composition comprises polymorph Form A of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form A (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form A).

[0237] In certain embodiments, the pharmaceutical composition comprises polymorph Form B of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form B (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form B).

[0238] In certain embodiments, the pharmaceutical composition comprises polymorph Form C of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form C (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form C).

[0239] In certain embodiments, the pharmaceutical composition comprises polymorph Form D of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form D (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form D).

[0240] In certain embodiments, the pharmaceutical composition comprises polymorph Form E of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form E (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form E).

[0241] In certain embodiments, the pharmaceutical composition comprises polymorph Form F of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form F (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form F).

[0242] In certain embodiments, the pharmaceutical composition comprises polymorph Form G of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form G (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form G).

[0243] In certain embodiments, the pharmaceutical composition comprises polymorph Form H of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form H (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form H).

[0244] In certain embodiments, the pharmaceutical composition comprises polymorph Form I of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form I (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form I).

[0245] In certain embodiments, the pharmaceutical composition comprises polymorph Form J of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form J (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form J).

[0246] In certain embodiments, the pharmaceutical composition comprises polymorph Form K of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form K (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form K).

[0247] In certain embodiments, the pharmaceutical composition comprises polymorph Form L of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form L (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form L).

[0248] In certain embodiments, the pharmaceutical composition comprises polymorph Form M of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form M (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form M).

[0249] In certain embodiments, the pharmaceutical composition comprises polymorph Form N of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form N (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form N).

[0250] In certain embodiments, the pharmaceutical composition comprises polymorph Form O of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form O (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form O).

[0251] In certain embodiments, the pharmaceutical composition comprises polymorph Form P of tucatinib as described in U.S. Patent No. 9,168,254. In certain embodiments, tucatinib in the pharmaceutical composition is substantially in Form P (e.g., at least 80%, at least 85%, at least 90%, or at least 95% Form P).

H. Articles of Manufacture and Kits

[0252] In another aspect, the present disclosure provides an article of manufacture or kit for treating or ameliorating the effects of a HER2 positive cancer in a subject, the kit comprising tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist (e.g., tucatinib, trastuzumab, paclitaxel, and ramucirumab).

[0253] The articles of manufacture or kits are suitable for treating or ameliorating the effects of HER2 positive and/or metastatic cancer in a subject. In some embodiments, the cancer is an advanced cancer. In some other embodiments, the cancer is a drug-resistant cancer. In some instances, the cancer is a multidrug-resistant cancer.

[0254] Materials and reagents to carry out the various methods of the present disclosure can be provided in articles of manufacture or kits to facilitate execution of the methods. As used herein, the term “kit” includes a combination of articles that facilitates a process, assay, analysis, or manipulation. In particular, kits of the present disclosure find utility in a wide range of applications including, for example, diagnostics, prognostics, therapy, and the like.

[0255] Articles of manufacture or kits can contain chemical reagents as well as other components. In addition, the articles of manufacture or kits of the present disclosure can include, without limitation, instructions to the user, apparatus and reagents for administering combinations of tucatinib, trastuzumab, a taxane, and a VEGFR-2 antagonist (e.g., tucatinib, trastuzumab, paclitaxel, and ramucirumab) or pharmaceutical compositions thereof, sample tubes, holders,

trays, racks, dishes, plates, solutions, buffers, or other chemical reagents. Articles of manufacture or kits of the present disclosure can also be packaged for convenient storage and safe shipping, for example, in a box having a lid.

III. Exemplary Embodiments

[0256] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, patent applications, and sequence accession numbers cited herein are hereby incorporated by reference in their entirety for all purposes.

[0257] The disclosure will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the disclosure. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

Example 1: Phase 2/3, study of tucatinib in combination with trastuzumab, a taxane, and a VEGFR-2 antagonist in subjects with previously treated, locally-advanced unresectable or metastatic HER2+ gastric or gastroesophageal junction adenocarcinoma (GEC).

[0258] Study Objectives

[0259] The Phase 2 portion of this study will determine the recommended dose of paclitaxel when administered in combination with tucatinib, trastuzumab, and ramucirumab, evaluate the safety and tolerability of tucatinib in combination with trastuzumab, ramucirumab, and paclitaxel, and evaluate the activity, and pharmacokinetics (PK) of the regimen in subjects with locally-advanced unresectable or metastatic HER2+ GEC who have received prior treatment with a HER2-directed antibody in the locally-advanced unresectable or metastatic disease setting. Specific objectives and corresponding endpoints for the study are summarized below (

[0260] Table 1).

Table 1: Objectives and corresponding endpoints (Phase 2)

Primary Objectives	Corresponding Endpoints
To determine the recommended dose of paclitaxel when administered in combination with tucatinib, trastuzumab, and ramucirumab	Frequency of dose-limiting toxicities (DLT) during the first cycle of treatment with tucatinib, trastuzumab, ramucirumab, and paclitaxel
To evaluate the safety and tolerability of tucatinib in combination with trastuzumab, ramucirumab, and paclitaxel	Type, incidence, severity, seriousness, and relatedness of adverse events (AEs) and laboratory abnormalities Vital signs and other relevant safety variables Frequency of dose holds, dose reductions, and discontinuations of tucatinib, paclitaxel, trastuzumab, and ramucirumab
Secondary Objectives	Corresponding Endpoints
To evaluate the preliminary activity of tucatinib in combination with trastuzumab, ramucirumab, and paclitaxel in subjects with previously treated locally-advanced unresectable or metastatic GEC that is HER2+ according to blood-based next generation sequencing (NGS) assay of circulating tumor DNA (ctDNA)	ORR (complete response [CR] or partial response [PR]) per Response evaluation criteria in solid tumors (RECIST) version 1.1 according to investigator assessment Confirmed ORR per RECIST version 1.1 according to investigator assessment PFS per RECIST version 1.1 according to investigator assessment Duration of response (DOR; CR or PR) per RECIST version 1.1 according to investigator assessment Disease control rate (DCR; CR or PR or stable disease/non-CR, non-progressive disease as best objective response) per RECIST version 1.1 according to investigator assessment
To evaluate the PK of tucatinib and the tucatinib metabolite ONT-993 To evaluate the PK of paclitaxel and its metabolites in the presence and absence of tucatinib	The PK parameters to be calculated for tucatinib, paclitaxel, and their respective metabolites (if applicable) may include but are not limited to: area under the plasma concentration-time curve (AUC) to the time of the last quantifiable concentration (AUC _{last}), maximum observed concentration (C _{max}), time of C _{max} (T _{max}), trough concentration (C _{trough}), and metabolic ratio based on AUC (MR _{AUC})
Exploratory Objectives	Corresponding Endpoints
To evaluate the preliminary activity of tucatinib in combination with trastuzumab, ramucirumab, and paclitaxel in subjects with disease	ORR per RECIST version 1.1 according to investigator assessment Confirmed ORR per RECIST version 1.1 according to investigator assessment

that is HER2-negative according to blood-based NGS assay but HER2+ according to tissue biopsy	PFS per RECIST version 1.1 according to investigator assessment Duration of response per RECIST version 1.1 according to investigator assessment Disease control rate per RECIST version 1.1 according to investigator assessment
To evaluate the correlation between HER2 alterations as detected by blood-based NGS assays and standard tissue-based assays To explore correlations between blood-based biomarkers and clinical outcomes To evaluate the PK of tucatinib and ONT-993 in subjects with and without gastrectomy	Frequency of HER2 alterations according to blood-based NGS assay and according to assays on biopsy samples obtained after progression on the most recent line of therapy Potential biomarkers of response, resistance, or toxicity may be evaluated in blood The PK parameters of tucatinib and ONT-993 in subjects with and without gastrectomies may include but are not limited to: AUC _{last} , C _{max} , T _{max} , C _{trough} , and MR _{AUC}

[0261] The Phase 3 portion will compare the efficacy, safety, patient-reported outcomes (PRO), and health care resource utilization (HCRU) of tucatinib and trastuzumab versus placebo in combination with ramucirumab and paclitaxel. It will also evaluate the activity of tucatinib combined with ramucirumab and paclitaxel. Specific objectives and corresponding endpoints for the study are summarized below (Table 2).

Table 2: Objectives and corresponding endpoints (Phase 3)

Primary Objective	Corresponding Endpoints
To compare the efficacy of tucatinib and trastuzumab versus placebo, in combination with ramucirumab and paclitaxel in subjects with previously treated, locally-advanced unresectable or metastatic HER2+ GEC	<i>Dual primary endpoints</i>
	OS
	PFS per RECIST version 1.1 according to investigator assessment
	<i>Key secondary endpoints</i>
	Confirmed ORR per RECIST version 1.1 according to investigator assessment
	<i>Other secondary endpoints</i>
	PFS per RECIST version 1.1 according to blinded independent central review (BICR) assessment
	Confirmed ORR per RECIST version 1.1 according to BICR assessment
	ORR per RECIST version 1.1 according to investigator assessment
	ORR per RECIST version 1.1 according to BICR assessment
	DOR per RECIST version 1.1 according to investigator assessment
	DOR per RECIST version 1.1 according to BICR assessment
	DCR per RECIST version 1.1 according to investigator assessment
	DCR per RECIST version 1.1 according to BICR assessment
Secondary Objectives	Corresponding Endpoints
To evaluate the safety and tolerability of tucatinib in combination with trastuzumab, ramucirumab, and paclitaxel	Type, incidence, severity, seriousness, and relatedness of AEs and laboratory abnormalities
	Vital signs and other relevant safety variables
	Frequency of dose holds, dose reductions, and discontinuations of tucatinib, trastuzumab, ramucirumab, and paclitaxel

To evaluate the anti-tumor activity of tucatinib in combination with ramucirumab and paclitaxel	<p><i>Secondary Endpoints</i></p> <p>Confirmed ORR per RECIST version 1.1 according to investigator assessment</p> <p>DOR per RECIST version 1.1 according to investigator assessment</p> <p><i>Exploratory Endpoints</i></p> <p>OS</p> <p>PFS per RECIST version 1.1 according to investigator assessment</p> <p>PFS per RECIST version 1.1 according to BICR assessment</p> <p>Confirmed ORR per RECIST version 1.1 according to BICR assessment</p> <p>ORR per RECIST version 1.1 according to investigator assessment</p> <p>ORR per RECIST version 1.1 according to BUCR assessment</p> <p>DOR per RECIST version 1.1 according to investigator assessment</p> <p>DCR per RECIST version 1.1 according to investigator assessment</p> <p>DCR per RECIST version 1.1 according to BICR assessment</p>
To assess PROs by treatment arm	<p>Time to deterioration of GEC symptoms as assessed by the European Organisation for Research and Treatment of Cancer (EORTC) quality of life questionnaire (QLQ)-C30 and EORTC QLQ-OG25 questionnaires</p> <p>Change from baseline in health-related quality of life (HRQoL)</p> <p>Utility index values as assessed by the EQ-5D-5L</p>
Exploratory Objectives	Corresponding Endpoints
To evaluate the safety and tolerability of tucatinib in combination with ramucirumab, and paclitaxel	<p>Type, incidence, severity, seriousness, and relatedness of AEs and laboratory abnormalities</p> <p>Vital signs and other relevant safety variables</p> <p>Frequency of dose holds, dose reductions, and discontinuations of tucatinib, ramucirumab, and paclitaxel</p>
To evaluate the PK of tucatinib	The PK parameter to be calculated for tucatinib (if applicable) includes: C_{trough}

To explore correlations between blood-based biomarkers and clinical outcomes	Potential biomarkers of response, resistance, or toxicity may be evaluated in blood
To assess HCRU by treatment arm	Cumulative incidence of healthcare resource utilization, including length of stay, hospitalizations, and emergency department visits

Number of Planned Subjects

Phase 2: Approximately 66 subjects will be enrolled and treated

Phase 3: Approximately 500 subjects will be randomized to 3 arms.

Investigational Plan

Summary of Study Design

[0262] This is an international, multicenter Phase 2/3 study in subjects with locally-advanced unresectable or metastatic HER2+ GEC who have received prior treatment with a HER2 directed antibody, and have received 1 prior line of therapy in the advanced disease setting.

[0263] The study is composed of the following parts:

Open-label Phase 2 portion:

[0264] Paclitaxel dose optimization stage: this single-arm stage will determine the recommended dose of paclitaxel when combined with tucatinib, trastuzumab, and ramucirumab

[0265] Dose expansion stage: this 2-cohort stage will enroll subjects to further evaluate the safety and activity of the regimen in a total of approximately 30 response evaluable subjects in each cohort, once the recommended paclitaxel dose is determined.

[0266] Randomized, double-blind, placebo-controlled Phase 3 portion: will compare the efficacy and evaluate the safety of tucatinib and trastuzumab versus placebo, in combination with ramucirumab and paclitaxel, and evaluate the efficacy and safety of tucatinib combined with ramucirumab and paclitaxel.

[0267] Dose reductions of tucatinib (or placebo), ramucirumab, and paclitaxel will be allowed. Dose reductions of trastuzumab (or placebo) will not be allowed; if trastuzumab cannot be restarted

after being held for an AE, it must be discontinued. If any study drug is discontinued, the subject can continue to receive study treatment with the remaining agents. Study treatment (defined as the administration of any of the 4 study drugs, without initiation of new anti-cancer treatment) will continue until unacceptable toxicity, disease progression, withdrawal of consent, death, or study closure. Disease response and progression will be assessed using RECIST version 1.1.

[0268] While on study treatment, radiographic disease evaluations will be done every 6 weeks for the first 36 weeks, and every 9 weeks thereafter, irrespective of dose holds or interruptions (FIG 1). All efforts should be made to continue treatment until unequivocal evidence of radiologic progression occurs according to RECIST version 1.1. If study treatment is discontinued before documentation of disease progression, radiographic evaluations will be performed at least every 9 weeks until the occurrence of progression, withdrawal of consent, or study closure. After occurrence of disease progression, subjects will continue to be followed for survival every 12 weeks, until death, consent withdrawal, or study closure.

Phase 2 - Safety Lead-in

Phase 2

Paclitaxel Dose Optimization Stage

[0269] In the paclitaxel dose optimization stage of the study, the initial paclitaxel dose will be 60 mg/m² intravenous (IV) on Days 1, 8, and 15 of each 28-day cycle, in combination with tucatinib 300 mg orally (PO) twice daily (BID), trastuzumab (6 mg/kg IV loading dose on cycle 1 day 1, 4 mg/kg IV on cycle 1 day 15 and days 1 and 15 of each cycle thereafter), and ramucirumab (8 mg/kg IV on days 1 and 15).

[0270] Six subjects will initially be enrolled and treated at paclitaxel 60 mg/m² (FIG. 2). Subjects can have centrally confirmed HER2+ disease according to a blood-based NGS assay of ctDNA done at screening or IHC/ISH assay of tissue biopsies obtained after progression on the most recent line of systemic therapy. Once 6 subjects are evaluable for DLT, enrollment will be paused and the Safety Monitoring Committee (SMC) will undertake an evaluation of safety and PK. If necessary, additional subjects will be enrolled to replace subjects inevaluable for DLT. If >2 DLTs are observed in the 6 subjects receiving 60 mg/m², the evaluation of the regimen will be halted, or an alternative dose level/schedule may be recommended by the SMC. If ≤2 DLT are observed, the

paclitaxel dose will be escalated to 80 mg/m² and evaluated in a further 6 subjects. If >2 DLTs are observed at the 80 mg/m² dose level, the 60 mg/m² paclitaxel dose will be declared the recommended dose, and evaluation of the regimen will continue in the Dose Expansion stage. Otherwise, 80 mg/m² will be the recommended dose. The SMC may also recommend the inclusion of additional subjects in any dose level or the evaluation of an alternative dose level/schedule, if necessary. The SMC will continuously monitor subjects for AEs, deaths, other serious adverse events (SAEs), dose modifications, and laboratory abnormalities, with a specific focus on DLTs.

[0271] Dose Expansion Stage

[0272] Following the paclitaxel dose optimization stage, two cohorts will be opened to enroll further subjects. Cohort 2A will enroll subjects with HER2+ disease as determined by HER2 amplification in a blood-based NGS assay of ctDNA performed at a central laboratory at screening. The exploratory Cohort 2B will enroll subjects whose disease did not show HER2 amplification in the blood based NGS assay, but did show centrally confirmed HER2 overexpression/amplification according to the package insert of FDA approved tests for immunohistochemistry (IHC) and in situ hybridization (ISH) in a biopsy obtained after progression on the most recent line of systemic therapy.

[0273] For each type of HER2 positivity evaluated (blood-based NGS assay or IHC/ISH assay of a tissue biopsy), approximately 30 response-evaluable subjects who have been treated at the recommended dose in either the dose optimization stage or the dose expansion stage will be enrolled. Consequently, approximately 24 to 30 response-evaluable subjects will be enrolled in each cohort during the dose expansion stage, in order to further evaluate the safety of the study regimen and undertake an initial assessment of anti-tumor activity. Subjects who are not evaluable for response will be replaced.

[0274] The SMC will evaluate the safety of the study regimen throughout the remainder of the Phase 2, in the 2 cohorts. At least 6 subjects with a history of prior gastrectomy (without maintenance of the pylorus) will be enrolled in either cohort to evaluate the PK of tucatinib and ONT-993 in this population; alternative dose levels/schedules may be explored depending on the PK of tucatinib in subjects with gastrectomy.

[0275] A formal efficacy analysis will be undertaken when 30 subjects with HER2+ disease according to blood based NGS assay from either Cohort 2A or the dose optimization stage have been treated at the recommended paclitaxel dose, are evaluable for response, and have been followed for at least 6 weeks. If the ORR per RECIST v1.1 according to investigator assessment is $\geq 36\%$, the SMC may recommend that the Phase 3 evaluation of the regimen be initiated in subjects with HER2 amplification in a blood-based NGS assay, given that it is safe and tolerable. Enrollment in Cohort 2B can continue after the initiation of the Phase 3.

Phase 3

[0276] Approximately 500 subjects who have HER2 amplification in a blood-based NGS assay will be randomized in approximately an 8:8:1 ratio to either Arm 3A (tucatinib, trastuzumab, ramucirumab, and paclitaxel; 235 subjects), Arm 3B (tucatinib placebo, trastuzumab placebo, ramucirumab, and paclitaxel; 235 subjects), or Arm 3C (tucatinib, trastuzumab placebo, ramucirumab, and paclitaxel; 30 subjects) (**Error! Reference source not found.**). Efficacy in Arms 3A and 3B will be formally compared to demonstrate the benefit of adding tucatinib plus trastuzumab to standard of care ramucirumab plus paclitaxel. Efficacy in Arm 3C will be analyzed separately. Randomization will be stratified by region (Asia vs Rest of World), time to progression on first-line therapy for locally-advanced unresectable or metastatic disease (<6 months vs ≥ 6 months), and history of prior gastrectomy (yes vs no). Subjects randomized to Arm 3B will receive a tucatinib placebo and a trastuzumab placebo; subject randomized to Arm 3C will receive a trastuzumab placebo. Subjects, investigators, and staff, and the sponsor study team will be blinded to study arm allocation. Subjects who are screened for the Phase 3 but are found to be HER2-negative according to the blood-based NGS assay, may be enrolled in Cohort 2B of the Phase 2 if it is still enrolling and the subject has HER2-positive disease according to assay of a biopsy obtained after progression on the most recent line of therapy.

[0277] In the Phase 3 portion of the study, an Independent Data Monitoring Committee (IDMC) will periodically review relevant aggregate safety data and will make recommendations to the sponsor on the conduct of the study. Safety will also be monitored in an ongoing basis by the sponsor throughout the study.

Method of Assigning Subjects to Treatment Groups

Phase 2

[0278] Following informed consent and screening assessments, eligible subjects will be assigned to the paclitaxel dose level currently enrolling during the paclitaxel dose optimization stage. Once the paclitaxel recommended dose is established, subjects will be assigned to either Cohort 2A or Cohort 2B based on the results of blood-based NGS assay or, if the former was negative, IHC/ISH assay of a tumor biopsy obtained after progression on the most recent line of systemic therapy.

Phase 3

[0279] Following informed consent and screening assessments, eligible subjects will be randomly assigned to Arms 3A, 3B, and 3C in an approximately 8:8:1 ratio. Randomization will be performed centrally using a system that will assign a unique subject randomization number but will not specify the actual treatment assignment. Randomization procedures are detailed in the Study Manual.

- Randomization will be stratified by:
- Region of inclusion: Asia versus Rest of World
- Time to progression on first-line therapy for locally-advanced unresectable or metastatic disease: <6 months versus ≥ 6 months
- History of prior gastrectomy: yes versus no

Blinding and Unblinding

[0280] Maintaining the blind of the study in the Phase 3 portion is crucial for achieving the study objectives. Unblinding an individual subject's treatment assignment may only occur when one of the following circumstances is applicable:

[0281] At the time of study closure, the study treatment assignment will be provided by sponsor to the investigator.

[0282] Unblinding a subject's treatment assignment prior to study closure must be limited to emergency circumstances where knowledge of the treatment assignment would affect decisions regarding the clinical management of the subject. In the event of such an emergency

circumstance, a formal unblinding procedure, carried out by a third-party organization will be followed to allow the investigator to immediately access a subject's treatment assignment (see Study Manual). Information on study treatment assignment should not be distributed to any other personnel involved in the clinical trial, apart from the study site pharmacist, who will be unblinded to treatment allocation. In the event of any emergency unblinding, the sponsor is to be notified within 24 hours of the occurrence.

[0283] Details regarding unblinding procedures are described in the Study Manual.

Unblinding for Safety Monitoring

[0284] Safety data in the Phase 3 portion is monitored by an IDMC. Unblinding of aggregate safety data for ongoing safety monitoring and risk/benefit assessment by the IDMC will be performed through an independent Data Coordinating Center to ensure the integrity of the study.

[0285] Suspected unexpected serious adverse reactions will be unblinded in accordance with local regulatory reporting requirements. Pre-specified personnel from the sponsor Drug Safety Department will unblind the identity of study medication for any unexpected (as per the Investigator's Brochure) SAEs that are considered to be related to the blinded study drugs (tucatinib, trastuzumab, or placebo).

Treatments

Treatments Administered

[0286] Subjects in the Phase 2 portion of the study will receive combination therapy of the investigational medicinal products tucatinib and trastuzumab combined with standard-of-care ramucirumab and paclitaxel. In the Phase 3, subjects will receive either tucatinib and trastuzumab (Arm 3A), tucatinib placebo and trastuzumab placebo (Arm 3B), or tucatinib and trastuzumab placebo (Arm 3C), all combined with ramucirumab and paclitaxel. Study treatment will be given on a 28-day cycle, with tucatinib (or placebo) every day, trastuzumab (or placebo) and ramucirumab on Days 1 and 15, and paclitaxel on Days 1, 8, and 15 (Table 3). In subjects in the Phase 2 undergoing cycle 1 PK assessments, the first tucatinib dose will be given in the evening on Day 1. In this study, subjects are considered to be on study treatment if they are receiving any of the study drugs (tucatinib/placebo, trastuzumab/placebo, ramucirumab, and/or

paclitaxel). Cycles are defined by paclitaxel administration, with a new cycle starting whenever the day 1 infusion of paclitaxel is administered. If paclitaxel is discontinued, cycles will be defined as occurring every 28 days from the last day 1 administration of paclitaxel.

[0287] In the Phase 2, tucatinib should be dosed at approximately the same time as the start of the paclitaxel infusion on cycle 1 day 8 and cycle 2 day 1, when both tucatinib and paclitaxel PK are assessed. The administration order of the IV study drugs is paclitaxel first, then trastuzumab and ramucirumab, or according to institutional standard of care.

Table 3: Treatment schedule

Agent	Dose	Route	Cycle	Daily frequency	Cycle Day		
					Day 1	Day 8	Day 15
Phase 2							
Tucatinib	300 mg	PO	All	BID	Every day, from Cycle 1 Day 1 ^a		
Trastuzumab ^b	4 mg/kg (6 mg/kg on Cycle 1 Day 1)	IV	All	Once	X		X
Ramucirumab	8 mg/kg	IV	All	Once	X		X
Paclitaxel	60 or 80 mg/m ² ^c	IV	All	Once	X	X	X
Phase 3							
Arm 3A							
Tucatinib	300 mg	PO	All	BID	Every day, from Cycle 1 Day 1		
Trastuzumab ^b	4 mg/kg (6 mg/kg on Cycle 1 Day 1)	IV	All	Once	X		X
Ramucirumab	8 mg/kg	IV	All	Once	X		X
Paclitaxel	Phase 2 recommended dose	IV	All	Once	X	X	X
Arm 3B							
Tucatinib placebo	Not applicable	PO	All	BID	Every day, from Cycle 1 Day 1		
Trastuzumab placebo ^b	Not applicable	IV	All	Once	X		X
Ramucirumab	8 mg/kg	IV	All	Once	X		X
Paclitaxel	80 mg/m ²	IV	All	Once	X	X	X

<i>Agent</i>	<i>Dose</i>	<i>Route</i>	<i>Cycle</i>	<i>Daily frequency</i>	<i>Cycle Day</i>		
					<i>Day 1</i>	<i>Day 8</i>	<i>Day 15</i>
<i>Arm 3C</i>							
Tucatinib	300 mg	PO	All	BID	Every day, from Cycle 1 Day 1		
Trastuzumab placebo ^b	Not applicable	IV	All	Once	X		X
Ramucirumab	8 mg/kg	IV	All	Once	X		X
Paclitaxel	Phase 2 recommended dose	IV	All	Once	X	X	X

- a In subjects in the Phase 2 undergoing cycle 1 PK assessments, the first tucatinib dose will be given in the evening on Day 1.
- b Trastuzumab may also be given on a weekly basis at 2 mg/kg IV, but only in circumstances where weekly infusions are required to resynchronize with the paclitaxel cycle.
- c The paclitaxel dose optimization stage will initially evaluate 60 mg/m² and potentially escalate to 80 mg/m². Alternative dose levels/schedules may be evaluated as recommended by the SMC

Investigational Product, Dose, and Mode of Administration

Tucatinib

[0288] Tucatinib, an investigational agent under study in this protocol, is a kinase inhibitor that selectively inhibits HER2, and displays limited activity against the related kinase EGFR.

[0289] In the Phase 2, tucatinib will be supplied in an open-label manner, by the sponsor. In the Phase 3, treatment allocation to tucatinib or tucatinib placebo will be double-blinded.

[0290] Detailed information describing the preparation, administration, and storage of tucatinib is located in the Pharmacy Instructions.

Description

[0291] Tucatinib drug product is supplied as both a coated yellow oval-shaped tablet in a 150 mg dosage strength and a coated yellow round convex tablet in a 50 mg dosage strength. The tablets are manufactured from a drug product intermediate amorphous dispersion of tucatinib in polyvinylpyrrolidone-vinyl acetate copolymer, which is then combined with the pharmaceutical excipients (microcrystalline cellulose, sodium chloride, potassium chloride, sodium bicarbonate, silicon dioxide, crospovidone, and magnesium stearate), and compressed into tablets.

Dose and Administration

[0292] Tucatinib will be administered according to the following:

[0293] Route of administration: PO. Dose: Tucatinib 300 mg will be administered PO BID from cycle 1 day 1 onwards.

[0294] Dosing schedule: BID on each day of study treatment. Tucatinib or tucatinib placebo should be taken once in the morning and once in the evening, with approximately 8 to 12 hours between doses in the same calendar day. In subjects in the Phase 2 undergoing cycle 1 day 1 PK assessments, tucatinib will not be administered in the morning of cycle 1 day 1; the first dose will be in the evening, after all PK samples have been collected.

[0295] In the Phase 3, subjects in Arm 3B receive a tucatinib placebo PO BID.

[0296] Dose modifications of tucatinib or placebo are described herein. Subjects will be instructed by the pharmacist or investigator as to the specific number of tablets required for each dose. At each visit during study treatment, subjects will be supplied with the appropriate number of tablets for the number of doses to be taken prior to the next scheduled visit.

[0297] It is recommended that if a subject misses a scheduled dose of tucatinib or placebo and less than 6 hours have passed since the scheduled dosing time, the dose should be immediately taken. It is recommended that if more than 6 hours have passed since the scheduled dosing time, the subject should not take the missed dose but should wait and take the next regularly scheduled dose. Tablets may be taken with or without food. Tablets must be swallowed whole and may not be crushed, chewed, or dissolved in liquid. On the day of dosing, the individual unit dose of the tucatinib or placebo tablet may be exposed to ambient temperature for up to 6 hours prior to dose.

[0298] Complete dosing instructions will be provided to the pharmacist prior to the initiation of the study. Complete dosing instructions will also be provided to study subjects and will include the minimum times between doses, dosing in relation to meals, and instructions for missed doses. Subject compliance with investigational study drug dosing instructions will be assessed with the use of subject diaries or pill count and study drug accountability.

Trastuzumab or Trastuzumab Placebo**Description**

[0299] Trastuzumab is a humanized immunoglobulin G1 (IgG1) kappa mAb which binds to the extracellular domain of HER2; it mediates antibody-dependent cellular cytotoxicity by inhibiting proliferation of cells which over express the HER2 protein. Trastuzumab is indicated for adjuvant treatment of HER2-overexpressing node positive or node negative breast cancer, in combination with paclitaxel for first-line treatment of HER2 overexpressing mBC, as a single agent for treatment of HER2-overexpressing breast cancer in patients who have received one or more chemotherapy regimens for metastatic disease, and in combination with cisplatin and capecitabine or 5-fluorouracil, for the treatment of patients with HER2-overexpressing metastatic GEC who have not received prior treatment for metastatic disease.

Method of Procurement

[0300] Trastuzumab is commercially available. In Phase 2, details regarding its sourcing may vary by site and/or region as outlined in other documents such as Clinical Trial Agreements.

[0301] In the Phase 3, treatment allocation to trastuzumab or trastuzumab placebo will be double blinded, and will be supplied.

Dose and Administration

[0302] Trastuzumab will be administered on Day 1 and 15 of every 28-day cycle. A loading dose of 6 mg/kg IV will be administered on cycle 1 day 1 followed by 4 mg/kg with each subsequent dose. Trastuzumab placebo will be used in Arms 3B and 3C of the Phase 3. Trastuzumab may also be given on a weekly basis at 2 mg/kg IV once weekly, in order to resynchronize administration to Day 1 and 15 of the 28-day paclitaxel cycle, after discussion with the medical monitor. If dosing of trastuzumab has been held for >4 weeks and the medical monitor has agreed to restart trastuzumab, the IV loading dose of 6 mg/kg should be given per approved dosing instructions. Trastuzumab infusion rates will be per institutional guidelines.

[0303] To maintain the blind, subjects assigned to receive trastuzumab placebo will receive an IV infusion that does not contain trastuzumab. Refer to Pharmacy Manual for additional instructions.

Ramucirumab**Description**

[0304] Ramucirumab (CYRAMZA®) is a recombinant human IgG1 mAb with an approximate molecular weight of 147 kDa, produced in genetically engineered mammalian NS0 cells. It is a VEGFR2 antagonist that specifically binds VEGFR2 and blocks binding of VEGFR ligands, VEGF-A, VEGF-C, and VEGF-D. As a result, ramucirumab inhibits ligand stimulated activation of VEGFR2, thereby inhibiting ligand induced proliferation, and migration of human endothelial cells. It is indicated for the treatment of subjects with advanced or metastatic GEC with disease progression on or after prior fluoropyrimidine- or platinum containing chemotherapy as a single agent or in combination with paclitaxel. It is also indicated for subjects with previously-treated metastatic non-small cell lung cancer in combination with docetaxel, in subjects with previously treated mCRC in combination with FOLFIRI, and in subjects with previously treated hepatocellular carcinoma.

Method of Procurement

[0305] Ramucirumab is commercially available and details regarding its sourcing may vary by site and/or region as outlined in other documents such as Clinical Trial Agreements.

Dose and Administration

[0306] Ramucirumab 8 mg/kg will be administered on Days 1 and 15 of each 28 day cycle. Ramucirumab will be administered IV per institutional guidelines, under the direction of the investigator. Ramucirumab is for IV infusion only. Do not administer as an IV push or bolus.

[0307] IRRs related to ramucirumab have been observed. To reduce the risk of IRRs with Ramucirumab, subjects will receive premedication/postmedication as described herein.

Paclitaxel**Description**

[0308] Paclitaxel is an antimicrotubule agent that promotes the assembly of microtubules from tubulin dimers and stabilizes microtubules by preventing depolymerization. This stability inhibits the normal dynamic reorganization of the microtubule network, which is essential for vital interphase and mitotic cellular functions. In addition, paclitaxel induces abnormal arrays or bundles of microtubules throughout the cell cycle and multiple asters of microtubules during mitosis.

Method of Procurement

[0309] Paclitaxel is commercially available and details regarding sourcing of paclitaxel may vary by site and/or region as outlined in other documents such as Clinical Trial Agreements.

Dose and Administration

[0310] Paclitaxel will be administered on Days 1, 8, and 15 of each 28-day cycle. Paclitaxel will be administered IV per institutional guidelines, under the direction of the investigator. The initial paclitaxel dose to be evaluated in the Phase 2 dose optimization stage is 60 mg/m²; an 80 mg/m² dose level may be explored. Additional dose levels/schedules may be evaluated as recommended by the SMC. In the Phase 3, paclitaxel will be administered at the recommended dose identified in Phase 2 in Arms 3A and 3C, and at 80 mg/m² in Arm 3B.

[0311] Subjects with a history of severe hypersensitivity reactions to products containing polyoxyl 35 castor oil (e.g., cyclosporin for injection concentrate, teniposide for injection concentrate) are not to be treated with paclitaxel.

[0312] Injection site and hypersensitivity reactions related to paclitaxel are common. To reduce the risk of these reactions, subjects will receive premedication as described herein. Given the possibility of extravasation, it is advisable to closely monitor the infusion site for possible infiltration during drug administration. Vital signs should be monitored frequently during the paclitaxel infusion.

[0313] Initiate concomitant G-CSF as clinically indicated.

[0314] Table 4 summarizes the conditions, in terms of AEs and laboratory test abnormalities that must be met for before administering paclitaxel. If the conditions are not met on the planned Day 1 of a cycle, then the paclitaxel infusion should be delayed until the conditions are met. If the conditions are not met on the planned Day 8 or 15, then the paclitaxel infusion should be skipped.

Table 4: Criteria for paclitaxel treatment on Day 1, 8, and 15 of each cycle

AE/abnormality	Day 1	Day 8 and 15
ANC:	$\geq 1.5 \times 10^3/\mu\text{L}$	$\geq 1.0 \times 10^3/\mu\text{L}$
Platelets:	$\geq 75 \times 10^3/\mu\text{L}$	
Bilirubin:	$\leq 1.5 \times \text{ULN}$	
AST/ALT:	$\leq 3 \times \text{ULN}^a$ ($< 5 \times \text{ULN}$ if liver metastases present)	
Paclitaxel-related AEs:	Grade ≤ 1 or baseline (except for alopecia) Anemia Grade ≤ 2	

^a On cycle 1 day 1, AST/ALT is to be $\leq 2.5 \times \text{ULN}$ ($< 5 \times \text{ULN}$ if liver metastases present).

Dose Modifications

[0338] Dose modification recommendations (including dose holds, dose reduction, or discontinuation of drugs) in response to AEs are described for tucatinib/placebo, for trastuzumab/placebo, for ramucirumab, and for paclitaxel. Dose reductions or treatment interruption/discontinuation for reasons other than those described in the following sections may be made by the investigator if it is deemed in the best interest of subject safety. Whenever possible, these decisions should first be discussed with the study medical monitor.

[0339] All AEs and clinically significant laboratory abnormalities should be assessed by the investigator for relationship to tucatinib/placebo, trastuzumab/placebo, ramucirumab, and paclitaxel. An AE may be considered related to any single study drug, any combination of study drugs, or to none of them. In the event that the relationship is unclear, discussion should be held with the study medical monitor, to determine which study drug(s) should be held and/or modified.

[0340] The beginning of each cycle is defined by the administration of the Day 1 infusion of paclitaxel. During paclitaxel cycle delays, ramucirumab and trastuzumab administration should continue as planned. When the new paclitaxel cycle starts, ramucirumab should be administered on Day 1, even if it was administered the previous week. Trastuzumab should not be administered on Day 1 if trastuzumab 4 mg/kg was administered the previous week; instead, to synchronize

trastuzumab to a delayed paclitaxel cycle, trastuzumab 2 mg/kg should be given on Day 8 followed by trastuzumab 4 mg/kg on Day 15. If paclitaxel cannot be administered on Day 8 or Day 15 of a cycle, that day is skipped, ramucirumab and trastuzumab are administered as scheduled, and the paclitaxel schedule continues unchanged. If paclitaxel is discontinued, protocol-defined visits will proceed using a 28-day cycle starting from the last paclitaxel Day 1, regardless of dose holds or delays.

[0341] Doses held for toxicity will not be replaced. Once reduced, the dose of a study drug should not be re-escalated. Any study drug that requires a delay >4 weeks should be discontinued, unless a longer delay is approved by the study medical monitor. If one or more study drugs are discontinued, study treatment can continue with the remaining study drugs.

Tucatinib/placebo Dose Modifications

[0342] Up to 3 dose reductions of tucatinib/placebo are allowed (Table 5). Subjects who would require a dose reduction to below 150 mg BID should discontinue treatment with tucatinib/placebo. Dose reductions of larger intervals than those described in Table 5 may be made at the discretion of the investigator with approval by the medical monitor, but dose reductions to below 150 mg BID are not allowed.

Table 5: Tucatinib/placebo: Recommended dose reduction schedule for adverse events

Dose Reduction Schedule	Tucatinib/Placebo Dose Level
Starting dose	300 mg PO BID ^a
1st dose reduction	250 mg PO BID
2nd dose reduction	200 mg PO BID
3rd dose reduction	150 mg PO BID
Requirement for further dose reduction	Discontinue tucatinib

^c Dose reductions of greater intervals than those recommended in this table (i.e., more than 50 mg per dose reduction) may be made if considered clinically appropriate by the investigator and approved by the medical monitor. However, tucatinib/placebo may not be dose reduced below 150 mg BID.

[0344] General dose modification guidelines for tucatinib/placebo are provided in Table 6 and Table 7. For subjects with documented Gilbert's disease, contact the medical monitor for guidance regarding dose modifications for LFT abnormalities.

Table 6: Dose modifications for clinical adverse events related to tucatinib/placebo

Adverse Reactions	Tucatinib Dose Modification
Diarrhea	
Grade 3 without anti-diarrheal treatment	Initiate or intensify appropriate medical therapy. Hold tucatinib until recovery to $\leq$ Grade 1 or baseline
Grade 3 with anti-diarrheal treatment	Initiate or intensify appropriate medical therapy. Hold tucatinib until recovery to $\leq$ Grade 1 or baseline Reduce tucatinib dose.
Grade 4 (life-threatening consequences; urgent intervention indicated)	Permanently discontinue tucatinib treatment.
Other adverse reactions	
Grade 3	Hold tucatinib until recovery to $\leq$ Grade 1 or baseline Reduce tucatinib dose
Grade 4	Permanently discontinue tucatinib.

Table 7: Dose modifications of tucatinib/placebo for LFT abnormalities, regardless of relationship to tucatinib/placebo

Laboratory Abnormality	Tucatinib Dose Modification
Bilirubin elevation $>1.5-3 \times \text{ULN}$	Hold tucatinib until recovery to $\leq 1.5 \times \text{ULN}$
Bilirubin elevation $>3-10 \times \text{ULN}$	Hold tucatinib until recovery to $\leq 1.5 \times \text{ULN}$ Reduce tucatinib dose.
Bilirubin elevation $>10 \times \text{ULN}$	Permanently discontinue tucatinib.
ALT or AST elevation $>5-20 \times \text{ULN}$	Hold tucatinib until recovery to $\leq 3 \times \text{ULN}$ or return to baseline level in subjects with known liver metastasis Reduce tucatinib dose.
ALT or AST elevation $>20 \times \text{ULN}$	Permanently discontinue tucatinib.
ALT or AST $>3 \times \text{ULN}$ AND bilirubin $>2 \times \text{ULN}$	Permanently discontinue tucatinib.

Trastuzumab/placebo Dose Modifications

[0345] In the event of Grade ≥ 3 trastuzumab-related AEs, hold trastuzumab until the AE has resolved to Grade ≤ 1 or pretreatment levels and initiate or intensify applicable medical therapy, as appropriate. Resume trastuzumab at the same dose; the trastuzumab/placebo dose may not be reduced. If dosing of trastuzumab is held for >4 weeks and the medical monitor has agreed to

restart trastuzumab, the IV loading dose of 6 mg/kg should be given per approved dosing instructions.

[0346] Trastuzumab dose modification guidelines for left ventricular dysfunction and cardiomyopathy, IRR, and for hypersensitivity reactions herein.

Left Ventricular Dysfunction and Cardiomyopathy

[0347] Trastuzumab can cause left ventricular cardiac dysfunction, arrhythmias, hypertension, disabling cardiac failure, cardiomyopathy, and cardiac death. Trastuzumab can also cause asymptomatic decline in LVEF.

[0348] Trastuzumab/placebo dose modification guidelines for left ventricular dysfunction, regardless of relationship to study drug, are provided in Table 8.

Table 8: Trastuzumab and trastuzumab placebo dose modification guidelines for left ventricular dysfunction

Symptomatic CHF	LVEF <40%	LVEF 40% to ≤45% and decrease ≥10% points from baseline	LVEF 40% to ≤45% and decrease <10% points from baseline	LVEF >45%
Discontinue trastuzumab/placebo	Do not administer trastuzumab/placebo	Do not administer trastuzumab/placebo	Continue treatment with trastuzumab/placebo	Continue treatment with trastuzumab/placebo
	Repeat LVEF assessment within 3 weeks.	Repeat LVEF assessment within 3 weeks.	Repeat LVEF assessment within 3 weeks	
	If LVEF <40% is confirmed, discontinue trastuzumab/placebo	If the LVEF has not recovered to within 10% points from baseline, discontinue trastuzumab/placebo		

Infusion-related Reactions

[0349] Symptoms of IRR occurring after trastuzumab administration include fever and chills, and on occasion included nausea, vomiting, pain (in some cases at tumor sites), headache, dizziness, dyspnea, hypotension, rash, and asthenia. In severe cases, symptoms have included bronchospasm, anaphylaxis, angioedema, hypoxia, and severe hypotension, usually reported during or immediately following the initial infusion. However, the onset and clinical course are variable, including progressive worsening, initial improvement followed by clinical deterioration, or delayed post-infusion events with rapid clinical deterioration. For fatal events, death occurred within hours to days following a serious IRR.

[0350] Interrupt trastuzumab infusion in all subjects experiencing dyspnea or clinically significant hypotension, and administer supportive therapy (which may include epinephrine, corticosteroids, diphenhydramine, bronchodilators, and oxygen). Subjects should be evaluated and carefully monitored until complete resolution of signs and symptoms. In subsequent infusions, premedicate subjects with antihistamines and/or corticosteroids.

[0351] Discontinue trastuzumab in subjects with Grade 3 to 4 IRR.

Hypersensitivity Reactions

[0352] Allergic/hypersensitivity reactions are characterized by adverse local or general responses from exposure to an allergen (NCI CTCAE version 5.0). For purposes of this study, allergic/hypersensitivity reactions are differentiated from IRRs by being defined as occurring >24 hours after infusion of trastuzumab. Allergic/hypersensitivity reactions may manifest in the same manner as IRRs, i.e., a combination of signs or symptoms including fever, rigors, flushing, itching, various types of rash, urticaria, dyspnea, nausea, vomiting, back or abdominal pain, and/or hypotension.

[0353] Anaphylaxis is a severe, life-threatening, generalized or systemic allergic/hypersensitivity reaction. Anaphylaxis is characterized by an acute inflammatory reaction resulting from the release of histamine and histamine-like substances from mast cells, causing a hypersensitivity immune response. Clinically, it presents with breathing difficulty, dizziness, hypotension, cyanosis, and loss of consciousness and may lead to death (Rosello 2017).

[0354] If anaphylaxis occurs, administration of trastuzumab should be immediately and permanently discontinued.

Ramucirumab Dose Modifications

[0355] Up to 2 dose reductions of ramucirumab will be allowed (Table 9). Subjects who would require a dose reduction to below 5 mg/kg should discontinue treatment with ramucirumab.

Table 9: Ramucirumab: Recommended dose reduction schedule for adverse events

Dose Reduction Schedule	Ramucirumab Dose Level
Starting dose	8 mg/kg
1st dose reduction	6 mg/kg
2nd dose reduction	5 mg/kg
Requirement for further dose reduction	Discontinue treatment

[0356] General dose modification guidelines for ramucirumab are provided in Table 10. Ramucirumab should be held for 28 days prior to any surgery, and resumed no sooner than 28 days after surgery, once the wound is fully healed and following discussion with the medical monitor.

[0357] Ramucirumab dose modification guidelines for hypertension, IRR, proteinuria, and nephrotic syndrome, impaired wound healing, and for reversible posterior leukoencephalopathy syndrome (RPLS) herein.

Table 10: Dose modifications for clinical adverse events

Clinical Adverse Event	Ramucirumab Dose Modification
Regardless of relationship to ramucirumab	
Grade 3-4 hemorrhage	Discontinue ramucirumab
Gastrointestinal perforation any grade	Discontinue ramucirumab
Wound healing complications of any grade that require medical intervention	Discontinue ramucirumab
Arterial Thromboembolic Events any grade	Discontinue ramucirumab
Grade 3-4 hypertension	Hold until controlled by medical management
Grade 3-4 hypertension not controlled by antihypertensive therapy	Discontinue ramucirumab
Hypertensive crisis or hypertensive encephalopathy	
Proteinuria	
– 1st occurrence (≥ 2 g per 24 hours)	Hold until < 2 g per 24 hours. Reduce dose

– Reoccurrence after dose reduction (≥ 2 g per 24 hours)	Hold until < 2 g per 24 hours. Reduce dose
– ≥ 3 g per 24 hours or in the setting of nephrotic syndrome	Discontinue ramucirumab
RPLS confirmed by MRI	Discontinue ramucirumab
Spontaneous development of fistula	Discontinue ramucirumab
Hepatic encephalopathy or hepatorenal syndrome	Discontinue ramucirumab
Related to ramucirumab	
IRR	
– Grade 1-2	Reduce infusion rate by 50%
– Grade 3-4	Discontinue ramucirumab

Hypertension

[0358] Control hypertension prior to initiating treatment with ramucirumab. Monitor blood pressure every two weeks or more frequently as indicated during treatment. Withhold ramucirumab for severe hypertension until medically controlled.

[0359] Permanently discontinue ramucirumab for medically significant hypertension that cannot be controlled with antihypertensive therapy or in subjects with hypertensive crisis or hypertensive encephalopathy.

Infusion-related Reactions

[0360] Symptoms of IRR occurring after ramucirumab administration have included rigors/tremors, back pain/spasms, chest pain and/or tightness, chills, flushing, dyspnea, wheezing, hypoxia, and paresthesia. In severe cases, symptoms included bronchospasm, supraventricular tachycardia, and hypotension.

[0361] Premedicate prior to each ramucirumab infusion. Monitor subjects during the infusion for signs and symptoms of IRR in a setting with available resuscitation equipment.

[0362] Reduce the infusion rate by 50% for Grade 1 to 2 IRR. Permanently discontinue ramucirumab for Grade 3 to 4 IRRs.

Proteinuria and Nephrotic Syndrome

[0363] Monitor proteinuria by urine dipstick and/or urinary protein creatinine ratio. If the result of the urine dipstick is 2+ or greater, perform a 24-hour urine collection for protein measurement.

Withhold ramucirumab for urine protein levels that are 2 or more grams over 24 hours. Reinitiate ramucirumab at a reduced dose once the urine protein level returns to less than 2 grams over 24 hours. Permanently discontinue ramucirumab for urine protein levels greater than 3 grams over 24 hours or in the setting of nephrotic syndrome.

Impaired Wound Healing

[0364] Withhold ramucirumab for 28 days prior to surgery. Do not administer ramucirumab for at least 28 days following a major surgical procedure, until the wound is fully healed, following discussion with the medical monitor. Discontinue ramucirumab in subjects who develop wound healing complications that require medical intervention.

Reversible Posterior Leukoencephalopathy Syndrome

[0365] In the event of RPLS, confirm the diagnosis of RPLS with magnetic resonance imaging (MRI) and permanently discontinue ramucirumab.

Paclitaxel Dose Modifications

[0366] The paclitaxel dose can be reduced by increments of 10 mg/m² (i.e., reductions to 70 mg/m² then to 60 mg/m² for a subject initially receiving 80 mg/m²); however, subjects who would require a dose reduction to below 60 mg/m² should discontinue treatment with paclitaxel. Dose reductions are implemented only at the start of a cycle, not on Day 8 or 15.

[0367] General dose modification guidelines for paclitaxel are provided in Table 11 and Table 12.

[0368] Paclitaxel dose modification guidelines for hepatotoxicity and for hypersensitivity herein.

Table 11: Paclitaxel dose modifications for clinical adverse events related to paclitaxel

Adverse Reaction	Paclitaxel Dose Modification
Paclitaxel-related non-hematological AEs	Delay Day 1 administration until recovery to Grade ≤ 1 or baseline or skip Day 8 or 15
– Grade ≥ 3	Reduce paclitaxel dose in next cycle
Grade 3-4 peripheral neuropathy	Delay Day 1 administration until recovery to Grade ≤ 1 or baseline or skip Day 8 or 15 Reduce paclitaxel dose in next cycle
Grade 3-4 hypersensitivity reaction	Discontinue paclitaxel

Table 12: Paclitaxel dose modifications for hematological abnormalities, regardless of relationship to paclitaxel

Hematological Abnormality	Paclitaxel Dose Modification
ANC $<1.5 \times 10^3/\mu\text{L}$	Delay Day 1 administration until recovery to $\geq 1.5 \times 10^3/\mu\text{L}$
ANC $<1.0 \times 10^3/\mu\text{L}$	Delay Day 1 administration recovery to $\geq 1.5 \times 10^3/\mu\text{L}$ or skip Day 8 or 15 administration
Platelet count $<75 \times 10^3/\mu\text{L}$	Delay Day 1 administration recovery to $\geq 75 \times 10^3/\mu\text{L}$ or skip Day 8 or 15 administration
Grade 4 hematological abnormalities	Reduce paclitaxel dose in next cycle

Hepatotoxicity

[0369] Dose modification for paclitaxel in the case of LFT abnormalities, regardless of relationship to study drug, are summarized in Table 13. A rise in indirect bilirubin with a normal direct bilirubin believed to be attributable to Gilbert's disease does not require change in dose or a drug hold.

Table 13: Paclitaxel dose modification guidelines for liver function abnormalities, regardless of relationship to paclitaxel

LFT abnormality	Action
Bilirubin elevation $>1.5-3 \times \text{ULN}$	Delay Day 1 administration until recovery to $\leq 1.5 \times \text{ULN}$ or skip Day 8 or 15 infusions
Bilirubin elevation $>3-10 \times \text{ULN}$	Delay Day 1 administration until recovery to $\leq 1.5 \times \text{ULN}$ or skip Day 8 or 15 infusions Reduce paclitaxel dose in next cycle
Bilirubin elevation $>10 \times \text{ULN}$	Permanently discontinue paclitaxel.
AST or ALT elevation $>5-20 \times \text{ULN}$	Delay Day 1 administration until recovery to $\leq 3 \times \text{ULN}$ or return to baseline level in subjects with known liver metastasis or skip Day 8 or 15 infusions Reduce paclitaxel dose
AST or ALT elevation $>20 \times \text{ULN}$	Permanently discontinue paclitaxel

ALT or AST >3 × ULN AND bilirubin >2 × ULN	Permanently discontinue paclitaxel
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Hypersensitivity Reactions

[0370] Paclitaxel treatment interruption is not required for minor symptoms of hypersensitivity, such as flushing, skin reactions, dyspnea, hypotension, or tachycardia.

[0371] Paclitaxel should be discontinued and aggressive symptomatic therapy applied in the event of severe reactions, such as hypotension requiring treatment, dyspnea requiring bronchodilators, angioedema, or generalized urticaria.

Required Premedication and Postmedication

[0372] Suggested premedication to be administered prior to each paclitaxel infusion is presented in Table 14. Paclitaxel premedication can be adjusted according to standard institutional practice.

Table 14: Paclitaxel premedication

Pre-medication	Dose	Administration time prior to paclitaxel
Dexamethasone	8–10 mg PO or 8–10 mg IV	30 to 60 min
Diphenhydramine or equivalent	12.5–50 mg IV	30 to 60 min
Ranitidine	50 mg IV	30 to 60 min

[0373] Ramucirumab pre-medication with an IV histamine-1 receptor antagonist (e.g., diphenhydramine hydrochloride) may be given prior to each ramucirumab infusion at the discretion of the investigator. For subjects who have experienced a Grade 1 or 2 IRR considered at least possibly related to ramucirumab, premedicate with a histamine-1 receptor antagonist, dexamethasone (or equivalent), and acetaminophen prior to each ramucirumab infusion.

[0374] Subjects who have experienced dyspnea or clinically significant hypotension related to trastuzumab during or following the previous infusion should be premedicated with antihistamines and/or corticosteroids prior to subsequent trastuzumab infusions.

Duration of Treatment

[0315] Study treatment will continue until unacceptable toxicity, disease progression, withdrawal of consent, death, or study closure. If a study drug (tucatinib/placebo, trastuzumab/placebo, ramucirumab, or paclitaxel) is discontinued, study treatment can continue with remaining study drug(s).

[0316] In the absence of clear evidence of radiographic progression per RECIST version 1.1, all efforts should be made to continue treatment until unequivocal evidence of radiologic progression per RECIST version 1.1 occurs. No crossover will be allowed.

Study Assessments

[0317] Screening/Baseline Assessments

[0318] Screening/Baseline assessments will be conducted to establish study baseline status and determine study eligibility.

[0319] Subject medical history includes a thorough review of significant past medical history, current conditions, any treatment for prior malignancies and response to prior treatment, and any concomitant medications.

[0320] The following assessments are required for all subjects at screening and/or baseline: physical exam, height, vital signs, weight, ECOG performance status, CT with contrast/PET-CT/MRI scan for baseline disease assessment, CBC with differential, serum chemistry panel, coagulation panel, urinalysis, ECG, echocardiogram/MUGA, Hepatitis B and C screening, blood sample for biomarker assay, and serum or urine β -hCG pregnancy test (for females of childbearing potential).

Evaluation of HER2 Status at Screening

[0321] A blood sample will be drawn to establish baseline HER2 amplification using an NGS assay, performed at a central laboratory prior to randomization.

[0322] Archival tumor blocks (or freshly-cut slides, following consultation with the medical monitor) sampled following progression during/after the most recent line of therapy, or other archival biopsies performed prior to the first line therapy for advanced disease, are to be collected at screening. Tissue samples obtained via resection, excision, punch (skin lesions only), or core

needle from a tumor site are suitable for testing. Fine needle aspiration, brushing, cell pellets from pleural effusion, forceps, and lavage samples are not acceptable. Tumor tissue should be of good quality based on total and viable tumor content; e.g., samples should contain a minimum of 100 tumor cells that preserve cellular context and tissue architecture, regardless of the needle gauge used to collect the sample or the retrieval method. See the Laboratory Manual for details concerning tissue samples.

[0323] HER2 expression in the biopsy will be evaluated using tissue-based NGS and according to the 2016 guideline of the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) "HER2 Testing and Clinical Decision Making in Gastroesophageal Adenocarcinoma", and the package insert of FDA-approved tests for IHC and ISH.

[0324] Response/Efficacy Assessments

[0325] Disease response to study treatment and the occurrence of disease progression will be determined according to RECIST version 1.1, as assessed by the investigator and by the BICR (Phase 3). Radiographic scans and additional imaging assessments (if applicable) will be performed at protocol-specified time points. Clinical management decisions will be based on local investigator assessment to ensure that treatment decisions are made in a timely manner; results of centralized review will not be available to investigators for clinical decision making.

[0326] Disease assessments will be performed at screening, and every 6 weeks for the first 36 weeks then every 9 weeks, irrespective of dose holds or interruptions. Subjects that discontinue study treatment for reasons other than documented progressive disease will continue to have disease assessments at least every 9 weeks until the occurrence of documented progression per RECIST version 1.1, death, withdrawal of consent, or study closure.

[0327] All known sites of metastatic or locally-advanced unresectable disease should be assessed by radiographic imaging at Screening/Baseline to document sites of disease and tumor burden. Imaging, preferably by high quality spiral contrast CT scan (with oral and/or IV contrast), should include the chest, abdomen, and pelvis, at a minimum; PET/CT (if high quality CT scan is included) and/or MRI scan may also be done as appropriate. If a CT scan with contrast is contraindicated (i.e., in subjects with contrast allergy or impaired renal clearance), a non-contrast CT scan of the chest may be performed instead, with MRI scans of the abdomen and pelvis. At the

investigator's discretion, other appropriate imaging (e.g., nuclear bone scan imaging for bone lesions) should be used to assess additional known sites of measurable disease. The same imaging modalities employed in Screening/Baseline should be used for all subsequent response assessments during study treatment and in the follow-up period, unless otherwise clinically indicated. If any other radiographic or assessment exam, including pathology from any on-study biopsies or procedures, is conducted per standard of care, the assessment information will be collected in the CRF. In the Phase 3, all imaging will be collected for retrospective BICR.

[0328] In the event of equivocal progression, for example a new lesion which is small in size (defined as an equivocal new lesion) and no imminent threat to subject safety, all efforts should be made to continue the subject until unequivocal radiologic progression or clinical progression is documented. Demonstration of an unequivocal new lesion constitutes disease progression.

[0329] In the Phase 2, subjects will be considered evaluable for response if they meet the following 3 criteria: (1) had baseline disease assessment, (2) received study treatment, and (3) had post-baseline disease assessment or discontinued treatment due to documented disease progression, clinical progression, treatment-related AE(s), or death.

[0330] In the Phase 3, all randomized subjects with measurable disease at baseline will be considered evaluable for response.

[0331] Subjects' clinical data must be available for CRF source verification. Copies of tumor images must be made available for review by the sponsor (or its designee) upon request. In the Phase 3, all imaging will be submitted or uploaded for retrospective BICR as soon as reasonably possible (e.g., within approximately 2 weeks) following the date of assessment. Refer to the Study Manual for instructions on collecting and submitting tumor imaging studies to the third-party imaging core laboratory for BICR.

[0332] Pharmacokinetic Assessments

[0333] Blood samples for tucatinib, ONT-993, paclitaxel, and paclitaxel metabolites PK assessment will be collected at protocol-defined timepoints; **Error! Reference source not found.** specifies PK and biomarker collection timepoints for all subjects in Phase 2 and Phase 3, while **Error! Reference source not found.** specifies additional PK collection timepoints for subjects in

the paclitaxel dose optimization stage and the first 6 subjects with and without gastrectomies (without maintenance of the pylorus).

[0334] In all subjects in the paclitaxel dose optimization stage and in the first 6 subjects in the Phase 2 dose expansion stage with a gastrectomy, tucatinib and ONT-993 concentrations will be sampled on cycle 1 day 8 and cycle 2 day 1; concentrations of paclitaxel and its metabolites will be sampled on cycle 1 days 1 and 8 and cycle 2 day 1. Subjects with gastrectomies can come from Cohort 2A or Cohort 2B.

[0335] In all subjects in the Phase 2 and Phase 3, tucatinib trough drug concentrations will be sampled on Day 1 of Cycles 2 to 6 prior to administration of tucatinib or placebo.

[0336] Plasma concentrations of tucatinib, ONT 993, paclitaxel, and its metabolites will be determined using validated liquid chromatography (LC)-mass spectrometry (MS)/MS methods. PK parameters of tucatinib, paclitaxel, and their respective metabolites will be calculated using standard noncompartmental methods. PK parameters to be estimated may include, but are not limited to: AUC_{last}, C_{max}, C_{trough}, T_{max}, and MR_{AUC}.

[0337] Trough PK samples should continue to be collected on schedule regardless of dose holds or interruptions. The cycle 1 and cycle 2 post-dose samples should not be collected during dose hold or interruptions.

PRO and HCRU Assessments

[0338] The EORTC QLQ-C30, EORTC QLQ- OG25, and EQ-5D-5L patient-reported outcome measures will be administered to assess GEC symptoms and HRQoL/health status information. PROs will be assessed, in the Phase 3 portion, predose on cycle 1 day 1, predose on Day 1 of every second cycle (Cycles 2, 4, 6, etc.) until discontinuation of all study treatment, at the end-of-treatment (EOT) visit, and at each follow-up visit until the occurrence of documented progression, death, withdrawal of consent, or study closure. HCRU data will also be collected during treatment and follow-up, including procedures that occur on study, length of stay, hospitalizations, ED visits, planned/unplanned provider visits, medication use, radiology, and other treatments or procedures.

Biomarker Assessments

[0339] HER2 status will be determined by blood-based NGS assay and by IHC/ISH assay of tumor biopsies (IHC3+ or IHC2+/ISH+). Additional biomarker assessments may include HER2 status

by tissue-based NGS as well as an exploratory assessment of HER2 mutations or other mutations as potential biomarkers of response. Additional exploratory analyses including but not limited to IHC and NGS analysis may be performed to interrogate biomarkers that are associated with tumor growth, survival, and resistance to targeted therapeutics. This assessment may enable the correlation of additional biomarkers with treatment outcome and may ultimately guide or refine patient selection strategies to better match tucatinib regimens with tumor phenotype/genotype in the future.

Safety Assessments

[0340] Safety assessments will include the surveillance and recording of AEs and SAEs, physical examination findings, vital signs including weight, electrocardiograms (ECGs), concomitant medications, pregnancy testing, and laboratory tests. Assessment of cardiac ejection fraction will be performed using MUGA scan or echocardiogram.

Statistical Methods

Sample Size Considerations

Phase 2:

[0341] The preliminary activity of the study regimen will be formally evaluated in approximately 30 response-evaluable subjects from the dose optimization stage or the dose expansion stage (Cohort 2A) who are HER2+ by the blood-based NGS assay and who were treated at the paclitaxel recommended dose.

[0342] The Phase 3 may be initiated if the observed ORR per investigator is $\geq 36\%$. With the sample size of 30, it is expected that at least 11 responders will be observed if the underlying ORR is $\geq 36\%$. The point estimate and 95% CI for ORR under different underlying ORR for the sample size of 30 is as follows:

ORR	Number of responses in 30 subjects	Lower bound of 95% CI	Upper bound of 95% CI
30%	9	14.7%	49.4%
36%	11	19.4%	55.5%
40%	12	22.7%	59.4%
47%	14	28.6%	66.0%
50%	15	31.3%	68.7%

[0343] Response-evaluable subjects in the Phase 2 include all subjects who meet all following criteria: (1) had baseline disease assessment, and (2) received study treatment, and (3) had post baseline assessment or discontinued treatment due to documented disease progression, clinical progression, toxicity, or death.

[0344] *Phase 3:*

[0345] The sample size for this portion of study was calculated based on maintaining 90% power for the dual primary endpoint of PFS with an alpha of 0.02 and 88% power for the dual primary endpoint of OS with an alpha of 0.03. For PFS, 317 events from Arm 3A or 3B are required with 90% power to detect a hazard ratio of 0.67 (4.5 months median PFS in Arm 3B versus 6.75 months in Arm 3A) using a 2-sided log-rank test and alpha of 0.02. For OS, 354 events from Arm 3A or 3B are required with 88% power to detect a hazard ratio of 0.70 (10 months median OS in Arm 3B versus 14.3 months in Arm 3A) using a 2-sided log-rank test and alpha of 0.03. The 2 primary endpoints will be evaluated using parallel testing, with alpha recycling if only one of them meets statistical significance.

[0346] Approximately 500 subjects will be randomized in approximately an 8:8:1 ratio to Arm 3A, Arm 3B, or Arm 3C. Approximately 470 subjects are expected to be randomized to the formal comparison of Arm 3A and Arm 3B. Assuming an accrual period of 30 months and a 5% yearly drop-out rate, it is expected that 317 PFS events and 354 OS events out of the 470 subjects will be observed approximately 25 and 39 months after first subject randomized, respectively.

Interim Analyses

[0347] The SMC will undertake safety and PK analyses once the first 6 subjects evaluable for DLT in each dose level in the paclitaxel dose optimization stage have been followed for at least 1 cycle. If alternative paclitaxel dose levels/schedule are evaluated, the SMC will undertake similar assessments.

[0348] In the Phase 3, there is no formal interim analysis planned for PFS. An interim efficacy analysis for OS is planned at the time of final analysis for PFS. Approximately 61% of the total OS events are expected to have occurred by the time of the interim analysis. The stopping boundary will be determined using Lan-DeMets spending functions with O'Brien and Fleming boundaries.

Analysis Methods

[0349] For Phase 2, "treatment group" will designate each dose level evaluated in the Phase 2 for analyses done in the All-Treated analysis set. For analyses using the Recommended Dose analysis set, 2 treatment groups will be presented: 1) subjects with HER2+ disease according to blood-based NGS assay treated at the paclitaxel recommended dose in the dose optimization stage or Cohort 2A, and 2) subjects who have HER2-negative disease according to blood-based NGS but HER2+ disease according to IHC/ISH assay of tumor biopsies treated at the paclitaxel recommended dose in the dose optimization stage or Cohort 2B.

[0350] For Phase 3, Arms 3A and 3B will be compared in the ITT analysis set and the Safety Analysis set; Arm 3C will be separately evaluated in these analysis sets.

[0351] In the Phase 2, efficacy will be summarized by treatment group in the Recommended Dose analysis set; safety will be summarized by treatment group in the All Treated analysis set. In the Phase 3, efficacy and PROs will be summarized by treatment group in the ITT analysis set; safety will be summarized by treatment group in the Safety Analysis set.

[0352] In the Phase 2, ORR, confirmed ORR, DOR, DCR and PFS per investigator will be summarized by treatment group. In the Phase 3, for the primary endpoints of PFS per investigator and OS, Arm 3A and Arm 3B will be compared using a 2-sided stratified log-rank test. Estimation of the hazard ratio will be based upon the stratified Cox regression model. PFS per investigator and OS will also be summarized using the Kaplan-Meier method, which will be used to estimate the time to event curves, including the median and milestone estimates. All subjects randomized to Arms 3A and 3B in the Phase 3 portion of the study will be included in the primary analysis of PFS and OS.

[0353] If only one of the two primary endpoints are statistically significant, the unused alpha can be passed to the other one. If both PFS per investigator and OS are statistically significant, then confirmed ORR per investigator among subjects with measurable disease in the Phase 3 portion will be formally compared between two treatment arms at the two-sided alpha level of 0.05, using a stratified Cochran-Mantel-Haenszel test.

[0354] The other secondary efficacy endpoints for the Phase 3 portion, including PFS, ORR per investigator, confirmed ORR per BICR, and DOR and DCR per BICR and per investigator, will

be summarized by treatment arm. The PFS per investigator, OS, and confirmed ORR per investigator will be summarized separately for Arm 3C.

[0355] In the Phase 3, assessments based on the EORTC-QLQ-C30, EORTC QLQ OG25, EQ 5D 5L and HCRU data will be summarized using descriptive statistics by treatment group for Arms 3A and 3B of the ITT set. PRO scores will be analyzed using longitudinal models. All subscales and individual item scores will be tabulated. Descriptive summaries of observed data at each scheduled assessment timepoint may be presented. Time to deterioration will be assessed in specific pre specified single items from either the EORTC QLQ-C30 or EORTC QLQ OG25; deterioration is defined as a 10-point increase from baseline in the symptom scales and a 10-point decrease from baseline for overall HRQoL.

[0356] Safety will be assessed through summaries of AEs, changes in laboratory test results, and changes in cardiac ejection fraction results. AEs will be classified by system organ class (SOC) and preferred term using the Medical Dictionary for Regulatory Activities (MedDRA); AE severities will be classified using the CTCAE version 5 criteria. All collected AE data will be listed.

[0357] Worst post-baseline laboratory values (hematology, coagulation, chemistry, and liver function) and change from baseline will be summarized. Abnormal laboratory values (relative to respective normal ranges) will be flagged in listings. The frequency and percentage of subjects with post-baseline clinically significant vital signs will be summarized. Cardiac ejection fraction data will be summarized for the all-treated subjects in Phase 2, by initial dose level, and for the Phase 3 by treatment group.

[0358] Extent of exposure for study drugs including frequency of dose holding, dose reductions, and dose discontinuations, as well as treatment compliance (percent of actual to planned dosing) will be summarized.

[0359] Statistical analysis methods for PK will include descriptive statistics on plasma concentrations and PK parameters, as well as exploratory analysis of the geometric mean ratios and 90% CIs of AUC_{last} and C_{max} for paclitaxel and its metabolites between cycle 1 day 1, cycle 1 day 8, and cycle 2 day 1, and exploratory analysis of the geometric mean ratios and 90% CIs of

tucatinib and ONT-993 between cycle 1 day 8 and cycle 2 day 1, and between subjects with and without gastrectomy.

Study Population

[0360] This study will enroll subjects with locally-advanced unresectable or metastatic HER2+ GEC who have received prior treatment with a HER2-directed antibody. Subjects will meet all of the enrollment criteria specified herein to be eligible for this study. Eligibility criteria may not be waived by the investigator and are subject to review in the event of a good clinical practice audit and/or health regulatory authority inspection.

Inclusion Criteria

1. Histologically or cytologically confirmed diagnosis of locally-advanced unresectable or metastatic HER2+ gastric or gastroesophageal junction adenocarcinoma
2. HER2+ disease at screening, as follows:
 - a. Phase 2 paclitaxel dose optimization stage: HER2 amplification in a blood-based NGS assay performed at a central laboratory or centrally-confirmed HER2 overexpression/amplification in a tumor biopsy obtained after progression on the most recent line of systemic therapy, evaluated following the package insert of FDA-approved tests for IHC and ISH (IHC3+ or IHC2+/ISH+)
 - b. Phase 2 dose expansion stage:
 - i. Cohort 2A: HER2 amplification in a blood-based NGS assay performed at a central laboratory
 - ii. Cohort 2B: centrally-confirmed HER2 overexpression/amplification in a tumor biopsy obtained after progression on the most recent line of systemic therapy, evaluated following the package insert of FDA-approved tests for IHC and ISH (IHC3+ or IHC2+/ISH+)
 - c. Phase 3: HER2 amplification in a blood-based NGS assay performed at a central laboratory
3. Can supply archival tumor tissue for central assay; if an archival sample is not available, the subject may be eligible, following approval by the medical monitor.
4. History of prior treatment with a HER2-directed antibody
5. Progressive disease during or after first-line therapy for locally-advanced unresectable or metastatic GEC

6. Phase 2: Measurable disease according to RECIST version 1.1
Phase 3: Measurable or non-measurable disease according to RECIST version 1.1
7. Age ≥ 18 years, or considered an adult by local regulations, at time of consent
8. Eastern Cooperative Oncology Group (ECOG) performance status score of 0 or 1
9. Life expectancy of at least 3 months, in the opinion of the investigator
10. Adequate hepatic function as defined by the following:
 - a. Total bilirubin $\leq 1.5 \times \text{ULN}$, except for subjects with known Gilbert's disease, who may enroll if the conjugated bilirubin is $\leq 1.5 \times \text{ULN}$
 - b. Transaminases (AST and ALT) $\leq 2.5 \times \text{ULN}$ ($\leq 5 \times \text{ULN}$ if liver metastases are present)
11. Adequate baseline hematologic parameters as defined by:
 - a. ANC $\geq 1.5 \times 10^3/\mu\text{L}$
 - b. Platelet count $\geq 100 \times 10^3/\mu\text{L}$; subjects with a stable platelet count from $75\text{--}100 \times 10^3/\mu\text{L}$ may be included with approval from the medical monitor
 - c. Hemoglobin ≥ 9 g/dL; subjects with hemoglobin $\geq 8\text{--}9$ g/dL may be included with approval from the Medical Monitor
 - d. In subjects transfused before study entry, transfusion must be ≥ 14 days prior to start of therapy to establish adequate hematologic parameters independent from transfusion support
12. Estimated glomerular filtration rate (GFR) ≥ 50 mL/min/1.73 m² using the Modification of Diet in Renal Disease (MDRD) study equation as applicable.
13. International normalized ratio (INR) ≤ 1.5 , and prothrombin time (PT) and partial thromboplastin time (PTT)/activated partial thromboplastin time (aPTT) $\leq 1.5 \times \text{ULN}$.
14. Left ventricular ejection fraction (LVEF) $\geq 50\%$ as assessed by echocardiogram or multi-gated acquisition scan (MUGA) documented within 4 weeks prior to first dose of study treatment.
15. Urinary protein of $\leq 1+$ on dipstick or routine urinalysis. If dipstick or routine analysis indicates proteinuria $\geq 2+$, then a 24-hour urine must be collected and must demonstrate <1000 mg of protein in 24 hours to allow participation in study
16. The subject must provide written informed consent
17. Subject must be willing and able to comply with study procedures, laboratory tests, and other requirements of the study

Exclusion Criteria

1. Subjects with squamous cell or undifferentiated GEC
2. Having received more than 1 line of prior systemic therapy for locally-advanced unresectable or metastatic disease
3. Having received taxanes ≤ 12 months prior to enrollment, prior treatment with ramucirumab, or prior treatment with tucatinib, lapatinib, neratinib, afatinib, or any other investigational anti-HER2 and/or anti-EGFR tyrosine kinase inhibitor, or with T-DM1, DS8201a, or any other HER2-directed antibody-drug conjugate
4. History of exposure to the following cumulative doses of anthracyclines:
 - e. Doxorubicin $> 360 \text{ mg/m}^2$
 - f. Epirubicin $> 720 \text{ mg/m}^2$
 - g. Mitoxantrone $> 120 \text{ mg/m}^2$
 - h. Idarubicin $> 90 \text{ mg/m}^2$
 - i. Liposomal doxorubicin (e.g. Doxil, Caelyx, Myocet) $> 550 \text{ mg/m}^2$
5. History of allergic reactions to trastuzumab, ramucirumab, paclitaxel, or compounds chemically or biologically similar to tucatinib, except for Grade 1 or 2 IRR to trastuzumab or ramucirumab that were successfully managed, or known allergy to any of the excipients in the study drugs or placebos
6. Phase 2 paclitaxel dose optimization stage only: history of prior partial or total gastrectomy
7. Treatment with any systemic anticancer therapy (including hormonal and biologic therapy), radiation, or an experimental agent, or participation in another interventional clinical trial ≤ 3 weeks prior to first dose of study treatment.
8. Major surgery within 28 days prior to enrollment or randomization, central venous access device placement within 7 days prior to enrollment or randomization, or planned major surgery following initiation of study treatment
9. Any toxicity related to prior cancer therapies that has not resolved to $\leq$ Grade 1, with the following exceptions:
 - Anemia
 - Alopecia

- Congestive heart failure (CHF), which must have been $\leq$ Grade 1 in severity at the time of occurrence, and must have resolved completely
10. Clinically significant cardiopulmonary disease such as:
- Ventricular arrhythmia requiring therapy
 - Symptomatic hypertension or uncontrolled asymptomatic hypertension $\geq 150/\geq 90$ mmHg despite standard medical management, as determined by the investigator
 - Any symptomatic history of CHF, left ventricular systolic dysfunction or decrease in ejection fraction
 - Severe dyspnea at rest (CTCAE Grade ≥ 3) due to complications of advanced malignancy or hypoxia requiring supplementary oxygen therapy, except when therapy is needed for obstructive sleep apnea
11. Known myocardial infarction or unstable angina within 6 months prior to first dose of study treatment
12. Known to be positive for hepatitis B by surface antigen expression. Known to be positive for hepatitis C infection (positive by polymerase chain reaction). Subjects who have been treated for hepatitis C infection are permitted if they have documented sustained virologic response of 12 weeks
13. Presence of known chronic liver disease
14. Phase 2: Known to be positive for human immunodeficiency virus (HIV)
Phase 3: Subjects known to be positive for HIV are excluded if they meet any of the following criteria:
- CD4+ T-cell count of < 350 cells/uL
 - Detectable HIV viral load
 - History of an opportunistic infection within the past 12 months
 - On stable antiretroviral therapy for < 4 weeks
15. Subjects who are pregnant, breastfeeding, or planning to become pregnant from time of informed consent until 7 months following the last dose of study drug

16. Have used a strong cytochrome P450 (CYP) 2C8 inhibitor within 5 half-lives of the inhibitor, or have used a strong CYP2C8 or CYP3A4 inducer within 5 days prior to first dose of study treatment.
17. History of malignancy other than GEC within 2 years prior to screening, with the exception of those with a negligible risk of metastasis or death (e.g., 5-year OS of $\geq 90\%$), such as adequately treated carcinoma in situ of the cervix, non-melanoma skin carcinoma, localized prostate cancer, ductal carcinoma in situ, or Stage I uterine cancer.
18. History of deep vein thrombosis, pulmonary embolism, or any other significant thromboembolism during the 3 months prior to enrollment or randomization.
19. Therapeutic anticoagulation with warfarin, low-molecular weight heparin or similar agents. Subjects receiving prophylactic, low-dose anticoagulation therapy are eligible provided that the coagulation parameters defined in the inclusion criteria ($[\text{INR} \leq 1.5 \text{ and } \text{PTT/aPTT} \leq 1.5 \text{ ULN}]$ or $[\text{PT} \leq 1.5 \text{ ULN and } \text{PTT/aPTT} \leq 1.5 \text{ ULN}]$) are met.
20. Chronic therapy with nonsteroidal anti-inflammatory agents (NSAIDs; e.g., indomethacin, ibuprofen, naproxen, or similar agents) or other anti-platelet agents (e.g., clopidogrel, ticlopidine, dipyridamole, anagrelide). Aspirin use at doses up to 325 mg/day is permitted.
21. Significant bleeding disorders, vasculitis, or had a significant bleeding episode from the gastrointestinal tract within 3 months prior to study entry.
22. History of any arterial thrombotic event, including myocardial infarction, unstable angina, cerebrovascular accident, or transient ischemic attack, within 6 months prior to enrollment or randomization.
23. History of gastrointestinal perforation and/or fistulae within 6 months prior to enrollment or randomization.
24. Serious non-healing wound or peptic ulcer or bone fracture within 28 days prior to enrollment or randomization
25. History of bowel obstruction, history or presence of inflammatory enteropathy or extensive intestinal resection (hemicolectomy or extensive small intestine resection with chronic diarrhea), Crohn's disease, ulcerative colitis or chronic diarrhea
26. Active or uncontrolled clinically serious infection

27. Known active central nervous system metastases. Irradiated or resected lesions are permitted, provided the lesions are fully treated and inactive, subject is asymptomatic, and no steroids have been administered for at least 30 days

Example 2: Combination of tucatinib and trastuzumab in colorectal cancer PDX models

[0361] In this example, the efficacy of tucatinib and trastuzumab was evaluated in PDX models of HER2 positive CRC. Mice were subcutaneously inoculated with CTG-0121, CTG-0784, or CTG-0383 cells, and subsequently treated with tucatinib, trastuzumab, or a combination of the two drugs (n = 10 per group). Tucatinib was administered orally at a dose of 50 mg/kg twice per day for 28 days (study days 0-27). Trastuzumab was administered intraperitoneally at a dose of 20 mg/kg once every three days. Nine doses of trastuzumab were administered, starting on study day 0. A vehicle-only group was included as a negative control.

[0362] As shown in FIGS. 5A-5C, both tucatinib and trastuzumab inhibited tumor growth in all three CRC PDX models. Furthermore, when a combination of the two drugs was administered, the inhibition of tumor growth was more pronounced than when either drug was used individually. In the CTG-0121 model, tucatinib, trastuzumab, and a combination of the two drugs produced tumor growth inhibition (TGI) indices of 104%, 109%, and 124%, respectively, at study day 29. In the CTG-0784 model, tucatinib, trastuzumab, and a combination of the two drugs produced TGI indices of 50%, 36%, and 103%, respectively, at study day 29. In the CTG-0383 model, tucatinib, trastuzumab, and a combination of the two drugs produced TGI indices of 117%, 80%, and 137%, respectively, at study day 29. Surprisingly, a synergistic effect was observed when a combination of the two drugs was administered in all three models. Of note, the activity of a combination of tucatinib and trastuzumab in each HER2 positive CRC PDX model was comparable to activity observed in a HER2 positive breast cancer model (BT-474).

Example 3: Combination of tucatinib and trastuzumab in esophageal cancer PDX model

[0363] In this example, the efficacy of tucatinib and trastuzumab was evaluated in PDX models of HER2 positive esophageal cancer. Mice were subcutaneously inoculated with CTG-0137 or CTG-0138 cells, and subsequently treated with tucatinib, trastuzumab, or a combination of the two drugs (n = 10 per group). Tucatinib was administered orally at a dose of 50 mg/kg twice per day

for 28 days (study days 0-27). Trastuzumab was administered intraperitoneally at a dose of 20 mg/kg once every three days. Nine doses of trastuzumab were administered, starting on study day 0. A vehicle-only group was included as a negative control.

[0364] In the CTG-0137 model, both tucatinib and trastuzumab inhibited tumor growth, exhibiting TGI indices at study day 15 of 49% and 55%, respectively (FIG. 6A). Furthermore, a synergistic effect was observed when a combination of the two drugs was administered, producing a TGI index of 85%.

[0365] In the CTG-0138 model, tucatinib inhibited tumor growth when administered as a single agent, producing a TGI index of 69% at study day 30 (FIG. 6B). However, a synergistic effect was observed when tucatinib and trastuzumab were administered in combination, producing a TGI index of 120%.

Example 4: Combination of tucatinib and trastuzumab in gastric cancer PDX models

[0366] In this example, the efficacy of tucatinib and trastuzumab was evaluated in PDX models of HER2 positive gastric cancer. Mice were subcutaneously inoculated with GXA 3038, GXA 3039, or GXA 3054 cells, and subsequently treated with tucatinib, trastuzumab, or a combination of the two drugs (n = 10 per group). Tucatinib was administered orally at a dose of 50 mg/kg twice per day for 28 days (study days 0-27). Trastuzumab was administered intraperitoneally at a dose of 20 mg/kg once every three days. Nine doses of trastuzumab were administered, starting on study day 0. A vehicle-only group was included as a negative control.

[0367] As shown in FIGS. 7A-7C, both tucatinib and trastuzumab inhibited tumor growth in all three gastric cancer PDX models. Furthermore, when a combination of the two drugs was administered, the inhibition of tumor growth was more pronounced than when either drug was used individually. In the GXA-3038 model, tucatinib, trastuzumab, and a combination of the two drugs produced TGI indices of 110%, 50%, and 116%, respectively, at study day 28. In the GXA-3039 model, tucatinib, trastuzumab, and a combination of the two drugs produced TGI indices of 48%, 38%, and 103%, respectively, at study day 29. In the GXA-3054 model, tucatinib, trastuzumab, and a combination of the two drugs produced TGI indices of 65%, 93%, and 136%, respectively, at study day 17. Surprisingly, a synergistic effect was observed when a combination of the two drugs was administered in all three models.

Example 5: Combination of tucatinib and trastuzumab in a cholangiocarcinoma PDX model

[0368] In this example, the efficacy of tucatinib and trastuzumab was evaluated in a PDX model of HER2 positive cholangiocarcinoma. Mice were subcutaneously inoculated with CTG-0927 cells and subsequently treated with tucatinib, trastuzumab, or a combination of the two drugs (n = 10 per group). Tucatinib was administered orally at a dose of 50 mg/kg twice per day for 28 days (study days 0-27). Trastuzumab was administered intraperitoneally at a dose of 20 mg/kg once every three days. Nine doses of trastuzumab were administered, starting on study day 0. A vehicle-only group was included as a negative control.

[0369] As shown in FIG. 8 both tucatinib and trastuzumab inhibited tumor growth. Furthermore, when a combination of the two drugs was administered, the inhibition of tumor growth was more pronounced than when either drug was used individually. At study day 28, the TGI indices for the tucatinib, trastuzumab, and combination therapy groups were 48%, 63%, and 86%, respectively.

Example 6: Combination of tucatinib and trastuzumab in NSCLC model

[0370] In this example, the efficacy of tucatinib and trastuzumab was evaluated in two different models of HER2 positive NSCLC. For these two studies, Calu-3 and NCI-H2170 cells were used, both of which express high levels of HER2, have gene amplification comparable to that of BT-474 breast cancer cells, and have previously demonstrated good responses to tucatinib *in vitro*.

[0371] Mice were subcutaneously inoculated with Calu-3 or NCI-H2170 cells and subsequently treated with tucatinib, trastuzumab, or a combination of the two drugs (n = 10 per group). For the Calu-3 study, tucatinib was administered orally at a dose of 50 mg/kg twice per day for 21 days, beginning on study day 7. Trastuzumab was administered intraperitoneally at a dose of 20 mg/kg once every three days, beginning on study day 7. Seven doses of trastuzumab were administered. A vehicle-only group was included as a negative control. Three individual animals received dose holidays (one in the negative control group and two in the combination therapy group).

[0372] For the NCI-H2170 study, tucatinib was administered orally at a dose of 50 mg/kg twice per day for 21 days, beginning on study day 18. Trastuzumab was administered intraperitoneally at a dose of 20 mg/kg twice per week, beginning on study day 18. A vehicle-only group was included as a negative control.

[0373] As shown in FIGS. 9A and 9B and Table 15, both tucatinib and trastuzumab inhibited tumor growth in both NSCLC models. Furthermore, when a combination of the two drugs was administered, the inhibition of tumor growth was more pronounced than when either drug was used individually. For the Calu-3 model, tucatinib, trastuzumab, and a combination of the two drugs produced tumor growth inhibition (TGI) indices of 63%, 86%, and 100%, respectively, at study day 28. Surprisingly, a synergistic effect was observed in the combination therapy group. For the NCI-2170 model, tucatinib, trastuzumab, and a combination of the two drugs produced TGI indices of 91%, 61%, and 98%, respectively, at study day 39.

Table 15

Tumor name	Cancer Type	Type of Xeno-graft	Vendor	Observed TGI (%)			Predicted % TGI
				Tucatinib	Trastuzumab	Tucatinib + Trastuzumab	Tucatinib + Trastuzumab
Calu-3	NSCLC	CDX	BioDuro	63	86	100	95
NCH-H2170	NSCLC	CDX	In house	91	61	98	97
CTG-0121	CRC	PDX	Champions Oncology	104	109	124	100
CTG-0784	CRC	PDX	Champions Oncology	50	36	103	68
CTG-0383	CRC	PDX	Champions Oncology	117	80	137	103
CTG-0137	Esophageal	PDX	Champions Oncology	49	55	85	77
CTG-0138	Esophageal	PDX	Champions Oncology	69	-34	120	59
CTG-0927	Cholangio-carcinoma	PDX	Champions Oncology	48	63	86	81
GXA-3038	Gastric carcinoma (Asian)	PDX	Oncotest	110	50	116	105
GXA-3039	Gastric carcinoma (Asian)	PDX	Oncotest	48	38	103	68

GXA-3054	Gastric carcinoma (Asian)	PDX	Oncotest	65	93	136	98
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CLAIMS

1. A method of treating a HER2 positive cancer in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a vascular endothelial growth factor receptor 2 (VEGFR-2) antagonist.
2. A method of treating cancer in a subject in need thereof, the method comprising:
 - (a) identifying the subject as having a HER2 positive cancer; and
 - (b) administering to the subject a therapeutically effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a vascular endothelial growth factor receptor 2 (VEGFR-2) antagonist.
3. The method of any one of claims 1 or 2, wherein the trastuzumab is administered to the subject at a dose of about 6 mg/kg.
4. The method of any one of claims 1 or 2, wherein the trastuzumab is administered to the subject at a dose of about 4 mg/kg.
5. The method of any one of claims 1-4, wherein the tucatinib is administered to the subject at a dose of about 150 mg to about 650 mg.
6. The method of any one of claims 1-5, wherein the tucatinib is administered twice daily.
7. The method of any one of claims 1-6, wherein the tucatinib is administered to the subject orally.
8. The method of any one of claims 1-7, wherein the VEGFR-2 antagonist is selected from the group consisting of bevacizumab, ramucirumab, aflibercept, cetuximab, panitumumab, regorafenib, sunitinib, sorafenib, pazopanib, vandetanib, axitinib, cediranib, vatalanib,

motesanib, lucatinib, intedanib, semaxanib, apatinib, lenvatinib, carbozantinib, and a combination thereof.

9. The method of any one of claims 1-8, wherein the VEGFR-2 antagonist is a monoclonal antibody selected from the group consisting of bevacizumab, ramucirumab, aflibercept, cetuximab, panitumumab, and combinations thereof.
10. The method of any one of claims 1-9, wherein the VEGFR-2 antagonist is ramucirumab.
11. The method of claim 10, wherein the ramucirumab is administered to the subject at a dose of about 8 mg/kg.
12. The method of any one of claims 1-11 wherein the taxane is selected from the group consisting of paclitaxel, docetaxel, cabazitaxel, larotaxel, BMS-184476, BMS-188797, BMS-275183, milataxel, ortaxel, TL-310, docosahexaenoic acid-paclitaxel (DHA-paclitaxel), nab paclitaxel, EndoTAG+paclitaxel, XRP9881, polymeric-micellar paclitaxel, RPR-109881A, a pharmaceutically acceptable salt or solvate thereof, and a combination thereof.
13. The method of any one of claims 1-12, wherein the taxane is paclitaxel.
14. The method of claim 13, wherein the paclitaxel is administered to the subject at a dose of about 50 mg/m² to about 100 mg/m².
15. The method of any one of claims 12-14, wherein the paclitaxel is administered to the subject at a dose of about 80 mg/m².
16. The method of any one of claims 1-15, wherein the HER2 positive cancer is selected from the group consisting of gastric adenocarcinoma, gastroesophageal junction (GEC) adenocarcinoma, esophageal adenocarcinoma, colorectal carcinoma (CRC), cholangiocarcinoma, gallbladder carcinoma, gastric cancer, lung cancer, biliary cancers, bladder cancer, esophageal

cancer, melanoma, ovarian cancer, liver cancer, prostate cancer, pancreatic cancer, small intestine cancer, non-small cell lung cancer, head and neck cancer, uterine cancer, cervical cancer, brain cancer, and breast cancer.

17. The method of any one of claims 1-16, wherein the HER2 positive cancer is gastric adenocarcinoma.

18. The method of any one of claims 1-17, wherein the HER2 positive cancer is gastroesophageal junction (GEC) adenocarcinoma.

19. The method of any one of claims 1-18, wherein the HER2 positive cancer is unresectable or metastatic.

20. The method of any one of claims 1-19, wherein the subject has been previously treated with a HER2-directed antibody.

21. The method of any one of claims 1-20, wherein the subject has not been previously treated with an anti-HER2 and/or an anti-EGFR tyrosine kinase inhibitor.

22. The method of any one of claims 1-21, wherein the subject has not been previously treated with a HER2-directed antibody-drug conjugate.

23. The method of claim 21, wherein the wherein the anti-HER2/EGFR tyrosine kinase inhibitor is selected from the group consisting of tucatinib, lapatinib, neratinib, or afatinib.

24. The method of claim 22, wherein antibody-drug conjugate is selected from the group consisting of ado-trastuzumab (T-DM1) or trastuzumab deruxtecan (DS8201a).

25. The method of any one of claims 1-24, wherein the subject has not been previously treated with an anthracycline.

26. The method of claim 25, wherein the anthracycline is selected from the group consisting of doxorubicin, epirubicin, mitoxantrone, idarubicin, liposomal doxorubicin, and combinations thereof.

27. The method of any one of claims 1-26, wherein the subject was previously treated with at least one anticancer therapy.

28. The method of claim 27, wherein the at least one anticancer therapy is selected from the group consisting of trastuzumab, lapatinib, trastuzumab and a taxane, pertuzumab, and combinations thereof.

29. The method of any one of claims 27 or 28, wherein the subject is refractory to the at least one anticancer therapy.

30. The method of any one of claims 27-29, wherein the subject developed a brain metastasis during the previous treatment with the at least one anticancer therapy.

31. A method for treating a HER2 positive cancer in a subject that has exhibited an adverse event after starting treatment with a combination therapy comprising tucatinib, trastuzumab, a taxane, and a vascular endothelial growth factor receptor 2 (VEGFR-2) antagonist at an initial dosage level, comprising administering to the subject at least one component of the combination therapy at a reduced dosage level.

32. The method of claim 31, wherein the taxane is selected from the group consisting of paclitaxel, docetaxel, cabazitaxel, larotaxel, BMS-184476, BMS-188797, BMS-275183, milataxel, ortaxel, TL-310, docosahexaenoic acid-paclitaxel (DHA-paclitaxel), nab paclitaxel, EndoTAG+paclitaxel, XRP9881, polymeric-micellar paclitaxel, RPR-109881A, a pharmaceutically acceptable salt or solvate thereof, and a combination thereof.

33. The method of any one of claims 31 or 32, wherein the taxane is paclitaxel.
34. The method of claim 33, wherein the paclitaxel is administered to the subject at an initial dose of about 50 mg/m² to about 100 mg/m².
35. The method of any one of claims 33 or 34, wherein the paclitaxel is administered to the subject at an initial dose of about 80 mg/m².
36. The method of any one of claims 33-35, wherein the paclitaxel is administered to the subject at a reduced dose of about 50 mg/m² to about 75 mg/m².
37. The method of any one of claims 33-36 wherein the paclitaxel is administered to the subject at a reduced dose of about 70 mg/m².
38. The method of any one of claims 33-36, wherein the paclitaxel is administered to the subject at a reduced dose of about 60 mg/m².
39. The method of claim 31-38, wherein the tucatinib is administered to the subject at an initial dose of about 150 mg to about 650 mg.
40. The method of claim 31-39, wherein the tucatinib is administered to the subject at an initial dose of about 300 mg.
41. The method of any one of claims 31-40, wherein the tucatinib is administered to the subject at a reduced dose of about 125 mg to about 275 mg.
42. The method of any one of claims 31-41, wherein the VEGFR-2 antagonist is selected from the group consisting of bevacizumab, ramucirumab, aflibercept, cetuximab, panitumumab, regorafenib, sunitinib, sorafenib, pazopanib, vandetanib, axitinib, cediranib, vatalanib,

motesanib, lucatinib, intedanib, semaxanib, apatinib, lenvatinib, carbozantinib, and a combination thereof.

43. The method of any one of claims 31-42, wherein the VEGFR-2 antagonist is ramucirumab.

44. The method of any one of claims 42 or 43, wherein the ramucirumab is administered to the subject at an initial dose of about 8 mg/kg.

45. The method of any one of claims 42-44, wherein the ramucirumab is administered to the subject at a reduced dose of about 6 mg/kg.

46. The method of any one of claims 42-44, wherein the ramucirumab is administered to the subject at a reduced dose of about 5 mg/kg.

47. A method of treating a HER2 positive cancer in a subject in need thereof, the method comprising:

- (a) administering to the subject a therapeutically effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a vascular endothelial growth factor receptor 2 (VEGFR-2) antagonist; and
- (b) administering an effective amount of an anti-diarrheal agent.

48. A method of reducing the severity or incidents of diarrhea, or preventing diarrhea in a subject having a HER2 positive cancer and being treated with an effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a vascular endothelial growth factor receptor 2 (VEGFR-2) antagonist, the method comprising administering an effective amount of an anti-diarrheal agent prophylactically.

49. A method of reducing the likelihood of a subject developing diarrhea, wherein the subject has a HER2 positive cancer and is being treated with an effective amount of a combination therapy comprising tucatinib, trastuzumab, a taxane, and a vascular endothelial growth factor

receptor 2 (VEGFR-2) antagonist the method comprising administering an effective amount of an anti-diarrheal agent prophylactically.

50. The method of any one of claims 47-49, wherein the combination therapy and the anti-diarrheal agent are administered concurrently.

51. The method of any one of claims 47-49, wherein the anti-diarrheal agent is administered prior to administration of the combination therapy.

52. The method of any one of claims 47-51, wherein the subject is exhibiting symptoms of diarrhea.

53. The method of any one of claims 47-51, wherein the subject is not exhibiting symptoms of diarrhea.

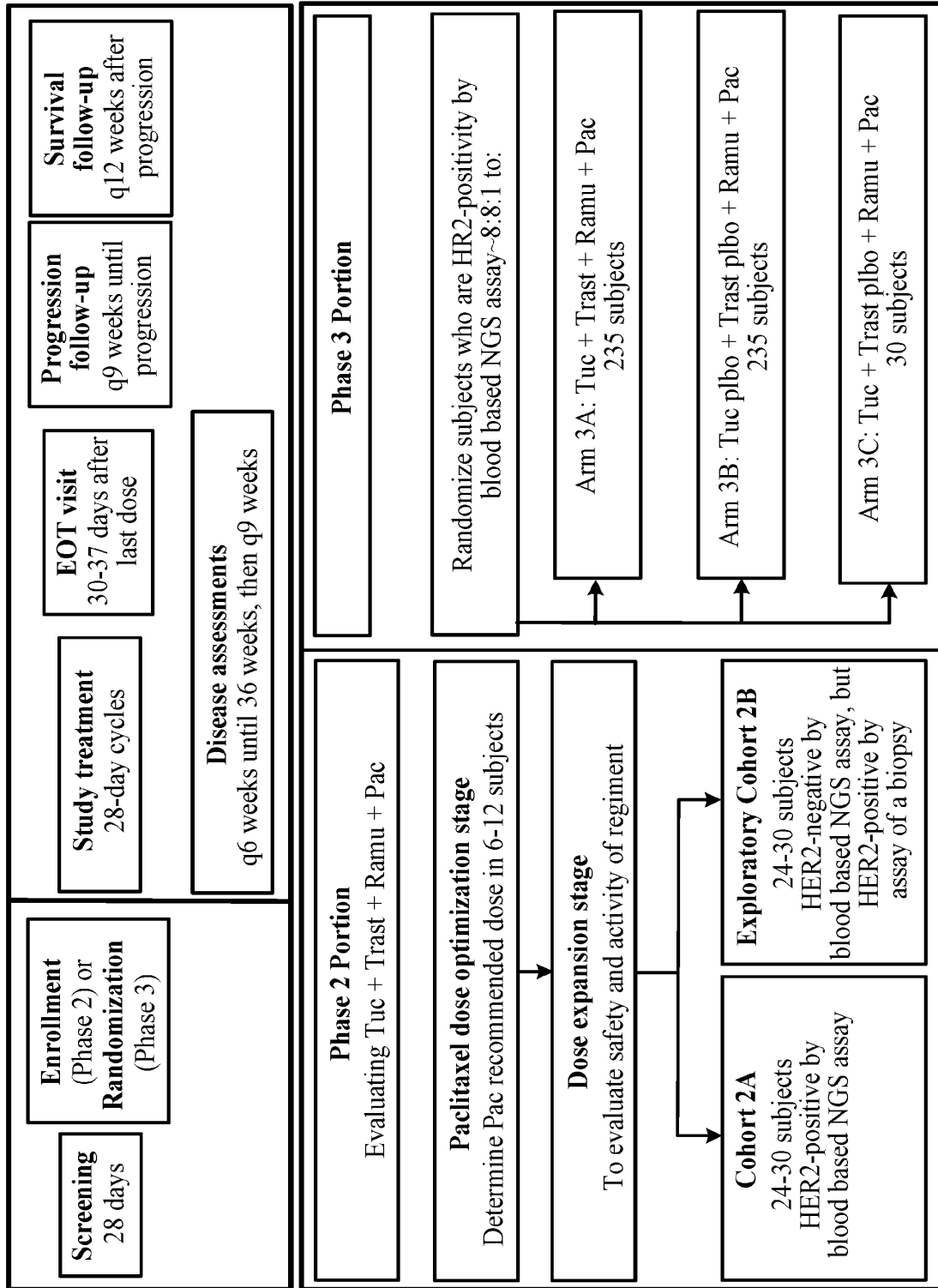
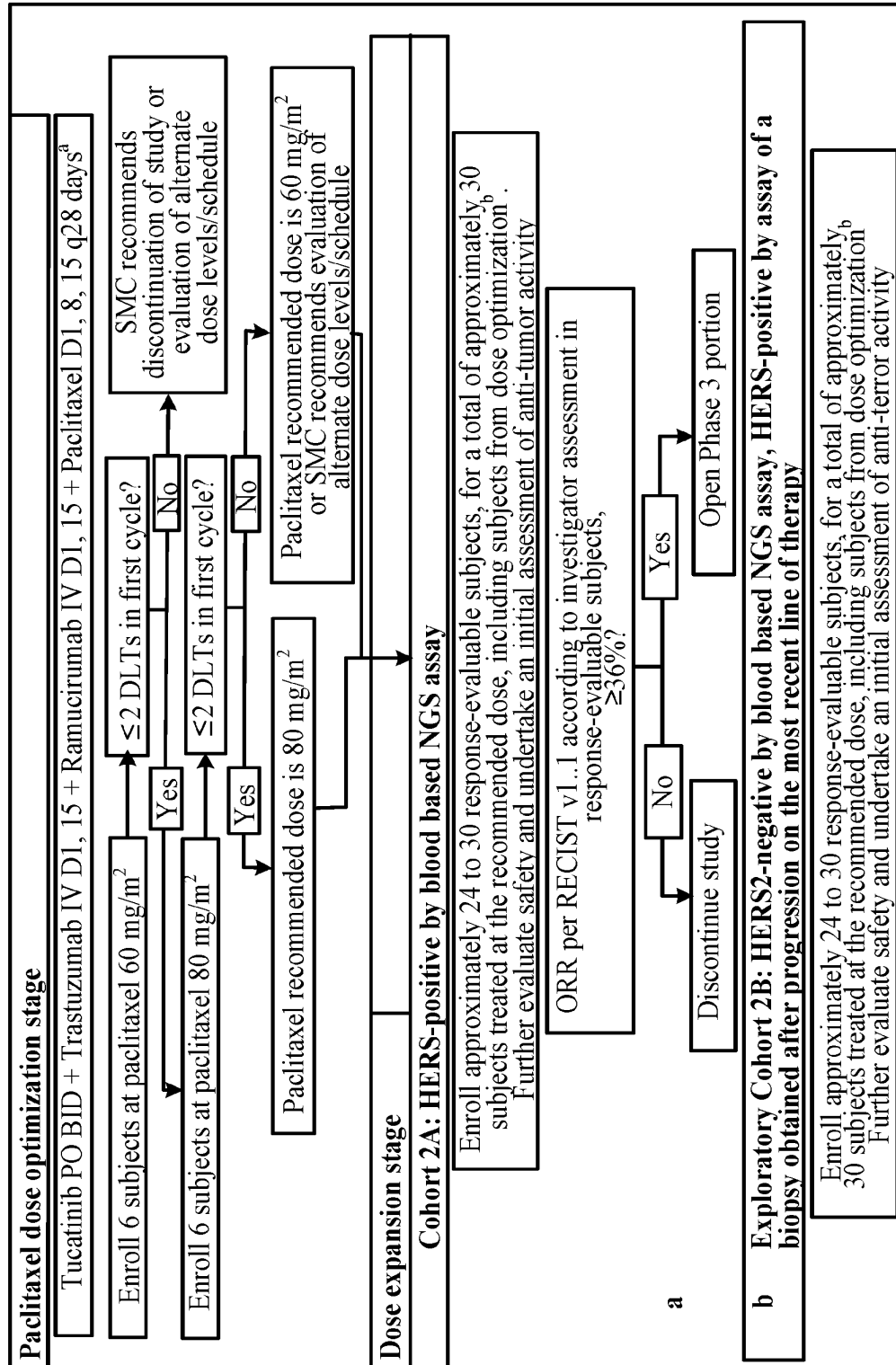


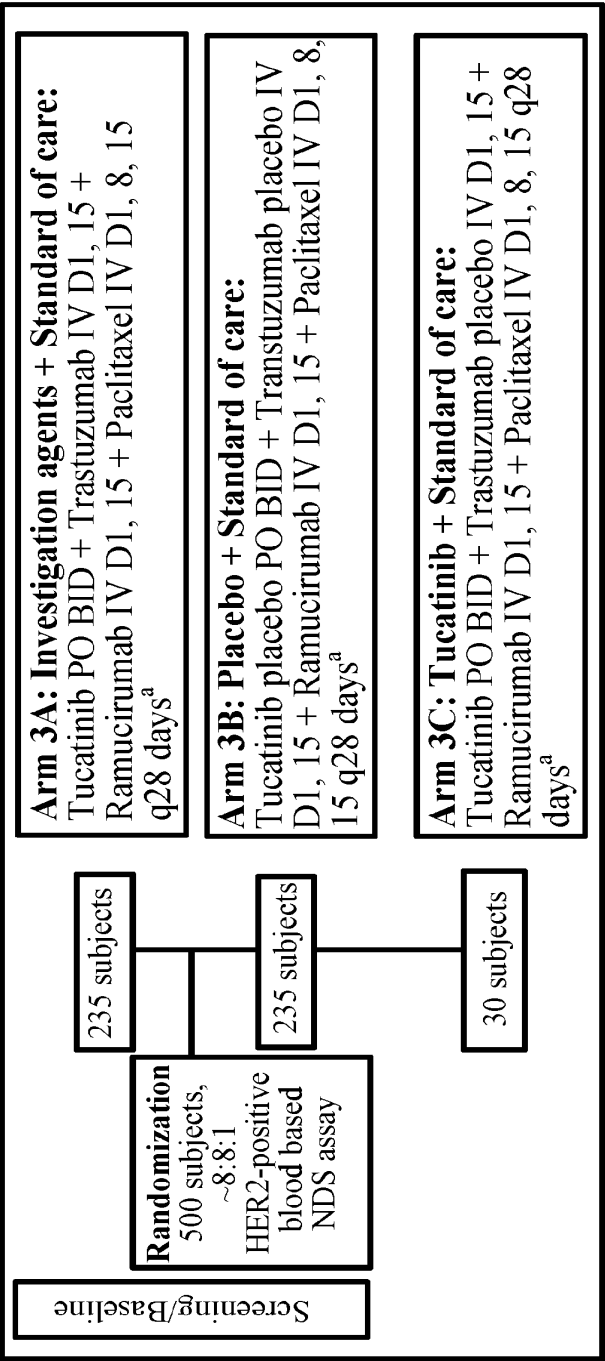
FIG. 1

Pac=paclitaxel; plbo=placebo; Ramu=ramucirumab; Tuc=tucatinib; Trast=trastuzumab.



Tucatinib will be administered at 300 mg BID PO; trastuzumab 6 mg/kg will be administered on Cycle 1 day 1, then 4 mg/kg in subsequent infusions; ramucirumab will be administered at a dose of 8 mg/kg per infusion; paclitaxel will be administered at a dose of 60 or 80 mg/m² per infusion, or an alternate dose/schedule upon recommendation of the SMC. Subjects evaluable for response will have a baseline disease assessment, receive study treatment, and have a Post-baseline assessment or discontinue treatment due to documented disease progression, clinical, progression, Treatment-related AE(s), or death.

FIG. 2



a. Tucatinib will be administered at 300 mg BID PO; trastuzumab 6 mg/kg will be administered on Cycle 1 day 1, then 4 mg/kg in subsequent infusions; ramucirumab will be administered at a dose of 3 mg/kg per infusion; paclitaxel will be administered at the recommended dose and schedule determined in the Phase 2 in Arms 3A and 3C and at 80 mg/m² in Arm 3B.

FIG. 3

SEQ. ID NO. 1 (trastuzumab heavy chain):

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT
RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT
LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP
AVLQSSGLYSLSVVTVPSSSLGTQYICNVNHHKPSNTKVDKKVEPKSCDKTHTCPPCPA
PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYCKVSNKALPAPIEKTISKAKGQPREPQV
YTLPPSRREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYS
KLTVDKSRWQQGNVVFSCSVMHAEALHNHYTQKSLSLSPG

SEQ. ID NO. 2 (trastuzumab light chain):

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVP
SRFSGRSRGTDFLTITSLQPEDFATYYCQHHYTPPTFGQGTKEIKRTVAAPSVFIFPPS
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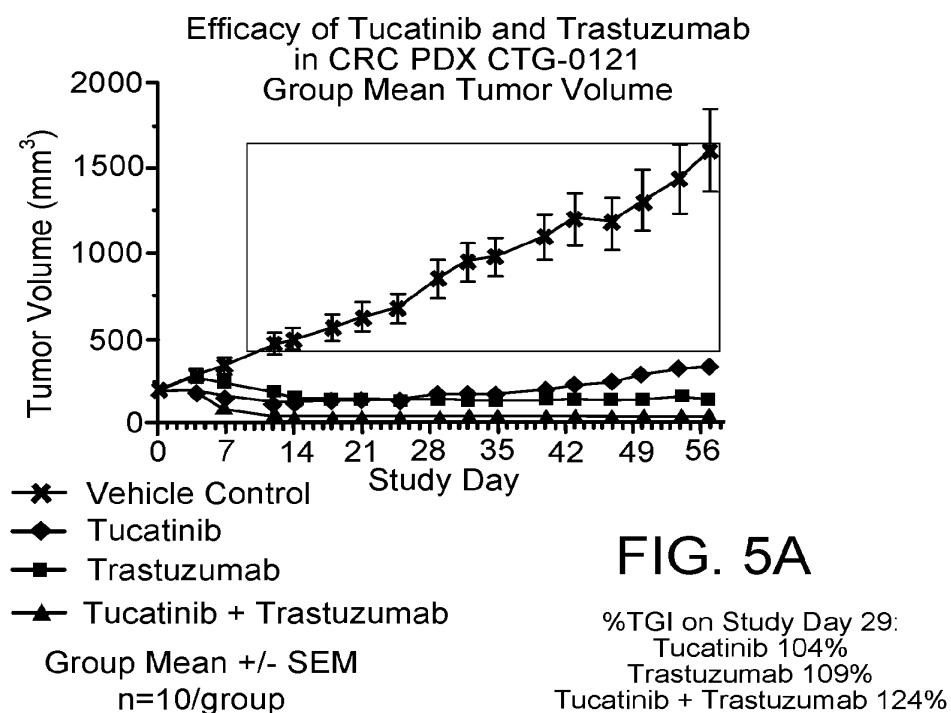
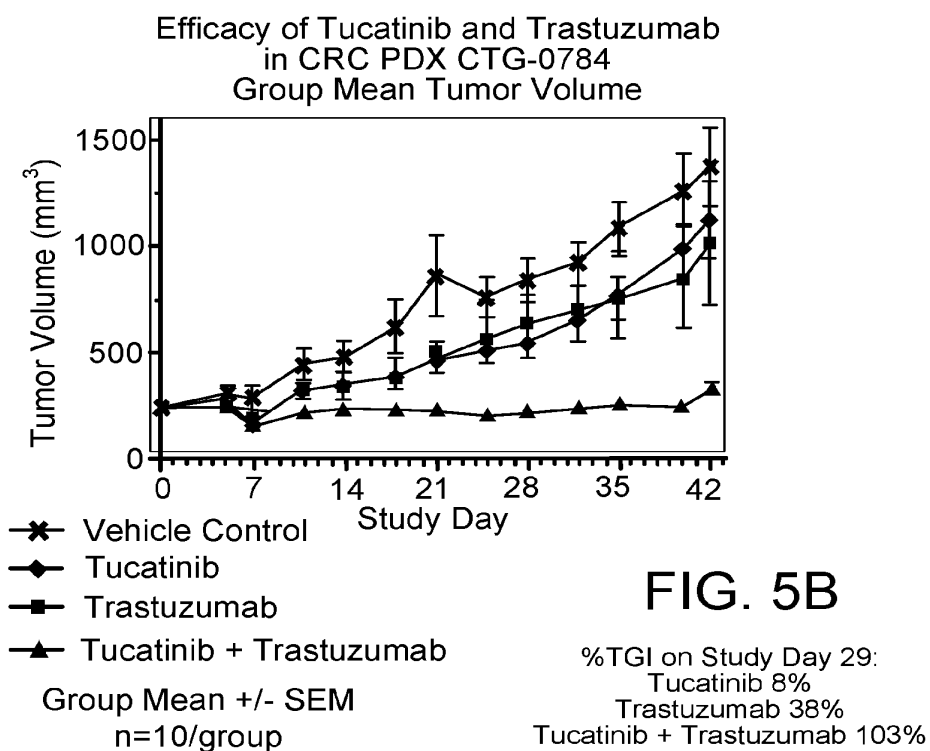
SEQ. ID NO. 3 (trastuzumab light chain variable domain):

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLESGVPS
RFSGRSRGTDFLTITSLQPEDFATYYCQHHYTPPTFGQGTKEIKRT

SEQ. ID NO. 4 (trastuzumab heavy chain variable domain):

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT
RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT
LVTVSS

FIG. 4



SUBSTITUTE SHEET (RULE 26)

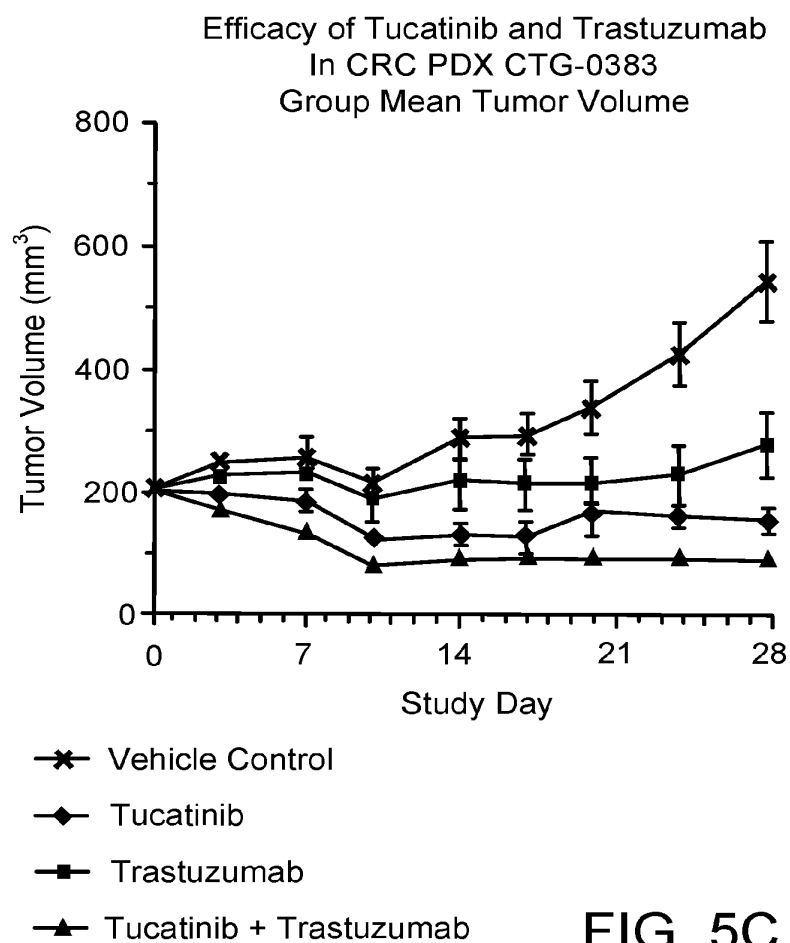
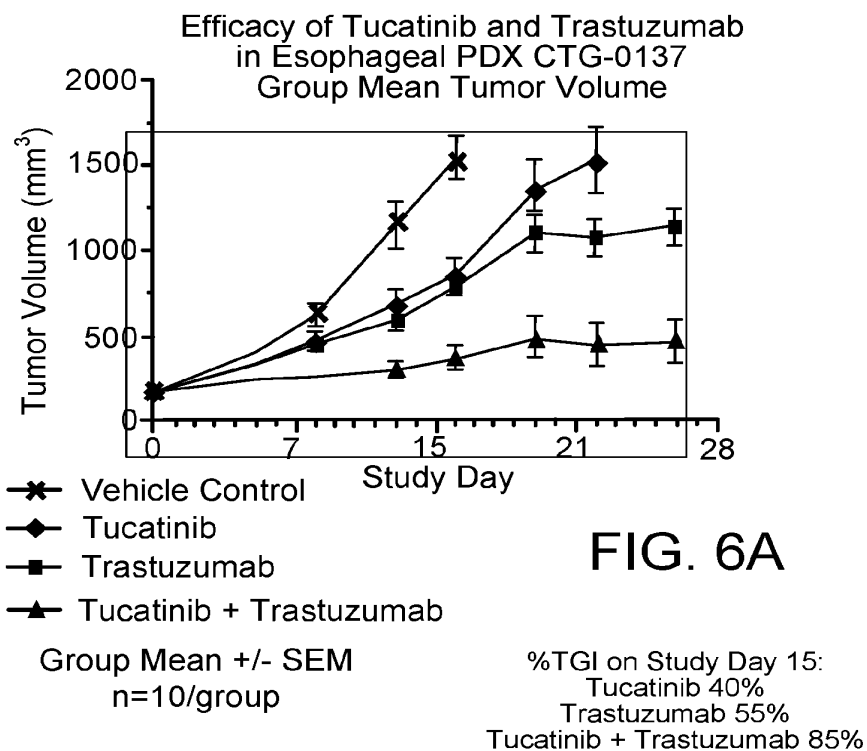
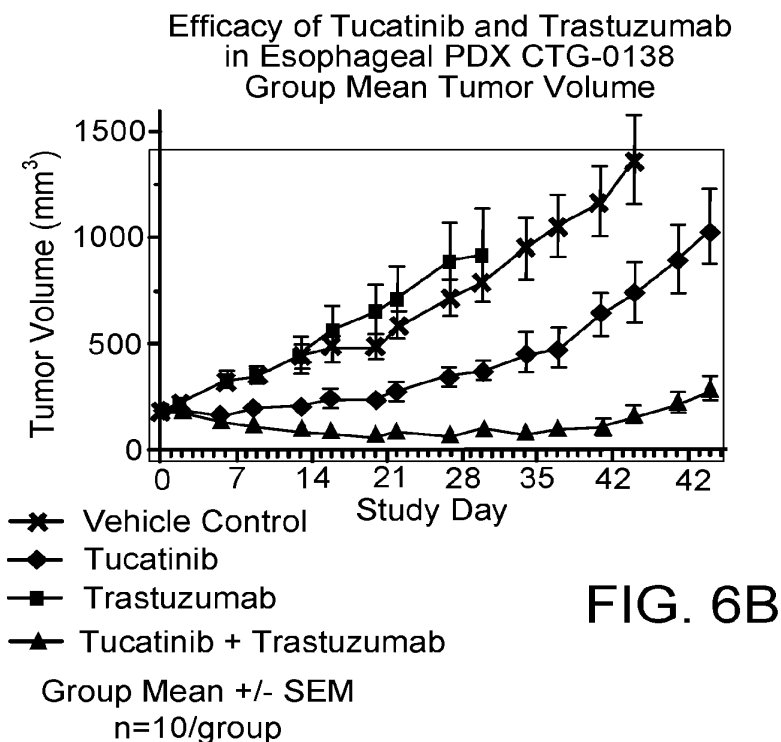


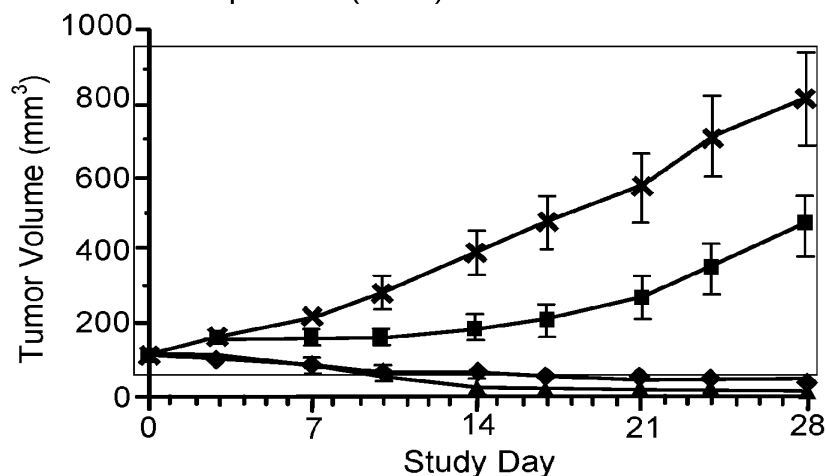
FIG. 5C

Group Mean +/- SEM
n=10/group



SUBSTITUTE SHEET (RULE 26)

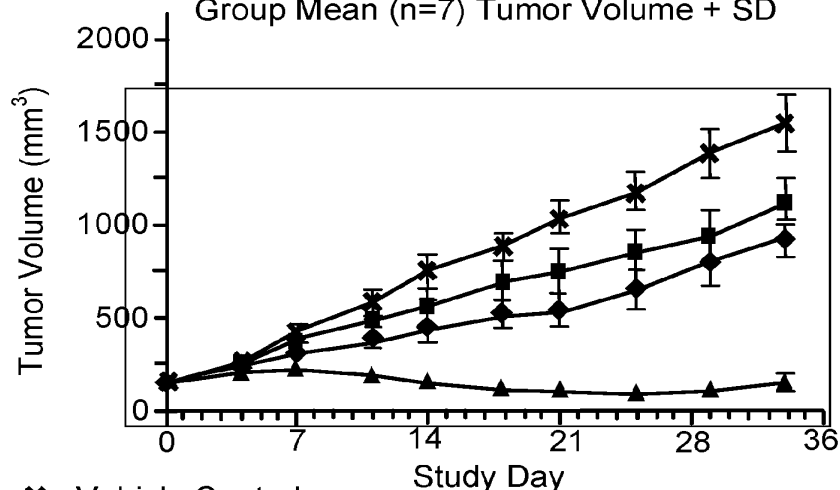
Effect on Tumor Growth of Tucatinib and Trastuzumab
in Gastric Cancer PDX GXA 3038
Group Mean (n=10) Tumor Volume + SD



- x— Vehicle Control
- ◆— Tucatinib: 50 mg/kg/dose, PO, BID x 28
- Trastuzumab: 20 mg/kg/dose, IP, QxD x 9
- ▲— Tucatinib + Trastuzumab

FIG. 7A

Effect on Tumor Growth of Tucatinib and Trastuzumab
in Gastric Cancer PDX GXA 3039
Group Mean (n=7) Tumor Volume + SD



- x— Vehicle Control
- ◆— Tucatinib: 50 mg/kg/dose, PO, BID x 28
- Trastuzumab: 20 mg/kg/dose, IP, QxD x 9
- ▲— Tucatinib + Trastuzumab

FIG. 7B

SUBSTITUTE SHEET (RULE 26)

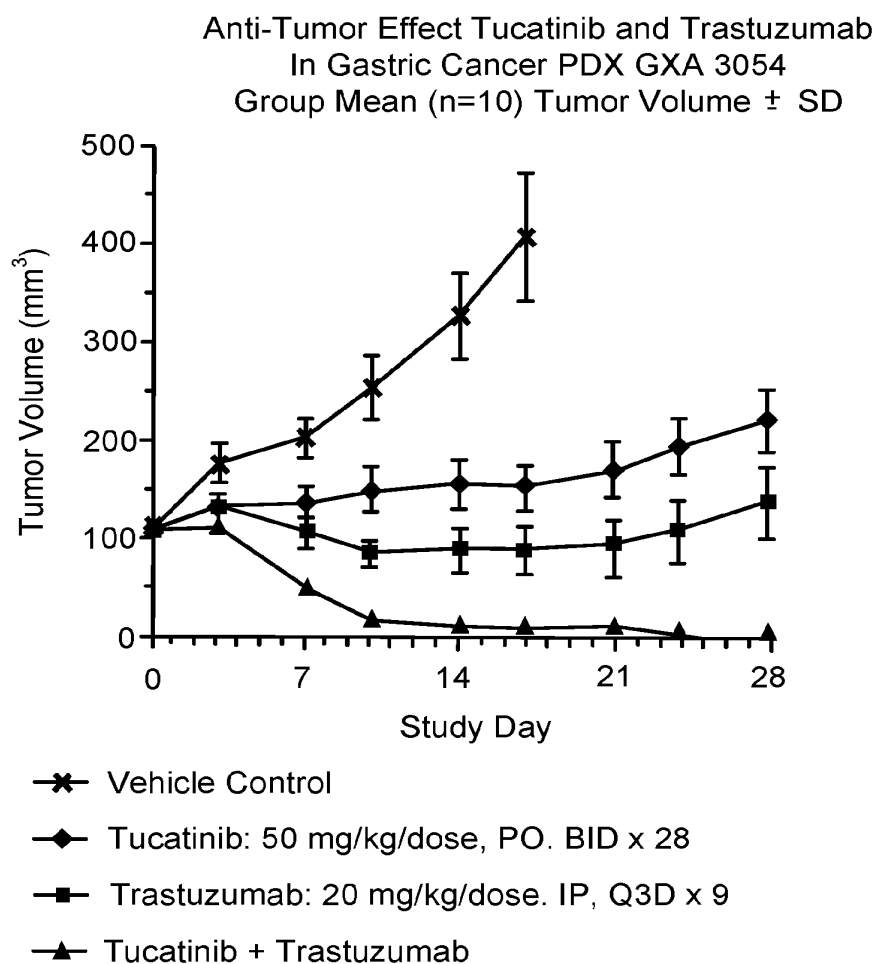


FIG. 7C

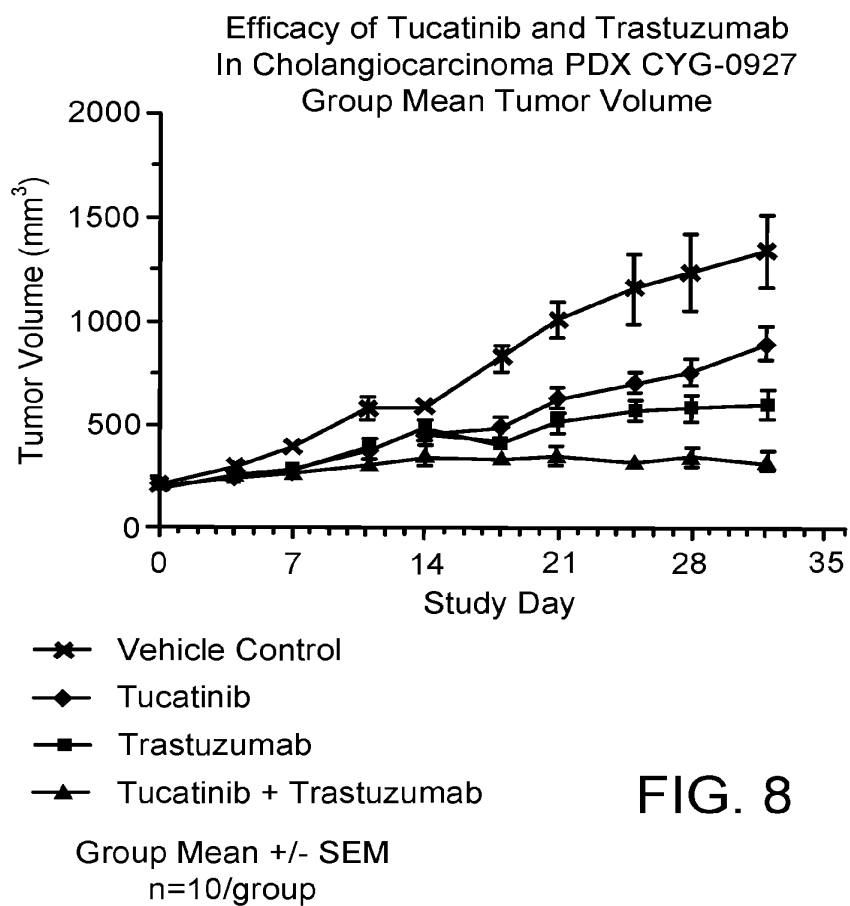


FIG. 8

Efficacy of Tucatinib and Trastuzumab
in Calu-3 Her2+ NSCLC Xenograft Model
Group Mean Tumor Volume

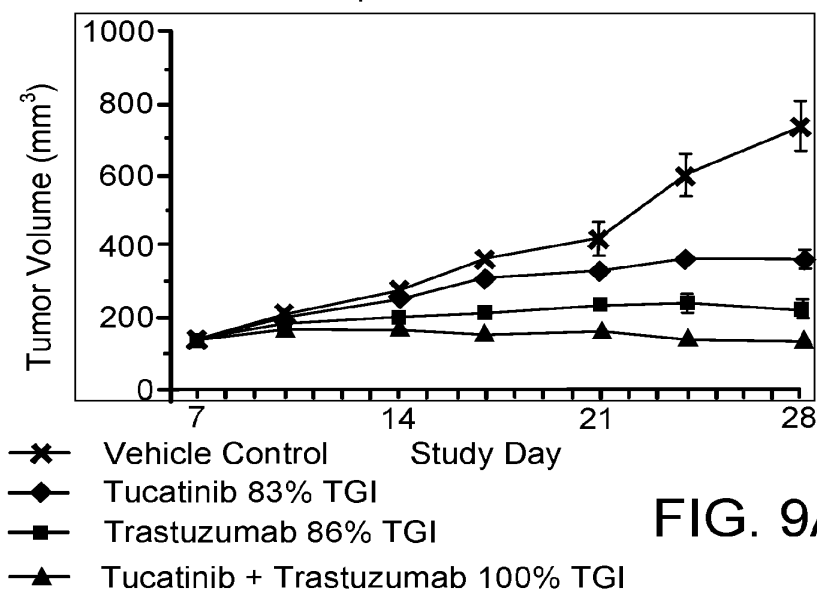


FIG. 9A

Efficacy of Tucatinib and Trastuzumab
in NCI-H2170 Her2+ NSCLC Xenograft Model
Group Mean Tumor Volume

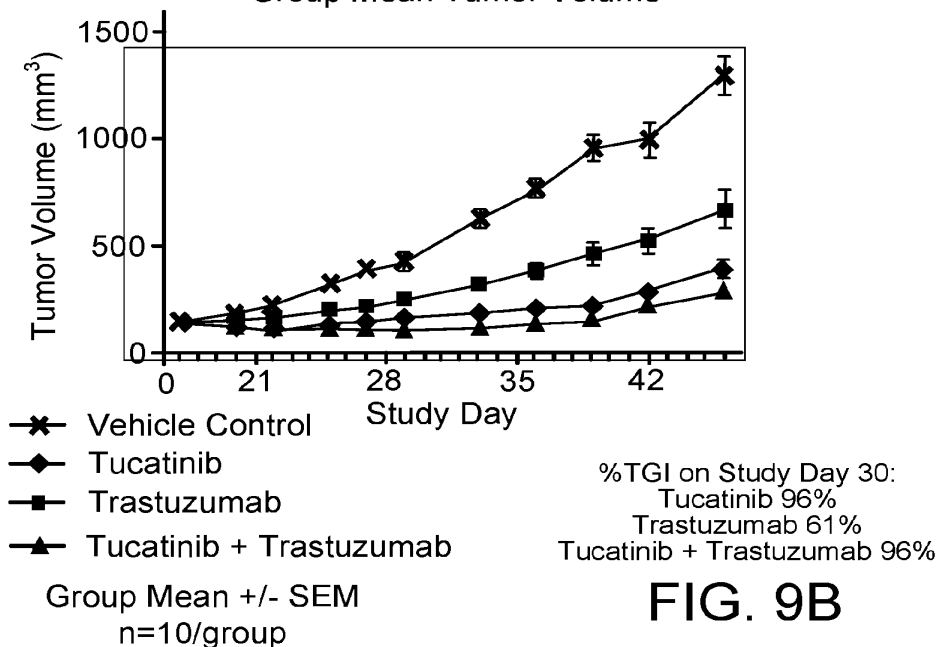


FIG. 9B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2021/043395

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/517 A61K39/395 A61K39/00 C07K16/32 A61P35/00
C07K16/28

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, EMBASE, BIOSIS, COMPENDEX, FSTA, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEE P ET AL: "Abstract nr 5104: In Vivo Activity of ARRY-380, a Potent, Small Molecule Inhibitor of ErbB2 in Combination with Trastuzumab, Docetaxel or Bevacizumab", THIRTY-SECOND ANNUAL CTSC-AACR SAN ANTONIO BREAST CANCER SYMPOSIUM / CANCER RESEARCH, vol. 69, no. (24 Suppl), 1 December 2009 (2009-12-01), pages 1-1, XP055863232, DOI: 10.1158/0008-5472.SABCS-09-5104 the whole document ----- -/--	1-53

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 November 2021

Date of mailing of the international search report

26/11/2021

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Gall-Truchot, A

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2021/043395

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KULUKIAN ANITA ET AL: "Preclinical Activity of HER2-Selective Tyrosine Kinase Inhibitor Tucatinib as a Single Agent or in Combination with Trastuzumab or Docetaxel in Solid Tumor Models", MOLECULAR CANCER THERAPEUTICS, vol. 19, no. 4, 1 April 2020 (2020-04-01), pages 976-987, XP055863296, US ISSN: 1535-7163, DOI: 10.1158/1535-7163.MCT-19-0873 the whole document abstract figures 3-5	1-53
X	----- WO 2013/170263 A2 (MERRIMACK PHARMACEUTICALS INC [US]) 14 November 2013 (2013-11-14) the whole document abstract claims 1, 3, 23-24 -----	1-53

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2021/043395

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2021/043395

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013170263 A2	14-11-2013	AU 2013259053 A1	15-01-2015
		CA 2873111 A1	14-11-2013
		CN 104755497 A	01-07-2015
		EP 2847226 A2	18-03-2015
		JP 2015520153 A	16-07-2015
		KR 20150030199 A	19-03-2015
		US 2015139936 A1	21-05-2015
		WO 2013170263 A2	14-11-2013

Form PCT/ISA/210 (patent family annex) (April 2005)



(51) International Patent Classification:

A61K 31/395 (2006.01) A61K 31/517 (2006.01)
A61K 31/444 (2006.01) A61K 45/06 (2006.01)
A61K 31/47 (2006.01) A61P 35/00 (2006.01)

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(25) Filing Language:

English

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English

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10 2020 005 002.6

17 August 2020 (17.08.2020) DE

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(74) Agent: **PRINZ & PARTNER MBB PATENTANWÄLTE RECHTSANWÄLTE**; Leipziger Platz 15, 10117 Berlin (DE).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

(54) Title: MEANS FOR THE THERAPY OF TUMOR DISEASES

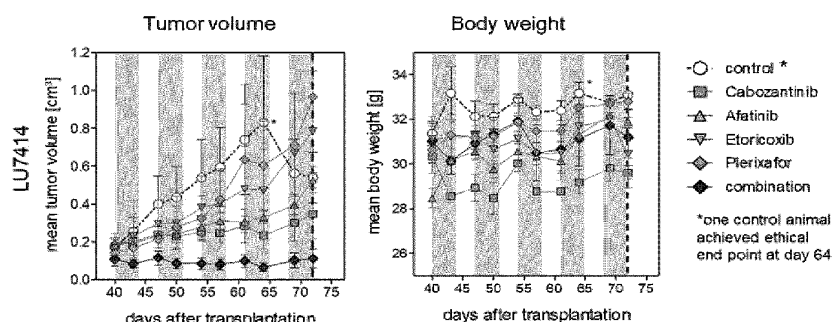


FIG. 2f)

(57) Abstract: The present invention relates to a pharmaceutical composition comprising a plurality of active agents respectively inhibiting VEGF's and/or VEGFR1-3; SDF1alpha and/or CXCR4; EGF and/or EGFR1-3; COX2 and/or PGE2 and/or EP; and HGF and/or HGFR (MET), in particular for use in the treatment or prophylaxis of tumor diseases, preferably selected from the group consisting of renal cell carcinoma (RCC), hepatocellular cancer (HCC), gastrointestinal stroma tumor (GIST), colorectal cancer (CRC), gastric cancer (GC) gastro-esophageal junction cancer (GEJ), neuro-endocrine tumor (NET), non-small cell lung cancer (NSCLC), chronic myeloid leukemia (CML) acute myeloid leukemia (AML), acute lymphatic leukemia (ALL), differentiated thyroid cancer (DTC) with or without driver mutations, such as EGFRm, KRAS, FGFR, ALK, cKIT, BRAF including a respective treatment method.

TITLE**MEANS FOR THE THERAPY OF TUMOR DISEASES**5 FIELD OF INVENTION

The present invention relates to a pharmaceutical composition comprising a plurality of active agents respectively inhibiting VEGF's and/or VEGFR1-3; SDF1alpha and/or CXCR4; EGF and/or EGFR1-3; COX2 and/or PGE2 and/or EP; and HGF and/or HGFR (MET), in particular for use in the treatment or prophylaxis
10 of tumor diseases, preferably selected from the group consisting of renal cell carcinoma (RCC), hepatocellular cancer (HCC), gastrointestinal stroma tumor (GIST), colorectal cancer (CRC), gastric cancer (GC) gastro-esophageal junction cancer (GEJ), neuro-endocrine tumor (NET), non-small cell lung cancer (NSCLC), chronic myeloid leukemia (CML) acute myeloid leukemia (AML), acute lymphatic
15 leukemia (ALL), differentiated thyroid cancer (DTC), breast cancer, ovarian cancer, cervical cancer, fallopian tube cancer, peritoneal cancer, sarcoma, pancreatic cancer, Head & Neck carcinoma, glioma, lymphoma, respectively with or without driver mutations, such as EGFRm, KRAS, FGFR, ALK, cKIT, BRAF, including a respective treatment method.

20 BACKGROUND

Drug-based tumor therapy has made immense progress in recent decades. A large number of very differentiated drugs are available for the therapy of tumor diseases.

As an example, Epidermal Growth Factor Receptor (EGFR) – Tyrosine Kinase Inhibitors
25 (TKIs), in particular EGFR1-3 can be used to target proliferation of malignant tumor cells, if present, to their driver mutations, such as EGFR-mutated tumors. Using EGFR1-3 tyrosine kinase inhibitors, such as afatinib, erlotinib, osimertinib, or gefitinib, for the first-line treatment of advanced NSCLC with activating EGFR

mutations is well established. However, treatment options after development of resistance for advanced squamous NSCLC, are limited, particularly in the second-line setting after progression. Vascular Endothelial Growth Factor (VEGF) inhibitors or Vascular Endothelial Growth Factor Receptors (VEGFR) inhibitors can be used to target tumor endothelial cells in order to decrease angiogenesis, tumor cell survival, and metastasis. Here also therapy resistances occur in view of VEGF / VEGFR inhibitors.

At the same time, the spectrum of side effects of therapy has expanded considerably. Overall, most antineoplastic substances belong to the drugs with the smallest therapeutic window.

Thus, there exists a need in providing a pharmaceutical composition effectively treating and/or preventing a tumor disease, wherein the therapy is safe, preferably provides a broader therapeutic window, results in less side effects and/or overcomes a therapeutic resistance to the therapy regime.

SUMMARY OF INVENTION

The aforementioned need is attended to at least in part by means of the claimed inventive subject matter. Advantages (preferred embodiments) are set out in the detailed description hereinafter and/or the accompanying figures as well as in the dependent claims.

Accordingly, a first aspect of this invention relates to a pharmaceutical composition comprising a plurality of active agents in a therapeutically effective amount and one or more pharmaceutical excipients, characterized in that

- a. at least one active agent inhibits VEGF's and/or VEGFR1-3 signaling,
- b. at least one active agent inhibits SDF1alpha and/or CXCR4 signaling,
- c. at least one active agent inhibits EGF and/or EGFR in particular EGFR1-3 signaling,
- d. at least one active agent inhibits COX2 and/or PGE2 and/or EP signaling, and
- e. at least one active agent inhibits HGF and/or HGFR (MET) signaling.

A second aspect of this invention relates to a pharmaceutical composition according to the first inventive aspect for use in the treatment or prophylaxis of a tumor disease, preferably selected from the group consisting of renal cell carcinoma (RCC), hepatocellular cancer (HCC), gastrointestinal stroma tumor (GIST), colorectal cancer (CRC), gastric cancer (GC) gastro-esophageal junction cancer (GEJ), neuro-endocrine tumor (NET), non-small cell lung cancer (NSCLC), chronic myeloid leukemia (CML) acute myeloid leukemia (AML), acute lymphatic leukemia (ALL), differentiated thyroid cancer (DTC), breast cancer, ovarian cancer, cervical cancer, fallopian tube cancer, peritoneal cancer, sarcoma, pancreatic cancer, Head & Neck carcinoma, glioma, lymphoma, respectively with or without driver mutations, such as EGFRm, KRAS, FGFR, ALK, cKIT, BRAF.

A third aspect of the invention relates to a method of treatment or prophylaxis of a tumor disease, preferably selected from the group consisting of renal cell carcinoma (RCC), hepatocellular cancer (HCC), gastrointestinal stroma tumor (GIST), colorectal cancer (CRC), gastric cancer (GC) gastro-esophageal junction cancer (GEJ), neuro-endocrine tumor (NET), non-small cell lung cancer (NSCLC), chronic myeloid leukemia (CML) acute myeloid leukemia (AML), acute lymphatic leukemia (ALL), differentiated thyroid cancer (DTC), breast cancer, ovarian cancer, cervical cancer, fallopian tube cancer, peritoneal cancer, sarcoma, pancreatic cancer, Head & Neck carcinoma, glioma, lymphoma, respectively with or without driver mutations, such as EGFRm, KRAS, FGFR, ALK, cKIT, BRAF.

The inventive aspects of the present invention as disclosed hereinbefore can comprise – in case it is reasonable for a person skilled in the art – any possible combination of the preferred inventive embodiments as set out in the dependent claims or disclosed in the following detailed description including the experimental section.

BRIEF DESCRIPTION OF DRAWINGS

Further aspects, characteristics and advantages of the invention will ensue from the following description of the embodiments with reference to the accompanying drawings, wherein

Fig. 1 is a simplified model of the inventive combination therapy regimen overcoming drug resistances in the therapy of tumor diseases.

FIGS. 2 a) to f) are diagrams displaying tumor volume and bodyweight during therapy regimen in the respective in vivo models LU 7406 (FIG. 2a), LU7414 (Fig. 2b), LU7177 (Fig. 2c), LU7766 (Fig. 2d), LU7860 (Fig. 2e), and LU7414 (Fig. 2f).

DETAILED DESCRIPTION OF EMBODIMENTS

As set out in more detail hereinafter, the inventors of the different aspects of this invention combine for the first time anti-tumor drugs from the group of VEGF and/or VEGFR inhibitors and EGF and/or EGFR, in particular EGFR1-3 inhibitors with active agents blocking the chemokine stromal-cell derived factor-1 (SDF-1alpha, also known as CXCL12) and C-X-C Motif Chemokine Receptor 4 (CXCR4) axis, and active agents inhibiting cyclooxygenase 2 (COX2) and/or prostaglandin E2 (PGE2) and/or E series of prostaglandin receptors (EP), as well as active agents inhibiting hepatocyte growth factor (HGF) and/or hepatocyte growth factor receptor (HGFR) (MET) in order to suppress a tumor disease already at lower concentrations compared to a respective monotherapy, in particular a tumor that is resistant to anti-tumor drugs from the group of VEGF and/or VEGFR inhibitors and EGF and/or EGFR, in particular EGFR1-3 inhibitors (see executive example below). In addition, the inventive combination regimen showed at the same time an excellent safety profile of the inventive pharmaceutical composition and might lead to less pronounced side effects in patients. Together with its excellent efficacy this inventive combination is deemed to be superior in comparison to a higher dose therapy regimen of a VEGFR inhibitor monotherapy, e.g. using cabozantinib (a multi-kinase inhibitor that targets MET, AXL, and VEGFR2), or a higher dose therapy regimen of a EGFR, in particular EGFR1-3 inhibitor monotherapy, e.g. using afatinib.

The fact, that the inventive pharmaceutical composition not only simultaneously inhibits VEGF / VEGFR, HGF / HGFR (MET) and EGF / EGFR in particular EGFR1-3 signaling pathways, but in addition simultaneously inhibits SDF-1 / CXCR4, and COX2 / PGE2 / EP signaling pathways seems to be necessary in order to suppress tumor growth by effectively target the paracrine signaling from

different cell types including immune cells, tumor endothelial cells, cancer associated fibroblasts and tumor cells forming a cellular tumorigenic network (see Fig. 1).

It is surprising, that the inventive combination of active agents overcomes tumor resistances, as it has previously been shown that a combination of cabozantinib and erlotinib, an EGFR inhibitor, did not appear to re-sensitize the resistant patients to erlotinib (Wakelee HA, et al., A phase Ib/II study of cabozantinib (XL184) with or without erlotinib in patients with non-small cell lung cancer. Cancer Chemother Pharmacol. 2017 May;79(5):923-932. doi: 10.1007/s00280-017-3283-z.).

Previous studies furthermore showed that plerixafor as an inhibitor of the SDF-1 – CXCR4 axis was found to abolish cabozantinib induced tumor growth inhibition (Patnaik A, et al. Cabozantinib Eradicates Advanced Murine Prostate Cancer by Activating Antitumor Innate Immunity. Cancer Discov. 2017 Jul;7(7):750-765. doi: 10.1158/2159-8290.CD-16-0778. Epub 2017 Mar 8. PMID: 28274958; PMCID: PMC5501767.).

In the context of the present invention the expression “*pharmaceutical composition comprising a plurality of active agents in a therapeutically effective amount*” means that the combination of inhibitory active agents a) to e) and optionally f) are comprised in the pharmaceutical composition in such an amount, that therapeutically suppresses tumor growth, i.e. wherein the volume and/or the weight of tumor or metastatic lesions remain constant or preferably decrease (i.e. as measured in the clinic by the revised RECIST criteria) and/or wherein the events of metastasis are decreased. In view of the synergistic effects provided by the inventive pharmaceutical composition, the concentrations in particular of the respective active agents a), b) and/or c) can be the same or preferably reduced in comparison to the concentration used when applying the respective active agents in monotherapy.

In the context of the present invention the term “active agent” may synonymously be used to “therapeutic agents”, “therapeutics”, “drugs”, “biologicals”, “targeted drugs”, “immunologicals”, “antibodies”, “small molecules” and the like.

The first aspect of the present invention thus relates to a pharmaceutical composition comprising a plurality of active agents in a therapeutically effective amount and one or more pharmaceutical excipients, characterized in that

- a. at least one active agent inhibits VEGF's and/or VEGFR1-3 signaling,
- 5 b. at least one active agent inhibits SDF1alpha and/or CXCR4 signaling,
- c. at least one active agent inhibits EGF and/or EGFR in particular EGFR1-3 signaling,
- d. at least one active agent inhibits COX2 and/or PGE2 and/or EP signaling, and
- 10 e. at least one active agent inhibits HGF and/or HGFR (MET) signaling.

According to an alternative or additive preferred embodiment of the present invention

- a. at least one VEGF's and/or VEGFR1-3 inhibitor is selected from the group consisting of cabozantinib, regorafenib, bevacizumab, ramucirumab, pazopanib, sunitinib, sorafenib, ponatinib, vandetanib, axitinib, lenvatinib, ziv-Aflibercept, tivozanib, golvatinib, and foretinib, preferably cabozantinib and regorafenib, and/or
- 15 b. at least one SDF1alpha and/or CXCR4 inhibitor is selected from the group consisting of plerixafor, motixafortide, LY2510924, and WZ811, preferably plerixafor, and/or
- 20 c. at least one EGF and/or EGFR in particular EGFR1-3 inhibitor is selected from the group consisting of afatinib, erlotinib, cetuximab, neratinib, necitumumab, gefitinib, lapatinib, osimertinib, canertinib, and saracatinib, preferably afatinib, cetuximab, neratinib, necitumumab, gefitinib, lapatinib, and osimertinib, more preferably afatinib, and/or
- 25 d. at least one COX2 and/or PGE2 and/or EP inhibitor is selected from the group consisting of etoricoxib, celecoxib, parecoxib, rofecoxib, and valdecoxib, preferably etoricoxib and celecoxib, and/or

- e. at least one active agent inhibiting HGF and/or HGFR (MET) is selected from the group consisting of cabozantinib, tivantinib, crizotinib, capmatinib, tepotinib, savolitinib, golvatinib, and foretinib, preferably cabozantinib and tivantinib.

5 Excluded from the present invention is an embodiment, wherein the plurality of active agents in the pharmaceutical composition is limited to active agents inhibiting a) VEGF and/or VEGFR, in particular wherein the respective inhibitors consist of bevacizumab, sunitinib, and sorafenib; b) SDF1alpha and/or CXCR4, in particular wherein the respective inhibitors consist of plerixafor; c) EGF and/or
10 EGFR, in particular wherein the respective inhibitors consist of afatinib, gefitinib, erlotinib, necitumumab, and osimertinib; and d) COX2 and/or PGE2 and/or EP, in particular wherein the respective inhibitors consist of celcoxib, and etoricoxib.

According to an alternative or additive preferred embodiment of the present invention Cabozantinib is selected in groups a) and e). In doing so, only four active
15 agents can be selected in order to inhibit the five signaling pathways a) to e).

According to an alternative or additive preferred embodiment of the present invention the inventive composition comprises a combination of active agents selected from the group consisting of

- i. cabozantinib, afatinib, plerixafor, and etoricoxib,
20 ii. cabozantinib, afatinib, plerixafor, and celecoxib,
iii. cabozantinib, osimertinib, plerixafor, and etoricoxib,
iv. cabozantinib, osimertinib, plerixafor, and celecoxib,
v. cabozantinib, necitumumab, plerixafor, and etoricoxib,
vi. cabozantinib, necitumumab, plerixafor, and celecoxib,
25 vii. cabozantinib, cetuximab, plerixafor, and etoricoxib,
viii. cabozantinib, cetuximab, plerixafor, and celecoxib,
ix. cabozantinib, neratinib, plerixafor, and etoricoxib,
x. cabozantinib, neratinib, plerixafor, and celecoxib,

- xi. cabozantinib, lapatinib, plerixafor, and etoricoxib ,
- xii. cabozantinib, lapatinib, plerixafor, and celecoxib,
- xiii. cabozantinib, gefitinib, plerixafor, and etoricoxib,
- xiv. cabozantinib, gefitinib, plerixafor, and celecoxib,
- 5 xv. regorafenib, necitumumab, plerixafor, etoricoxib and tivantinib,
- xvi. regorafenib, necitumumab, plerixafor, celecoxib and tivantinib,
- xvii. regorafenib, cetuximab, plerixafor, etoricoxib and tivantinib,
- xviii. regorafenib, cetuximab, plerixafor, celecoxib and tivantinib,
- xix. regorafenib, osimertinib, plerixafor, etoricoxib and tivantinib,
- 10 xx. regorafenib, osimertinib, plerixafor, celecoxib and tivantinib,
- xxi. regorafenib, afatinib, plerixafor, etoricoxib and tivantinib,
- xxii. regorafenib, afatinib, plerixafor, celecoxib and tivantinib,
- xxiii. regorafenib, cetuximab, plerixafor, etoricoxib and cabozantinib,
- xxiv. regorafenib, cetuximab, plerixafor, celecoxib and cabozantinib,
- 15 xxv. regorafenib, necitumumab, plerixafor, etoricoxib and crizotinib, and
- xxvi. regorafenib, necitumumab, plerixafor, celecoxib and crizotinib.

Combinations i) to xiv) are specifically preferred, as they comprise only four active agents in order to inhibit the five signaling pathways. In doing so, the overall amount of active agents and, thus, the body burden can be reduced, which in turn increases the safety profile of the inventive pharmaceutical composition and may decrease the risk of showing drug related side effects.

According to an alternative or additive preferred embodiment of the present invention the inventive composition further comprises

- 25 f. at least one active agent inhibiting programmed death 1 receptor (PD-1) and/or programmed death ligand 1 (PD-L1), wherein the PD-1 and/or PD-L1 inhibitor is preferably selected from the group consisting of

pembrolizumab, nivolumab, durvalumab, and atezolizumab, preferably durvalumab.

The following table 1 summarizes the information on the selected active agents effectively inhibiting the respective signaling pathways including their presently approved indications including approved dosing as well as a potential dosing in the inventive pharmaceutical combination:

Tab. 1 Active agents suitable to be used within the inventive pharmaceutical composition including approved indications and dosing and potential dosing in a combination regimen

Molecular paracrine signaling pathway	Drug	Approved indications (in subgroups thereof)	Approved dosing	Potential dosing in combination regimen
VEGFA-VEGFR2	cabozantinib	RCC, HCC	60mg/day	20-55mg/day
	regorafenib	CRC, HCC, GIST	160mg/day	50-150mg/day
	bevacizumab	CRC, breast cancer, NSCLC, RCC, ovarian cancer, peritoneal cancer cervical cancer, fallopian tube cancer	15mg/kg/ 3 weeks	5-15mg/kg/ 3weeks
	ramucirumab	GC, GEJ cancer, CRC, NSCLC	8mg/kg/2 weeks	4-7mg/kg/ 2 weeks
	pazopanib	RCC, Sarcoma	800mg/day	250-750mg/day
	sunitinib	GIST, NETs, RCC	50mg/day	20-45mg/day
	sorafenib	HCC, RCC, DTC, AML	800mg/day	250-750mg/day
	ponatinib	CML, ALL	45mg/day	15-40 mg/day
	vandetanib	Thyroid carcinoma	300mg/day	100-250mg/day
	axitinib	RCC	10mg/day	10mg/day
	lenvatinib	RCC, HCC, thyroid carcinoma	18-24mg/day	18-24mg/day
	ziv-aflibercept	CRC	4 mg per kg every two weeks	4 mg per kg every two weeks
	tivozanib	Not approved		
	golvatinib	Not approved		
	foretinib	Not approved		
EGF-EGFRwt	afatinib	NSCLC	40mg/day	10-35mg/day
	erlotinib	NSCLC, pancreatic cancer	150mg/day	50-145mg/day
	cetuximab	CRC, Head & Neck carcinoma	250mg/m ² / week	125-240 mg/m ² /week

	neratinib	Breast cancer	240mg/day	150-230mg/day
	necitumumab	NSCLC	800mg/day 1 and 8 in 3 week cycles	300-750mg/day 1 and 8 in 3 week cycles
	gefitinib	NSCLC	250mg/day	75-245mg/day
	lapatinib	Breast cancer	1250mg/day	500-1200 mg/day
	osimertinib	NSCLC	80mg/day	20-75mg/day
	canertinib	Not approved		
	saracatinib	Not approved		
SDF1a- CXCR4	plerixafor	Stem cell mobilization prior transplantation	20mg/day/5 days	5-15 mg/day/5days
	motixafortide	Not approved		
	LY2510924	Not approved		
	WZ811	Not approved		
COX2- PGE2	etoricoxib	Arthrosis, rheumatoid arthritis, gout, morbus bechterew, tooth pain	60mg/day	10-55mg/day
	celecoxib	Arthrosis, rheumatoid arthritis, morbus bechterew	200mg/day	50-185mg/day
	parecoxib	Post-surgery pain	40mg/day	20-35mg/day
	rofecoxib	Not approved		
	valdecoxib	Not approved		
HFG-MET	cabozantinib	RCC, HCC	60mg/day	20-55mg/day
	crizotinib	NSCLC	500mg/day	100-450mg/kg
	tivantinib	NSCLC	450mg/day	100-445mg/kg
	capmatinib	Not approved		
	tepotinib	Not approved		
	savolitinib	Not approved		
	golvatinib	Not approved		
	foretinib	Not approved		

Generally, the present invention the inventive composition may contain one, two or more separate pharmaceutical dosage formulations, wherein each dosage formulation contains at least one active agent. The respective pharmaceutical dosage forms may comprise one or more dosage units. In case the pharmaceutical composition includes two or more separate pharmaceutical dosage formulations, the combination of dosage formulations may be commercialized in one kit /

package comprising the inventive pharmaceutical composition including the different dosage formulations. As an example, in case one dosage formulation may represent a solid composition, such as a tablet, and one dosage formulation may represent a liquid composition, such as an injection solution, the respective
5 inventive pharmaceutical composition may comprise in the commercialized kit / package one or more units (single tablets) of the solid dosage formulation and one or more units (vials comprising the injection solution) of the liquid dosage formulation. Accordingly, the one, two or more pharmaceutical dosage formulations may independently of each other be the same or different, and may
10 be selected from the group consisting of

- i. solid dosage forms for oral or enteral application, wherein solid dosage forms are preferably include tablets, capsules, spheroids, mini-tablets, pellets, granules, and pills;
- 15 ii. semi-solid dosage forms for topical application to skin or mucous membranes, wherein semi-solid dosage forms are preferably include creams, gels, ointments, and suppositories; and
- 20 iii. liquid dosage forms for oral, external, or parenteral application, wherein liquid dosage forms preferably include mixtures, linctuses, elixirs, syrups, drops, lotions, liniments, collodions, nasal drops, nasal sprays, eye drops, inhalations, aerosols, and injections, e.g., *sub cutan* (s.c.) injections, *intra muscular* (i.m.) injections, *intra venous* (i.v.) injections, *intra peritoneal* (i.p.), *intra pleural* (i.pl.), *intra tumoral* (i.t.), *intra thecal* (i.th.), *intra cerebral / intra cranial* (i.c.) injections.

The one or more pharmaceutical excipients for the respective pharmaceutical
25 formulations may readily be selected as necessary from suitable excipients classes including fillers, diluents, binders, disintegrants, antiadherent, lubricants, glidants, coatings, sorbents, preservatives, antioxidants, flavoring agents, sweetening agents, coloring agents, solvent & co-solvent, buffering agents, chelating agents, viscosity imparting agents, surface active agents, humectants and the like. The technical
30 content regarding the aforementioned solid, semi-solid and liquid dosage forms as well as the aforementioned respective exemplary excipient classes and their respective constituents disclosed in Hand book of pharmaceutical excipients, Edition-Nine, Edited by-Raymond Crowe Paul J Sheskey and Marian E Quinn,

Publisher-Pharmaceutical Press; Aulton's Pharmaceutics The design and manufacture of medicines, Edition-Six, Edited by- Michael E.Aulton, Publisher-Elsevier; Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Edition- Twelve, Author Loyd Allen Jr, Timothy B. McPherson, Publisher- Lippincott

5 Williams and Wilkins; Pharmazeutische Technologie: Mit Einführung in Biopharmazie und Biotechnologie, Authors Kurt-Heinz Bauer, Karl-Heinz Frömming and Claus Führer is incorporated herein by reference. As an example, a respectively suitable solid dosage form may be a tablet or a capsule composition and preferably may exhibit in addition to the one or more active agents

10 pharmaceutically acceptable excipients selected from a diluent, a binder, a disintegrant, a lubricant, and optionally a coating. As a further example, a suitable liquid dosage form may be an injection solution, preferably a s.c. injection solution, which may comprise in addition to one or more active agents a suitable solvent, such as water for injections, and a suitable buffer agent / pH adjusting agent.

15 According to an alternative or additive preferred embodiment of the present invention, the inventive composition may comprise a first pharmaceutical dosage formulation that contains at least one active agent inhibiting b) SDF1alpha and CXCR4 signaling pathway, and at least a second pharmaceutical dosage formulation that contains two or more, preferably all of the at least one active

20 agents respectively inhibiting a) VEGF's and/or VEGFR1-3, c) EGF and/or EGFR in particular EGFR1-3, d) COX2 and/or PGE2 and/or EP, e) HGF and/or HGFR (MET), and optionally f) PD-1 and/or PD-L1 signaling pathways. More preferably, the first pharmaceutical formulation may be a liquid dosage formulation, preferably an s.c. injection dosage form and in particular, and/or wherein the second

25 pharmaceutical dosage form is a solid dosage form, preferably a tablet or capsule and may contain as an example one or more of the remaining active agents. As an example, the first pharmaceutical dosage formulation may be an s.c. injection solution comprising plerixafor in particular in an amount of 24 mg or less per dosage unit, the dosage unit may comprise 1.2 ml. The second pharmaceutical

30 dosage formulation may be selected from a solid dosage formulation, preferably a tablet or a capsule comprising one or more of the remaining active agents, in particular cabozantinib, afatinib, and etoricoxib or celecoxib. In case one or more of the remaining active agents may physically or chemically interact with each other in the respective dosage formulation, e.g. solid dosage formulation, suitable

counter measures will be taken as available in the art, such as separating the respective active agents in different compartments of the dosage formulation, masking the respective active agents, etc. and/or separating the respective interacting active agents in separate pharmaceutical dosage formulations.

- 5 Regarding a suitable dosing regimen, the active agents of the inventive pharmaceutical composition containing one or more pharmaceutical dosage formulations may generally be administered daily, wherein preferably in this case at least active agents a), b) and/or c) may be administered in a concentration, which is lower than the concentration commonly used in their respective
10 monotherapy in order to decrease side effects. In addition or alternatively, one or more of the active agents of the inventive pharmaceutical composition containing one or more pharmaceutical dosage formulations may be administered less than every day, which may also decrease the risk of side effects.

- As shown in the example section, during the complete treatment duration, no
15 tumor outgrowth was observed in either PDX model, indicating a strong therapy efficacy by preventing development of an adaptive resistance against this regimen. After treatment discontinuation growth suppression remained up to 18 days (LU7860) with only slight increase in tumor growth. This indicates a long-lasting and sustained effect of the combination therapy on the tumor biology. In other
20 words, a preferred dose regimen includes the administration of the inventive pharmaceutical composition in intervals from e.g. weeks to months, such as administration of inventive pharmaceutical composition for one or more, preferably five or more days, followed by a one or more, preferably two or more days without administration of the inventive pharmaceutical composition (also called "*drug holiday*"). Such interval dose regimen may improve the patient compliance in
25 particular due to a reduced risk of side effects.

 According to an alternative or additive preferred embodiment of the present invention, the dose regimen may comprise or consist of

- 30 a. at least one active agent inhibiting VEGF's and/or VEGFR1-3 is administered once daily for one, two, three, four or five days followed by at least one, preferably two days without administration,
- b. at least one active agent inhibiting SDF1alpha and/or CXCR4 is administered once daily for one, two, three, four or five days, preferably for

one day followed by at least one, preferably five days without administration,

5 c. at least one active agent inhibiting EGF and/or EGFR in particular EGFR1-3 is administered once daily for one, two, three, four or five days followed by at least one, preferably two days without administration,

d. at least one active agent inhibiting COX2 and/or PGE2 and/or EP is administered once daily for one, two, three, four or five days followed by at least one, preferably two days without administration,

10 e. at least one active agent inhibiting HGF and/or HGFR (MET) is administered once daily for one, two, three, four or five days followed by at least one, preferably two days without administration, and optionally

f. at least one active agent inhibiting PD-1 and/or PD-L1 that is administered once daily for one, two, three, four or five days followed by at least one, preferably two days without administration.

15 The aforementioned (preferred) embodiments of the first aspect of the present invention may be combined throughout. In particular, the different preferred embodiments of the first aspect of the present invention can alternatively or in addition be combined with each other.

20 All aforementioned embodiments including the combination of preferred embodiments in relation to the first aspect of the present invention can also be used for combination with (preferred) embodiments of the second and third aspect of the present invention.

25 The second aspect of the present invention relates to a pharmaceutical composition according to the first inventive aspect for use in the treatment or prophylaxis of a tumor, preferably selected from the group consisting of renal cell carcinoma (RCC), hepatocellular cancer (HCC), gastrointestinal stroma tumor (GIST), colorectal cancer (CRC), gastric cancer (GC) gastro-esophageal junction cancer (GEJ), neuro-endocrine tumor (NET), non-small cell lung cancer (NSCLC), chronic myeloid leukemia (CML) acute myeloid leukemia (AML), acute lymphatic
30 leukemia (ALL), differentiated thyroid cancer (DTC), breast cancer, ovarian cancer, cervical cancer, fallopian tube cancer, peritoneal cancer, sarcoma, pancreatic

cancer, Head & Neck carcinoma, glioma, lymphoma, respectively with or without driver mutations, such as EGFRm, KRAS, FGFR, ALK, cKIT, BRAF. Preferably, the tumor is a non-small cell lung cancer (NSCLC), more preferably without driver mutations.

5 According to a preferred embodiment the pharmaceutical composition, in particular comprising one or more pharmaceutical formulations is/are respectively provided facilitating enteral administration, preferably oral administration, and/or intranasal administration and/or sub-lingual administration and/or buccal administration. Such an embodiment is preferred, as it may facilitate treatment at
10 home and would not require hospitalization and may improve patient compliance.

As shown in the example section, during the complete treatment duration, no tumor outgrowth was observed in either PDX model, indicating a strong therapy efficacy by preventing development of an adaptive resistance against this regimen. After treatment discontinuation growth suppression remained up to 18 days
15 (LU7860) with only slight increase in tumor growth. This indicates a long-lasting and sustained effect of the combination therapy on the tumor biology. In other words, a preferred dose regimen includes the administration of the inventive pharmaceutical composition in intervals from e.g. weeks to months, such as administration of inventive pharmaceutical composition for one or more, preferably
20 five or more days, followed by a one or more, preferably two or more days without administration of the inventive pharmaceutical composition (also called "*drug holiday*"). Such interval dose regimen may improve the patient compliance in particular due to a reduced risk of side effects.

The respective dosing regimens disclosed with respect to the first inventive
25 aspect also apply with respect to the third inventive aspect. In particular, all aforementioned embodiments including a (sub)combination of preferred embodiments disclosed in relation to the first aspect of the present invention can also be used in combination with respect to the second aspect of the present invention.

30 The third aspect of the present invention relates to a method of treatment or prophylaxis of a tumor disease, preferably selected from the group consisting of renal cell carcinoma (RCC), hepatocellular cancer (HCC), gastrointestinal stroma tumor (GIST), colorectal cancer (CRC), gastric cancer (GC) gastro-esophageal

junction cancer (GEJ), neuro-endocrine tumor (NET), non-small cell lung cancer (NSCLC), chronic myeloid leukemia (CML) acute myeloid leukemia (AML), acute lymphatic leukemia (ALL), differentiated thyroid cancer (DTC), breast cancer, ovarian cancer, cervical cancer, fallopian tube cancer, peritoneal cancer, sarcoma, pancreatic cancer, Head & Neck carcinoma, glioma, lymphoma, respectively with or without driver mutations, such as EGFRm, KRAS, FGFR, ALK, cKIT, BRAF. Preferably, the tumor is a non-small cell lung cancer (NSCLC), more preferably without driver mutations.

As already set out with respect to the second inventive aspect, a preferred embodiment of the inventive method of treatment requires the enteral administration, preferably oral administration, and/or intranasal administration and/or sub-lingual administration and/or buccal administration of an inventive pharmaceutical composition that comprises one or more pharmaceutical formulations. Such an embodiment is preferred, as it may facilitate treatment at home and would not require hospitalization and thereby may improve patient compliance.

Moreover, an interval administration of the inventive pharmaceutical composition may be preferred as set out with respect to the second inventive aspect, as the experimental data shows that the tumor growth suppression maintains constant within 18 days after administration of the last dose. Such an interval administration from weeks to months, in particular from one or more, preferably five or more days followed by an interval without administration of inventive pharmaceutical composition of one or more days, in particular two or more days is preferred.

The respective dosing regimens disclosed with respect to the first inventive aspect also apply with respect to the third inventive aspect. In particular, all aforementioned embodiments including a (sub)combination of preferred embodiments disclosed in relation to the first aspect of the present invention can also be used in combination with respect to the third aspect of the present invention.

The present invention is explained further with the aid of the following non-limiting examples, illustrating the parameters of and compositions employed within

the present invention. Unless stated otherwise, all data, in particular percentages, parts and ratios are by weight.

According to the present invention the individual features of the exemplary embodiments of the inventive pharmaceutical tablet as disclosed in the detailed description or claims of the present application can respectively be separately combined with features of the exemplary embodiments herein below.

EXECUTIVE EAMPLES

The following embodiments show the results of treatments of patient-derived NSCLC xenografts (PDX) treated with various anti-cancer monotherapies including chemotherapies, small molecules and monoclonal antibodies and the combination therapy listed within the scope of the present invention (Therapy). Efficacy was measured using the Response Evaluation Criteria in Solid Tumors (RECIST) and according to the measured ratio of the treated tumor (T) versus a placebo control (C) with the largest difference that occurred (optimal).

PD: progressive disease; SD: stable disease; PR: partial response.

10 Methods

Patient-Derived Xenograft (PDX) lung cancer models

The establishment and characterization of PDX mouse models and lung PDX models in particular, was previously described (Tab. 2) (Fichtner et al., 2008, Rolff et al., 2016). Generation of the PDX has been approved by the local ethics committee at Charite Berlin and patients have provided informed consent.

In brief, fresh tumor samples were cut into pieces of 3 x 3 mm and were subcutaneously transplanted to Rj:NMRI-Foxn1nu/nu nude recipient mice. Once subcutaneous (s.c.) tumors became palpable, tumor size was measured twice weekly with a digital caliper. Individual tumor volumes (TV) were calculated by the formula $V = (\text{length} \times \text{width}^2) / 2$ and related to the values at the first day of treatment (relative tumor volume, RTV). In addition, therapeutic efficacy was assessed by applying Response Evaluation Criteria In Solid Tumors (RECIST) (Eisenhauer et al., 2009). According to these criteria a reduction in tumor volume of at least 30% was defined as partial response (PR), an increase in tumor volume of at least 20% was defined as progressive disease (PD). Stable disease (SD) was defined as neither sufficient reduction to qualify for PR nor sufficient increase to qualify for PD. The Objective Response Rate (ORR) includes all tumors with Complete Response (CR) and PR and the Clinical Benefit Rate includes all tumors with CR, PR and SD expressed in percentage. The body weight (BW) of mice was recorded regularly at least two times per week and the change in body weight was utilized as variable for tolerability.

Tab. 2. Characterization of selected NSCLC tumor models

		Tumor Models				
		LU7406	LU7414	LU7177	LU7766	LU7860
Characterization	Histology	Adeno Ca	SCC	SCC	SCC	SCC
	Gender	Female	Female	Male	Male	Male
	Age	53	64	42	71	71
	TNM Classification	ypT2 ypN2 cM0 G3 R0	pT2 pN0 cM0 G3 R0	pT2 pN2 M0 G3 R0	pT2 pN2 cM0 G3 R0	pT1 pN0 cM0 G3 R0
	Stage	IIIA	IIA	IIIA	IIIA	IA
	Best Response for all Therapies Tested (RECIST)	PD	PD	CR (gemcита bine)	PR (paclitaxel)	SD (paclitaxel)
	Identified Driver Mutation	none	none	none	none	none

Legend: Adeno CA: adenocarcinoma; SCC: squamous cell carcinoma; PD: progressive disease; CR: complete response; SD: stable disease; PR: partial response

- 5 After 25 to 40 days, the mean tumor volume reached the indicated starting volume (150-200 mm³). Mice were randomly assigned to control and treatment groups (1-4 mice per group) and treatment was started on the day of randomization.
- 10 The mice were treated using the following drug dosages and treatment schedules: cabozantinib 15 mg/kg, afatinib 15 mg/kg, etoricoxib 10 mg/kg orally and plerixafor 5 mg/kg intra-peritoneally for 5 days on treatment and 2 days off treatment for four cycles in total (Tab. 3a). Common dosing regimes in the

respective model for monotherapeutic applications require the once daily application throughout of all active ingredients optionally in a higher dose, such as of cabozantinib 30-60 mg/kg (Paratala et al., 2018; Scott et al., 2018), afatinib 20 mg/kg (Floc'h N et al., 2018), etoricoxib 10 mg/kg (Floc'h N et al., 2018) orally and plerixafor 5 mg/kg intra-peritoneally (Singla et al., 2015). Placebo control mice were treated with corresponding vehicles only (p.o. with 0.5% methyl cellulose and s.c. with aqua ad iniectabilia) or with respective monotherapy at the same dosages. At the end of the experiments, tumors were excised and one half snap frozen and stored at -80 °C for further analyses. The second half was fixed in formalin fixed and embedded in paraffin (FFPE).

All mice were handled in accordance with the Guidelines for the Welfare and Use of Animals in Cancer Research (Workman et al., 2010) and according to the German Animal Protection Law, approved by the responsible local authorities.

Tab. 3a. Characteristics of the Low Dose Combination Drug Regimen

Target	Drug*	Route of Administration	Dosing in Combination Therapy (Fig. 2a-e) and in Monotherapy Controls (Fig. 2f)	
			Dosing	Schedule
VEGFR2 (KDR) / MET (HGFR)	Cabozantinib	oral	15mg/kg	5 days on / 2 days off for 4 cycles
CXCR4	Plerixafor	intra-peritoneal	5mg/kg	
EGFRwt	Afatinib	oral	15mg/kg	
COX2 (PTGS2)	Etoricoxib	oral	10mg/kg	

* In combination therapy cabozantinib, afatinib and etoricoxib were administered in a single combined gavage dose and plerixafor was administered separately intra-peritoneal

Table 3b. Characteristics of the Drug Regimen of Monotherapy according to state of the art

Target	Drug*	Route of Adminis-tration	Common Dosing <i>in vivo</i> Models (state of the art)	
			Dosing	Schedule
VEGFR2 (KDR) / MET (HGFR)	Cabozantinib	oral	30-60mg/kg (Paratala et al., 2018; Scott et al., 2018)	once daily
CXCR4	Plerixafor	intra-peritoneal	5mg/kg (Singla et al., 2015)	
EGFRwt	Afatinib	oral	20mg/kg (Floc'h N et al., 2018)	
COX2 (PTGS2)	Etoricoxib	oral	10mg/kg (Jayaraman et al., 2010)	

Drawings

- 5 **Fig. 1** displays a simplified model of the inventive combination therapy regimen overcoming drug resistances by simultaneous targeting interdependent signaling in a cellular tumorigenic network of NSCLC tumors.

In Fig. 1 the following reference signs have the meaning:

- (1) Immune cell;
- 10 (2) Tumor endothelial cell;
- (3) Cancer associated fibroblast;
- (4) Tumor cell;
- (10) Evading immune destruction;
- (20) angiogenesis, proliferation, migration, cell survival and
- 15 vascular permeability;
- (30) secretion of protumorigenic factors;
- (40) proliferation, cell survival, metastasis and secretion of VEGF;

- (11) active agents inhibiting PD-1-PD-L1 signalling
- (21) active agents inhibiting VEGF-VEGFR signalling
- (41) active agents inhibiting COX2-PGE2-EP signalling
- (42) active agents inhibiting EGF-EGFR signalling
- 5 (43) active agents inhibiting SDF1alpha-CXCR4 signalling
- (44) active agents inhibiting HGF-MET signalling
- PGE2 prostaglandin E2
- VEGFR vascular endothelial growth factor receptor;
- EGFR epidermal growth factor receptor;
- 10 COX2 (PTGS2) cyclooxygenase 2
- HGF hepatocyte growth factor.

Signaling axes of PD-1-PD-L1, VEGF-VEGFR, COX2-PGE2-EP, EGF-EGFR, SDF-1-CXCR4, and HGF-MET are exemplary shown for some of the known paracrine pathways binding the cellular tumorigenic network in NSCLC tumors using respective inhibiting active agents (11), (21), (41), (42), (43), and (44), which are inventively selected as follows:

- (11) nivolumab, pembrolizumab, stezolizumab, durvalumab;
- (21) cabozantinib, regorafenib, bevacizumab, ramucirumab, pazopanib, sunitinib, sorafenib, ponatinib, vandetanib, axitinib, lenvatinib, ziv-aflibercept, tivozanib, golvatinib, foretinib
- 20 (41) atoricoxib, celecoxib, parecoxib, rofecoxib, valdecoxib
- (42) afatinib, erlotinib, cetuximab, neratinib, necitumumab, gefitinib, lapatinib, osimertinib, canertinib, saracatinib
- (43) plerixafor, motixafortide, LY2510924 and WZ811
- 25 (44) cabozantinib, crizotinib, tivantinib, capmatinib, tepotinib, savolitinib, golvatinib, foretinib

Figs. 2a) to 2f) display *in vivo* efficacy testing of innovative combination treatment regime in selected NSCLC PDX mouse models *versus* monotherapy regiment of the state of the art. In all trials, treatment with the innovative combination was highly effective in preventing tumor propagation and tumor out-
5 growth achieving stable disease and partial response as best response criteria in all experiments. Stable body weight throughout the complete trials indicate distinguished safety profile of the applied combination treatment.

Resistance profiles of all tumor models against carboplatin, paclitaxel, gemcitabine, etoposide, cetuximab, erlotinib and bevacizumab was evaluated in
10 the initial characterization as follows: Fig. 2a. LU7406 and Fig. 2b. LU7414 showed progressive disease (PD) for all tested therapies. Fig. 2c. LU177 showed PD for all but one therapies tested. For gemcitabine complete response (CR) was determined. Fig. 2d. LU7766 showed for four out of the seven tested therapies PD. For carboplatin stable disease (SD), for paclitaxel partial response (PR) and for
15 cetuximab SD was observed. Fig. 2e. LU7860 showed PD for all but one therapies tested. For paclitaxel SD was observed.

In the experiment LU7414 displayed in Fig. 2f. with compounds administered as monotherapies at the same drug dosages used for the innovative combination therapy *versus* control and the innovative combination groups reveal a superior
20 effect of the innovative combination therapy.

Results

Complete suppression of tumor growth and absence of toxicity in all NSCLC PDX models treated with the combination therapy regimen

25 In total, 16 nude mice bearing one of the selected NSCLC PDX tumors were randomized for treatment with the innovative combination therapy regimen consisting of low dose cabozantinib, afatinib, plerixafor and etoricoxib for at least four cycles with 5 days on and 2 days off treatment controlled by a placebo (N=18) *versus* state of the art monotherapy treatment (N=12) group (Tab. 3a). The safety
30 profile of this combination regimen was assessed by the continuous measurement of body weight (BW) of treated mice compared to the placebo group as an indicator for toxicity.

Throughout the experiments only in the LU7414 trial a BW reduction between 5-13% was detected for the innovative combination during the second treatment cycle after day 4 resulting in drug holiday from day 5-7 in this cycle. However, the cumulative dose of these mice was the same compared to all other animals treated with the combination since the first cycle was tested with 6 days on and 1 day off treatment. In none of the remaining mice at any time point a significant difference in BW between the verum and the placebo or the monotherapy group was observed. In general, all mice included in these experiments either gained weight during the course of treatment or remained at the baseline BW level with minimal variances. None of the mice had to be discontinued from treatment, died or had to be euthanized during the course of treatment. This observation reflects the beneficial safety profile of this inventive combination regimen and is in alignment with the low dose therapy compared to regular state of the art concentrations used of these drugs in in vivo experiments (Tab. 3a).

16 out of 16 NSCLC PDX tumors combined from all models showed a treatment response for the applied innovative combination regimen as evaluated per RECIST analysis (Figs. 2a) to 2f)). In 13 tumors a partial response (PR) and in 3 tumors a stable disease (SD) was induced. In contrast, in 29 out of 30 placebo or monotherapy treated tumors progressive disease (PD) was observed. Only for a single tumor of the cabozantinib monotherapy group a partial response was observed and transformed into progressive disease during the later course of treatment (Fig. 2f). Thus, the ORR is 81% and the CBR is 100% across all NSCLC PDX tumors treated with the combination regimen. In none of the mice treated with the innovative combination regime a tumor outgrowth was observed during the treatment period. In contrast, all tumors in the placebo- or monotherapy group showed a progressive disease at the last day of treatment.

In addition, the growth of all tumors in the innovative combination regimen group remained suppressed in the follow up period for up to 18 days (LU7860) (Fig. 2e).

Overall, these data indicate that innovative combined targeting of the paracrine cellular tumorigenic network elicits strong anti-tumoral effects in NSCLC PDX tumors which are characterized by profound resistances to standard therapies.

Discussion

Despite some recent progress in the treatment of NSCLC, no impactful advances have been in the state of the art when treating tumors without an actionable driver mutation and without the expression of PD-L1 >50% (Herbst et al., 2018).

For the first time, the present inventors showed that the inventive targeting of paracrine signaling from different cell types of the cellular tumorigenic network breaks the intercellular crosstalk and demonstrates a highly effective treatment of tumors that are resistant to monotherapies of chemotherapeutics and targeted drugs against EGFR1-3 and VEGFR. In all treated NSCLC tumors randomly selected from a panel of 38 NSCLC tumors, growth was completely suppressed by the applied low dose inventive combination regimen. Among them were two highly treatment resistant tumors that did not respond to any tested therapy previously (LU7406 and LU7414). During the complete treatment duration, no tumor outgrowth was observed in either PDX model, indicating a strong therapy efficacy by preventing development of an adaptive resistance against this regimen. After treatment discontinuation growth suppression remained up to 18 days (LU7860) with only slight increase in tumor growth. This indicates a long-lasting and sustained effect of the combination therapy on the tumor biology.

The inventive combination therapy regimen can be considered as a low dose treatment regimen. The two drugs with a more pronounced safety profile in this therapy regimen are cabozantinib and afatinib. Cabozantinib is approved for the treatment of renal cell carcinoma and hepatocellular carcinoma and is efficacious in different tumor models at dosages between 30-60mg/kg once a day (You et al., 2011; Paratala et al., 2018; Kato et al., 2018). Therefore, the inventive drug combination use in our study only includes 50% or less of typically used cabozantinib concentrations in recently published preclinical studies. Afatinib is approved for the treatment of NSCLC either with activating EGFR mutation or in squamous cell carcinoma under or after progression on platinum-based chemotherapy and is efficacious in tumor models at dosages from 20mg/kg or higher once a day (Floc'h N et al., 2018). In the inventive combination therapy regimen used in this preclinical study, both drugs were used significantly below these state of the art drug doses, each at 15mg/kg and in addition to that paused after every 5 days of treatment. Plerixafor, approved for stem cell mobilization, and

etoricoxib, approved for pain treatment and degenerative and auto-inflammatory diseases, were used at state of the art concentration levels in the respective *in vivo* models (Singla et al., 2015; Jayaraman et al., 2010). The usage of this inventive combination regimen showed an excellent safety profile of the inventive pharmaceutical composition and might lead to less pronounced side effects in patients. Together with its excellent efficacy this inventive combination would be superior to a higher prescribed medication of single cabozantinib or afatinib.

Treatment with the inventive combination therapy does not seem to change the histopathological composition of tumors when compared to placebo treatment regarding the extent of the tumor and the stroma compartment as well as the number of blood vessels. Since the studies have been performed in nude mice, it remains open whether this treatment regimen impacts the infiltration, composition and effector phenotype of T cells. However, our studies also revealed that the expression of PD-L1, whose therapeutic targeting has essentially improved the prognoses of even metastasized NSCLC patients, was not affected by the inventive combination therapy, offering the opportunity of innovative combination therapies including immune checkpoint inhibitors.

Taken together the inventive pharmaceutical composition including the combination of active agents simultaneously inhibiting pathways forming a cellular tumorigenic network in NSCLC tumors can overcome resistance mechanisms of targeted therapy drugs. All of the active agents used in this inventive combination regimen are approved for different indications, and therefore their clinical profiles such as pharmacokinetics, pharmacodynamics and toxicities are well known. They are readily available as study drugs for usage in a clinical trial. In order to identify NSCLC patient subgroups that may respond to this therapeutic concept, inclusion criteria should require the proof of expression of the respective target gene set. Collected tumor specimens can easily be used for mRNA expression analysis by RNA sequencing or by a customized gene expression array. The analysis of mRNA expression as a predictive biomarker is already in clinical development (Schuler et al., 2019).

FURTHER EMBODIMENTS OF THE PRESENT INVENTION

1. Agents for the therapy of tumor diseases, comprising the combination therapy for the simultaneous inhibition of the signaling pathways (a) VEGFs and VEGFR1-3, (b) SDF1alpha and CXCR4, (c) EGF and EGFR, (d) COX2, PGE2 and EP, and (e) HGF and HGFR (MET).

2. Agents according to embodiment 1, additionally comprising a further combination of (1) with a PD-L1 or PD-1 inhibitor.

3. Agents according to embodiment 1, comprising a specific combination therapy of cabozantinib (against VEGFR2 and HGFR), afatinib (against EGFR), plerixafor (against CXCR4), and etoricoxib (against COX2) at doses equal to or below the approved monotherapies for each drug.

4. Agents according to embodiment 3, comprising a further combination with pembrolizumab or nivolumab or durvalumab or another PD-1/PD-L1 inhibitor targeting PD-1 or PD-L1.

The indication(s) of the claimed agent(s) are for:

Non-small cell lung cancer (NSCLC) of any histology (adenocarcinoma or squamous cell carcinoma).

Non-small cell lung cancer (NSCLC) with or without driver mutations such as EGFRm, KRAS, FGFR, ALK, cKIT, BRAF.

Other lung tumor diseases including small cell lung cancer and large cell lung cancer with or without driver mutation.

Tumor diseases of any origin with or without driver mutation.

The further embodiments as mentioned above relate to a therapeutic anti-cancer regimen comprising small molecules, e.g., tyrosine kinase inhibitors, and/or antibodies that target the following signal transduction pathways in a simultaneous, combined therapeutic approach: (a) VEGFs and VEGFRs, (b) SDF1alpha and CXCR4, (c) EGF and EGFR, (d) COX2, PGE2 and EP, and (e) HGF and HGFR. An example of the present invention is the combination therapy of the FDA and EMA approved drugs of (a) cabozantinib (or bevacizumab or sunitinib or sorafenib) with (b) afatinib (or gefitinib or osimertinib or cetuximab or erlotinib) with (c)

plerixafor and with (d) etoricoxib (or celecoxib) for the treatment of patients with non-small cell lung cancer.

The applied therapeutic dose of each drug for this combination treatment is either equal to the dose for each approved indication of that drug or at a therapeutic
5 dose below the approved drug doses.

Treatment of highly resistant patient-derived NSCLC tumors with combination therapy of cabozantinib, afatinib, plerixafor, and etoricoxib results in complete inhibition of tumor growth at low doses and throughout the duration of treatment.

10

CLAIMS:

1. A pharmaceutical composition comprising a plurality of active agents in a therapeutically effective amount and one or more pharmaceutical excipients, characterized in that
 - 5 a. at least one active agent inhibits VEGF's and/or VEGFR1-3 signaling,
 - b. at least one active agent inhibits SDF1alpha and/or CXCR4 signaling,
 - c. at least one active agent inhibits EGF and/or EGFR1-3 signaling,
 - d. at least one active agent inhibits COX2 and/or PGE2 and/or EP signaling, and
 - 10 e. at least one active agent inhibits HGF and/or HGFR (MET) signaling.
2. The pharmaceutical composition according to claim 1, wherein the
 - a. at least one VEGF's and/or VEGFR1-3 inhibitor is selected from the group consisting of cabozantinib, regorafenib, bevacizumab, ramucirumab, pazopanib, sunitinib, sorafenib, ponatinib, vandetanib, axitinib, lenvatinib,
15 ziv-Aflibercept, tivozanib, golvatinib, and foretinib, preferably cabozantinib and regorafenib, and/or
 - b. at least one SDF1alpha and/or CXCR4 inhibitor is selected from the group consisting of plerixafor, motixafortide, LY2510924, and WZ811, preferably plerixafor, and/or
 - 20 c. at least one EGF and/or EGFR1-3 inhibitor is selected from the group consisting of afatinib, erlotinib, cetuximab, neratinib, necitumumab, gefitinib, lapatinib, osimertinib, canertinib, and saracatinib, preferably afatinib, cetuximab, neratinib, necitumumab, gefitinib, lapatinib, and osimertinib, more preferably afatinib, and/or
 - 25 d. at least one COX2 and/or PGE2 and/or EP inhibitor is selected from the group consisting of etoricoxib, celecoxib, parecoxib, rofecoxib, and valdecoxib, preferably etoricoxib and celecoxib, and/or
 - e. at least one active agent inhibiting HGF and/or HGFR (MET) is selected from the group consisting of cabozantinib, tivantinib, crizotinib, capmatinib,

tepotinib, savolitinib, golvatinib, and foretinib, preferably cabozantinib and tivantinib.

3. The pharmaceutical composition according to claim 1 or 2, wherein the composition comprises a combination of active agents selected from the group consisting of
- 5
- i. cabozantinib, afatinib, plerixafor, and etoricoxib,
 - ii. cabozantinib, afatinib, plerixafor, and celecoxib,
 - iii. cabozantinib, osimertinib, plerixafor, and etoricoxib,
 - iv. cabozantinib, osimertinib, plerixafor, and celecoxib,
 - 10 v. cabozantinib, necitumumab, plerixafor, and etoricoxib,
 - vi. cabozantinib, necitumumab, plerixafor, and celecoxib,
 - vii. cabozantinib, cetuximab, plerixafor, and etoricoxib,
 - viii. cabozantinib, cetuximab, plerixafor, and celecoxib,
 - ix. cabozantinib, neratinib, plerixafor, and etoricoxib,
 - 15 x. cabozantinib, neratinib, plerixafor, and celecoxib,
 - xi. cabozantinib, lapatinib, plerixafor, and etoricoxib ,
 - xii. cabozantinib, lapatinib, plerixafor, and celecoxib,
 - xiii. cabozantinib, gefitinib, plerixafor, and etoricoxib,
 - xiv. cabozantinib, gefitinib, plerixafor, and celecoxib,
 - 20 xv. regorafenib, necitumumab, plerixafor, etoricoxib and tivantinib,
 - xvi. regorafenib, necitumumab, plerixafor, celecoxib and tivantinib,
 - xvii. regorafenib, cetuximab, plerixafor, etoricoxib and tivantinib,
 - xviii. regorafenib, cetuximab, plerixafor, celecoxib and tivantinib,
 - xix. regorafenib, osimertinib, plerixafor, etoricoxib and tivantinib,
 - 25 xx. regorafenib, osimertinib, plerixafor, celecoxib and tivantinib,

- xxi. regorafenib, afatinib, plerixafor, etoricoxib and tivantinib,
- xxii. regorafenib, afatinib, plerixafor, celecoxib and tivantinib,
- xxiii. regorafenib, cetuximab, plerixafor, etoricoxib and cabozantinib,
- xxiv. regorafenib, cetuximab, plerixafor, celecoxib and cabozantinib,
- 5 xxv. regorafenib, necitumumab, plerixafor, etoricoxib and crizotinib, and
- xxvi. regorafenib, necitumumab, plerixafor, celecoxib and crizotinib.
4. The pharmaceutical composition according to any one of the preceding claims, wherein the composition further comprises
- 10 g. at least one active agent inhibiting PD-1 and/or PD-L1, wherein the PD-1 and/or PD-L1 inhibitor is preferably selected from the group consisting of pembrolizumab, nivolumab, durvalumab, and atezolizumab, preferably durvalumab.
5. The pharmaceutical composition according to any one of the preceding claims, wherein the pharmaceutical composition contains one, two or more separate
- 15 pharmaceutical dosage formulations, wherein each dosage formulation contains at least one active agent.
6. The pharmaceutical composition according to claim 5, wherein the one, two or more pharmaceutical dosage formulations are independently of each other the same or different, selected from the group consisting of
- 20 i. solid dosage forms for oral or enteral application, wherein solid dosage forms are preferably include tablets, capsules, spheroids, mini-tablets, pellets, granules, and pills;
- ii. semi-solid dosage forms for topical application to skin or mucous membranes, wherein semi-solid dosage forms are preferably include
- 25 creams, gels, ointments, and suppositories; and
- iii. liquid dosage forms for oral, external, or parenteral application, wherein liquid dosage forms preferably include mixtures, linctuses, elixirs, syrups, drops, lotions, liniments, collodions, nasal drops, nasal sprays, eye drops, inhalations, aerosols, and injections.

7. The pharmaceutical composition according to claim 5 or 6, wherein a first pharmaceutical dosage formulation contains the at least one active agent inhibiting b) SDF1alpha and CXCR4 and at least a second pharmaceutical dosage formulation contains two or more of the at least one active agents respectively inhibiting a) VEGF's and/or VEGFR1-3, c) EGF and/or EGFR1-3, d) COX2 and/or PGE2 and/or EP, e) HGF and/or HGFR (MET), and optionally f) PD-1 and/or PD-L1.
8. The pharmaceutical composition according to claim 7, wherein the first pharmaceutical formulation is a liquid dosage form, preferably an injection dosage form, and/or wherein the second pharmaceutical dosage form is a solid dosage form, preferably a tablet or capsule.
9. The pharmaceutical composition according to any of the preceding claims, wherein the dose regimen may comprise or consist of
- a. At least one active agent inhibiting VEGF's and/or VEGFR1-3 is administered once daily for up to five days followed by at least one, preferably two days without administration,
- b. At least one active agent inhibiting SDF1alpha and/or CXCR4 is administered once daily for up to five days, preferably for one day followed by at least one, preferably five days without administration,
- c. At least one active agent inhibiting EGF and/or EGFR1-3 is administered once daily for up to five days followed by at least one, preferably two days without administration,
- d. At least one active agent inhibiting COX2 and/or PGE2 and/or EP is administered once daily for up to five days followed by at least one, preferably two days without administration,
- e. At least one active agent inhibiting HGF and/or HGFR (MET) is administered once daily for up to five days followed by at least one, preferably two days without administration, and optionally
- f. At least one active agent inhibiting PD-1 and/or PD-L1 that is administered once daily for up to five days followed by at least one, preferably two days without administration.

10. A pharmaceutical composition according to any one of the preceding claims for use in the treatment or prophylaxis of a tumor, preferably selected from the group consisting of renal cell carcinoma (RCC), hepatocellular cancer (HCC), gastrointestinal stroma tumor (GIST), colorectal cancer (CRC), gastric cancer (GC) gastro-esophageal junction cancer (GEJ), neuro-endocrine tumor (NET), non-small cell lung cancer (NSCLC), chronic myeloid leukemia (CML) acute myeloid leukemia (AML), acute lymphatic leukemia (ALL), differentiated thyroid cancer (DTC), breast cancer, ovarian cancer, cervical cancer, fallopian tube cancer, peritoneal cancer, sarcoma, pancreatic cancer, Head & Neck carcinoma, glioma, lymphoma, respectively with or without driver mutations, such as EGFRm, KRAS, FGFR, ALK, cKIT, BRAF.
11. A method of treatment or prophylaxis of a tumor disease, preferably selected from the group consisting of renal cell carcinoma (RCC), hepatocellular cancer (HCC), gastrointestinal stroma tumor (GIST), colorectal cancer (CRC), gastric cancer (GC) gastro-esophageal junction cancer (GEJ), neuro-endocrine tumor (NET), non-small cell lung cancer (NSCLC), chronic myeloid leukemia (CML) acute myeloid leukemia (AML), acute lymphatic leukemia (ALL), differentiated thyroid cancer (DTC), breast cancer, ovarian cancer, cervical cancer, fallopian tube cancer, peritoneal cancer, sarcoma, pancreatic cancer, Head & Neck carcinoma, glioma, lymphoma, respectively with or without driver mutations, such as EGFRm, KRAS, FGFR, ALK, cKIT, BRAF, comprising administering to a patient in need a pharmaceutical composition in accordance with any one of claims 1 to 10.
12. The pharmaceutical composition according to claim 10, or the method of treatment or prophylaxis of a tumor disease according to claim 11, wherein the pharmaceutical composition is administered by one or more of the administration routes selected from enteral administration, preferably oral administration, and/or intranasal administration and/or sub-lingual administration and/or buccal administration of the inventive pharmaceutical composition that comprises one or more pharmaceutical formulations.
13. The pharmaceutical composition according to claim 10 or 12, or the method of treatment or prophylaxis of a tumor disease according to claim 11 or 12, wherein the inventive pharmaceutical composition is administered in an interval

without administration of the pharmaceutical composition, preferably in an interval comprising one or more, preferably five or more days of administration of the pharmaceutical composition followed by one or more days without administration of pharmaceutical composition.

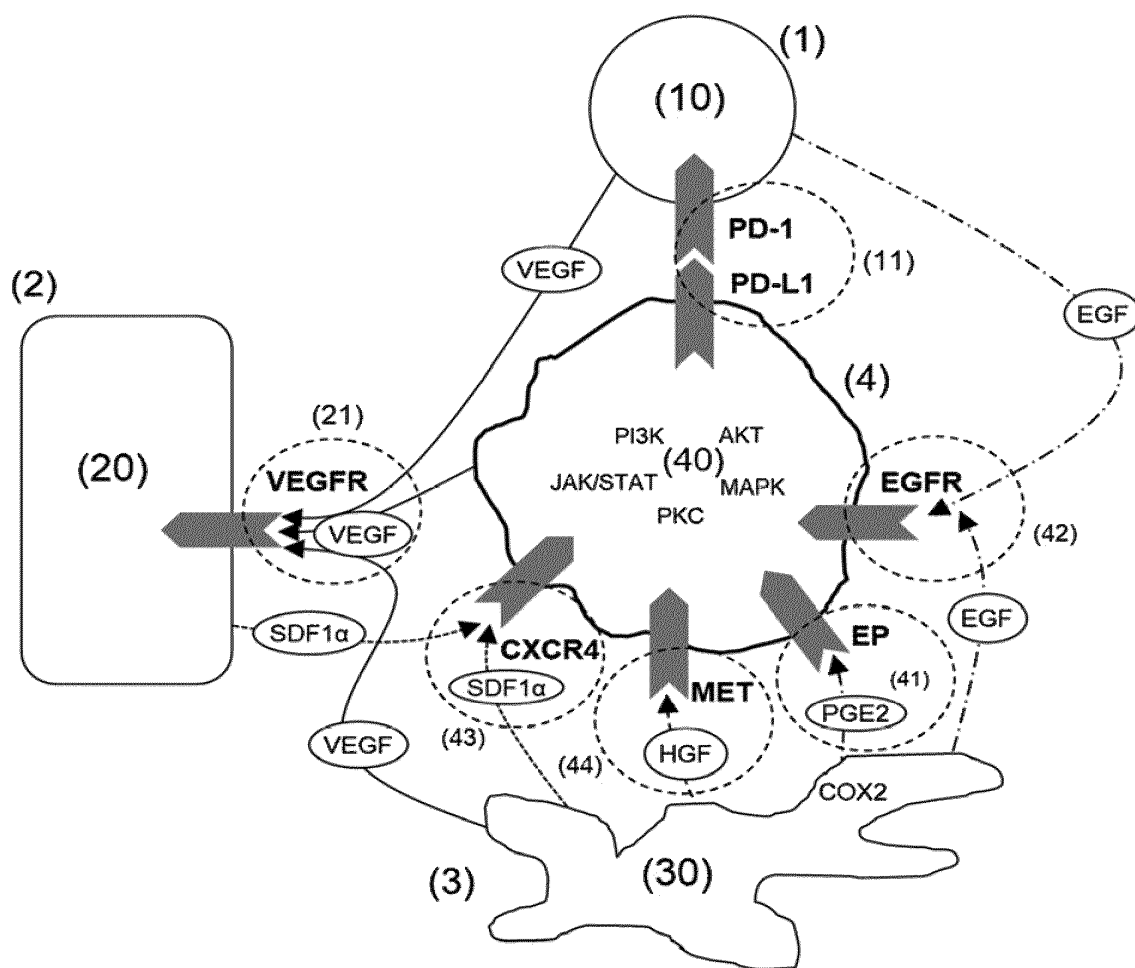


FIG. 1

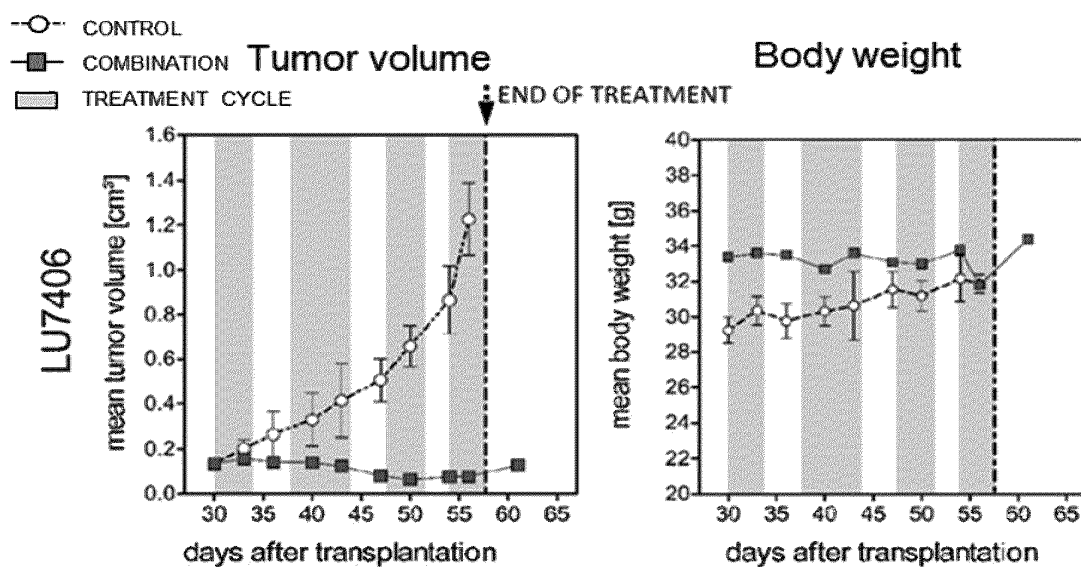


FIG. 2a)

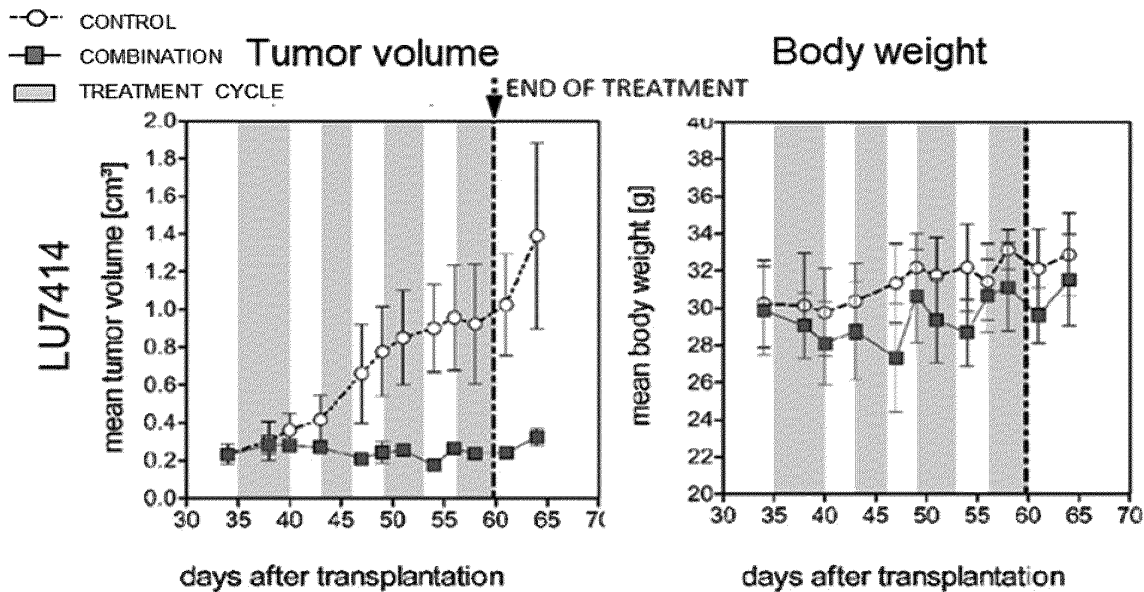


FIG. 2b)

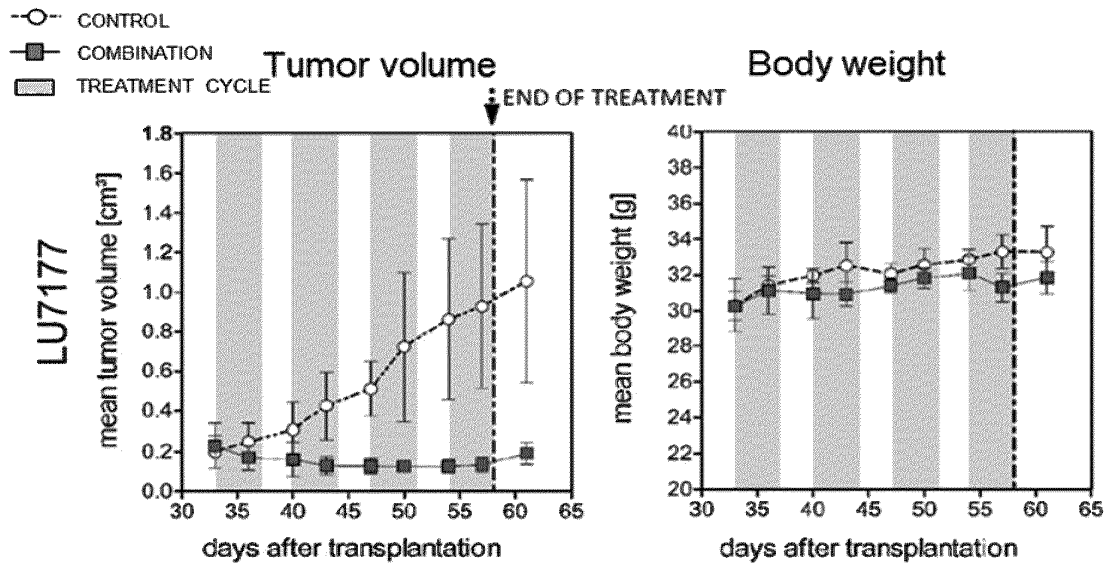


FIG. 2c)

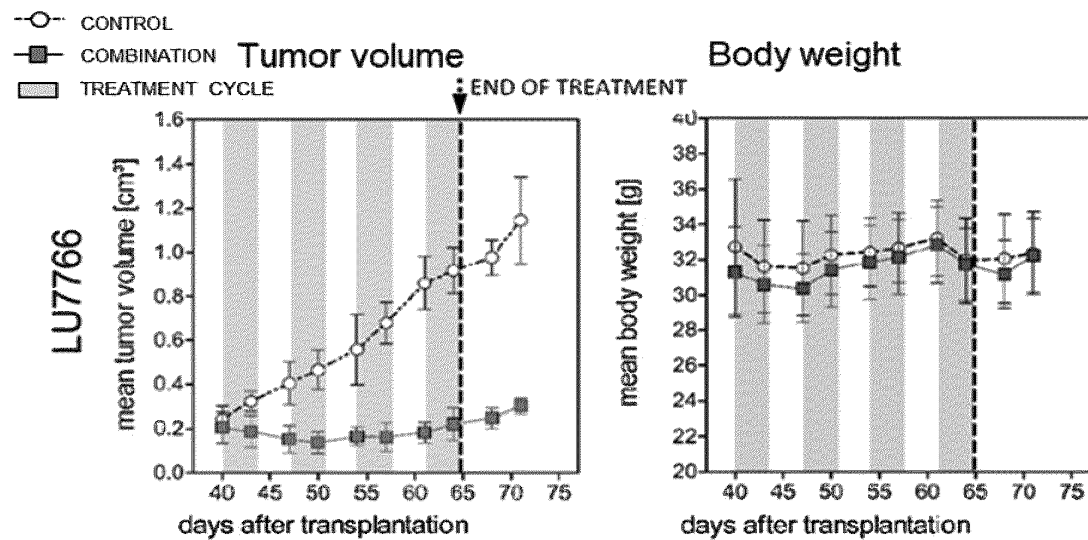


FIG. 2d)

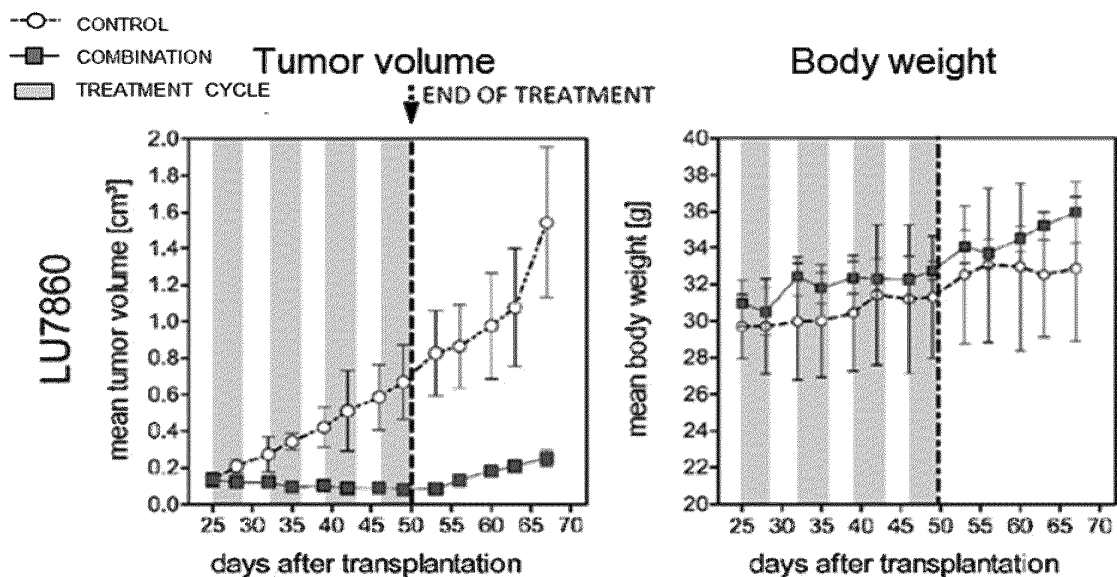


FIG. 2e)

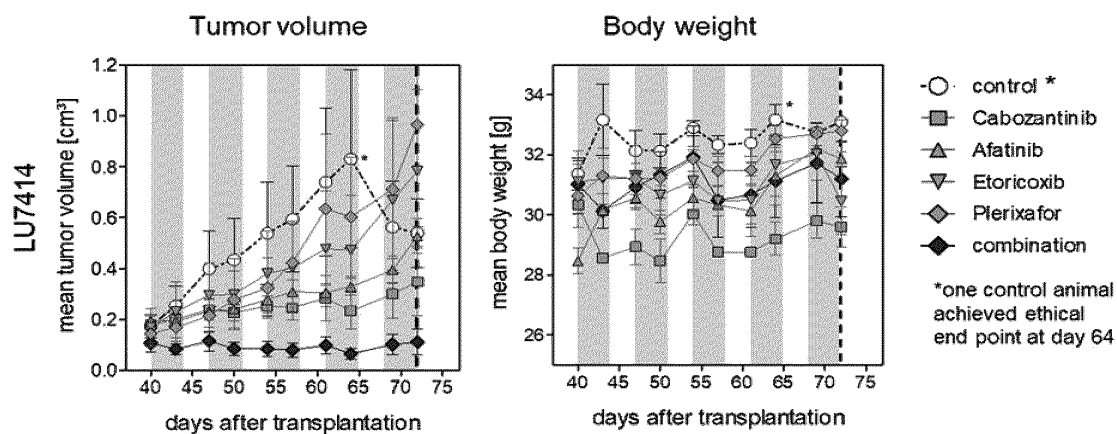


FIG. 2f)



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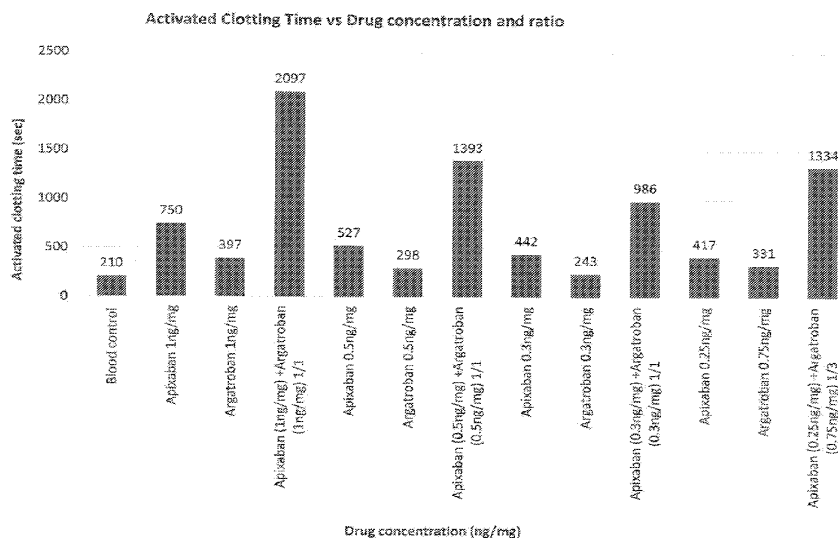


FIG. 2A

(57) Abstract: Devices, formulations, compositions, and methods are provided for inhibiting an inflammatory ophthalmic disease or eye condition in a patient. A therapeutic composition comprising a direct factor Xa inhibitor and/or a direct factor II inhibitor is provided. A therapeutically effective dose of the therapeutic composition is delivered to a site of the inflammatory ophthalmic disease or condition in the patient's eye(s). The therapeutic composition may be formulated for delivery to the patient by via injection, an implant, an eye drop, an emulsion, a suspension, an ointment, a nanomicelle, a nanoparticle, a nanosuspension, a liposome, an in-situ gel, a contact lens, or a microneedle or the like. The therapeutic composition may include one or more additional therapeutically active substances and/or one or more additional pharmaceutical agents.

[Continued on next page]

HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

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ANTICOAGULANT COMPOUNDS, METHODS AND DEVICES FOR OPHTHALMIC USE

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application No. 63/193,013 (Attorney Docket No. 32016-722.102), filed May 25, 2021, and of U.S. Provisional Application No. 63/077,170 (Attorney Docket No. 32016-724.101), filed September 11, 2020, each of which is incorporated herein by reference for all purposes in its entirety.

BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention. The present disclosure relates generally to anticoagulants and derivatives thereof and their use in therapeutic applications, and in particular with their use in ophthalmic applications.

[0003] As we age or as a result of eye condition, inflammatory disease of the eye or other disease conditions such as hypertension or diabetes, can manifest itself in the eye in various forms including but not limited to wet macular degeneration, dry macular degeneration, diabetic retinopathy, glaucoma, dry eye, etc. In some instances, drug therapies are available and have had some success in treating symptoms of the condition or disease but often fail to address the underlying inflammatory disease condition. For example, anti-angiogenic agents can be used to treat wet macular degeneration, and about 50% of patients respond to such therapy, but such agents do not adequately address the inflammatory cause of the disease condition and in many cases further deterioration of visual acuity or the eye occurs.

[0004] Systemic drugs have had limited success in addressing these conditions or in long term effectiveness to address such conditions.

[0005] It is therefore an objective of the present invention to provide improved compositions, methods, and devices for treating wet macular degeneration, dry macular degeneration, diabetic retinopathy, glaucoma, dry eye, and other inflammatory conditions in the eye. At least some of these objectives will be met by the inventions described and claimed herein.

[0006] 2. Background Art. Ophthalmic solutions incorporating anti-inflammatory and anti-coagulant substances are described in WO2005/077344 and WO2007056218. Other background art includes US6,500,855; US8,409,272; US8,946,219; US2003/0158120; US2005/0064006; US2009/0075949; US2010/0003542; US2010/0130543; US2010/0184729; US2018/0000490; EP1849434; CA2464290; and WO2013/007840.

[0007] The subject matter of this application is also related to that of the following commonly owned applications: PCT/US2021/34108 (Attorney Docket No. 32016-720.601), filed May 25, 2021 and PCT/US2021/44414 (Attorney Docket No. 32016-723.601), filed August 3, 2021, the full disclosures of which are incorporated herein by reference.

SUMMARY OF THE DISCLOSURE

[0008] In many cases, inflammation, chronic inflammation, formation or increase of fluid build-up, fibrosis, thrombin, cell apoptosis, and/or fibrin/clot formation occur, thereby damaging the eye or ocular tissues. The condition continues to upregulate the inflammatory and/or immune reaction pathway and generate excessive amounts of one of thrombin, fibrin, immune reaction which in turn can lead to or increasing the likelihood of morbidities.

[0009] There is still a need to develop specialized therapeutic compositions for ophthalmic applications which can prevent, reduce, dissolve, resolve, inhibit, or eliminate one or more of fibrin and/or clot, thrombin, immune reaction, glaucoma, and inflammation and reduce associated eye morbidities and mortalities/blindness.

[0010] It would therefore be desirable to provide a formulation or a devices that locally deliver one or more agents to inhibit or dissolve thrombin/clot formation, inflammation, immune response, and glaucoma and optionally other types of biologically active agents (e.g., anti-fibrotic agents, other anti-inflammatory agents, antiviral agents, antibiotic agents, anti-proliferative agents, immune suppressant agents, etc.), to the eye to inhibit, reduce, and/or prevent coagulation, fibrin formation, thrombin formation, inflammation, glaucoma, immune reaction, and/or clot formation in the eye(s), and/or fibrosis of the ocular tissue, and/or inflammation of the ocular fluid or tissue, and/or to treat viral infections, and/or to treat bacterial infections.

[0011] In one aspect, a method of treating an inflammatory ophthalmic condition or disease in a patient is provided. The method comprises providing a therapeutic composition comprising a direct factor Xa inhibitor and/or a direct factor IIa inhibitor; and delivering a therapeutically effective dose of the therapeutic composition to a site of the inflammatory ophthalmic condition or disease in the patient's eye.

[0012] In some examples, the therapeutically effective dose of the therapeutic composition is effective to suppress or prevent initiation, progression, or relapses of disease, including the progression of established disease.

[0013] In some examples, delivering the therapeutically effective dose of the therapeutic composition comprises administering the therapeutically effective dose via injection, an implant (such as an shunt or stent), an eye drop, an emulsion, a suspension, an ointment, a nanomicelle,

a nanoparticle, a nanosuspension, a liposome, an in-situ gel, a contact lens, a reservoir inside or outside the eye, or a microneedle.

[0014] In some examples, the inflammatory ophthalmic condition or disease comprises one or more of glaucoma, angiogenesis, leaking blood vessel, protein deposition, fibrosis, extracellular matrix (ECM) deposition, thrombin generation, clot formation, or fibrin formation.

[0015] In some examples, the site of the inflammatory ophthalmic condition or disease is selected from the group consisting of an eyelid, a meibomian gland, a trabecular meshwork, Schlemm's canal, a cornea, a sclera, a vitreous, a choroid, a uvea, and a retina.

[0016] In some examples, the inflammatory ophthalmic condition or disease is selected from the group consisting of wet age-related macular degeneration (AMD), dry AMD, diabetic retinopathy, glaucoma, uveitis, cataracts, conjunctivitis, and dry eye disease.

[0017] In some examples, the method comprises diagnosing the patient as having the inflammatory ophthalmic condition or disease prior to delivering the therapeutically effective dose of the therapeutic composition.

[0018] In some examples, the direct factor Xa inhibitor is selected from the group consisting of apixaban, betrixaban, edoxaban, otamixaban, razaxaban, rivaroxaban, (r)-n-(2-(4-(1-methylpiperidin-4-yl)piperazin-1-yl)-2-oxo-1-phenylethyl)-1h-indole-6-carboxamide(LY-517717), daraxaban (YM-150), 2-[(7-carbamimidoylnaphthalen-2-yl)methyl-[4-(1-ethanimidoylpiperidin-4-yl)oxyphenyl]sulfamoyl]acetic acid (YM-466 or YM-60828), eribaxaban (PD 0348292), 2-(5-carbamimidoyl-2-hydroxy-phenyl) 4-[5-(2,6-dimethylpiperidin-1-yl)-pentyl]-3-oxo-3, 4-dihydro-quinoxaline-6-carboxylic acid(PD0313052), (S)-3-(7-carbamimidoylnaphthalen-2-yl)-2-(4-(((S)-1-(1-iminoethyl)pyrrolidin-3-yl)oxy)phenyl)propanoic acid hydrochloride pentahydrate(DX9065a), letaxaban (TAK-442), GW813893, JTV-803, KFA-144, DPC-423, RPR-209685, MCM-09, or antistasin. For example, the direct factor Xa inhibitor may comprise rivaroxaban, or a salt, isomer, solvate, analog, derivative, metabolite, or prodrugs thereof. Alternatively, or in combination, the direct factor Xa inhibitor may comprise apixaban,

[0019] In some examples, an IC_{50} of the direct factor Xa inhibitor is within a range of about 0.01 nM to about 1000 nM. For example, the IC_{50} of the direct factor Xa inhibitor is within a range of about 0.1 nM to about 1000 nM, about 1 nM to about 1000 nM, or about 10 nM to about 1000 nM.

[0020] In some examples, the therapeutically effective dose of the direct factor Xa inhibitor is within a range of about 1 microgram to 10mg, more preferably is within a range of about 50 micrograms to about 10 mg. For example, the therapeutically effective dose of the

direct factor Xa inhibitor may be within a range of about 0.1 mg to about 10 mg or about 1 mg to about 5 mg.

[0021] In some examples, the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 0.001 ng/g tissue to about 100 mg/g tissue, preferably, the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 0.01 ng/g tissue to about 100 mg/g tissue, more preferably, the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue. For example, the therapeutically effective dose may be sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 0.2 ng/g tissue to about 100 mg/g tissue, about 0.5 ng/g tissue to about 100 mg/g tissue, about 1 ng/g tissue to about 100 mg/g tissue, about 10 ng/g tissue to about 100 mg/g tissue, or about 100 ng/g tissue to about 100 mg/g tissue; in about 1 day, 30 days, 60 days, 90 days, or 120 days after introducing the therapeutically effective dose. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor Xa inhibitor which is less than about 200 ng/ml, 100 ng/ml, 50 ng/ml, 25 ng/ml, or 10 ng/ml. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor Xa inhibitor which is less than a systemic therapeutic concentration of the direct factor Xa inhibitor for any systemic indication. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor Xa inhibitor which is smaller than a median maximum serum concentration (C_{max}) of the direct factor Xa inhibitor generated by systemic delivery of the direct factor Xa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor Xa inhibitor which does not exceed a median maximum serum concentration (C_{max}) of the direct factor Xa inhibitor generated by systemic delivery of the direct factor Xa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease for more than about 6 hours to about 3 days.

[0022] In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of the direct factor Xa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 120 days, for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0023] In one aspect, a method of treating an inflammatory ophthalmic condition or disease in a patient is provided. The method comprises providing a therapeutic composition comprising a direct factor IIa inhibitor; and delivering a therapeutically effective dose of the

therapeutic composition to a site of the inflammatory ophthalmic condition or disease in the patient's eye.

[0024] In some examples, the therapeutically effective dose of the therapeutic composition is effective to suppress or prevent initiation, progression, or relapses of disease, including the progression of established disease.

[0025] In some examples, delivering the therapeutically effective dose of the therapeutic composition comprises administering the therapeutically effective dose via injection, an implant (such as an ocular shunt or stent), an eye drop, an emulsion, a suspension, an ointment, a nanomicelle, a nanoparticle, a nanosuspension, a liposome, an in-situ gel, a contact lens, or a microneedle.

[0026] In some examples, the inflammatory ophthalmic condition or disease comprises one or more of glaucoma, angiogenesis, leaking blood vessel, protein deposition, fibrosis, extracellular matrix (ECM) deposition, thrombin generation, clot formation, or fibrin formation.

[0027] In some examples, the site of the inflammatory ophthalmic condition or disease is selected from the group consisting of an eyelid, a meibomian gland, a trabecular meshwork, Schlemm's canal, a cornea, a sclera, a vitreous, a choroid, a uvea, and a retina.

[0028] In some examples, the inflammatory ophthalmic condition or disease is selected from the group consisting of wet age-related macular degeneration (AMD), dry AMD, diabetic retinopathy, glaucoma, uveitis, cataracts, conjunctivitis, and dry eye disease.

[0029] In some examples, the method comprises diagnosing the patient as having the inflammatory ophthalmic condition or disease prior to delivering the therapeutically effective dose of the therapeutic composition.

[0030] In some examples, the factor IIa therapeutic composition is selected from the group consisting of argatroban, dabigatran, ximelagatran, melagatran, efegatran, inogatran, atecagatran metoxil (AZD-0837), hirudin, hirudin analogs, bivalirudin, desirudin, or lepirudin. In some examples, the direct factor IIa inhibitor comprises argatroban or a salt, isomer, solvate, analog, derivative, metabolite, or prodrugs thereof.

[0031] In some examples, an IC_{50} of the direct factor Xa inhibitor is within a range of about 0.01 nM to about 1000 nM. For example, the IC_{50} of the direct factor Xa inhibitor is within a range of about 0.1 nM to about 1000 nM, about 1 nM to about 1000 nM, or about 10 nM to about 1000 nM.

[0032] In some examples, the therapeutically effective dose of the direct factor Xa inhibitor is within a range of about 1 microgram to 10mg, more preferably is within a range of about 50 micrograms to about 10 mg. For example, the therapeutically effective dose of the

direct factor Xa inhibitor may be within a range of about 0.1 mg to about 10 mg or about 1 mg to about 5 mg.

[0033] In some examples, the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 0.001 ng/g tissue to about 100 mg/g tissue, preferably, the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 0.01 ng/g tissue to about 100 mg/g tissue, more preferably, the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue. For example, the therapeutically effective dose may be sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 0.2 ng/g tissue to about 100 mg/g tissue, about 0.5 ng/g tissue to about 100 mg/g tissue, about 1 ng/g tissue to about 100 mg/g tissue, about 10 ng/g tissue to about 100 mg/g tissue, or about 100 ng/g tissue to about 100 mg/g tissue; in about 1 day, 30 days, 60 days, 90 days, or 120 days after introducing the therapeutically effective dose. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which is less than about 200 ng/ml, 100 ng/ml, 50 ng/ml, 25 ng/ml, or 10 ng/ml. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which is less than a systemic therapeutic concentration of the direct factor IIa inhibitor for any systemic indication. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which is smaller than a median maximum serum concentration (C_{max}) of the direct factor IIa inhibitor generated by systemic delivery of the direct factor IIa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which does not exceed a median maximum serum concentration (C_{max}) of the direct factor IIa inhibitor generated by systemic delivery of the direct factor IIa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease for more than about 6 hours to about 3 days.

[0034] In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of the direct factor IIa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 120 days, for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0035] In some examples, the therapeutic composition comprises at least two therapeutic active substances comprising a direct factor Xa inhibitor and a direct factor IIa inhibitor.

[0036] In some examples, the therapeutic composition comprising a factor Xa inhibitor further comprises at least one additional therapeutically active substance. In some examples, the

at least one additional therapeutically active substance comprises a direct factor IIa inhibitor selected from the group consisting of argatroban, dabigatran, ximelagatran, melagatran, efegatran, inogatran, atecagatran metoxil (AZD-0837), hirudin, hirudin analogs, bivalirudin, desirudin, or lepirudin. In some examples, the direct factor IIa inhibitor comprises argatroban. or a salt, isomer, solvate, analog, derivative, metabolite, or prodrugs thereof. In some examples, the direct factor Xa inhibitor comprises apixaban and the direct factor IIa inhibitor comprises argatroban. In some examples, the therapeutically effective dose of the direct factor IIa inhibitor is within a range of about 50 micrograms to about 10 mg. In some examples, the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue. For example, the therapeutically effective dose may be sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 0.2 ng/g tissue to about 100 mg/g tissue, about 0.5 ng/g tissue to about 100 mg/g tissue, about 1 ng/g tissue to about 100 mg/g tissue, about 10 ng/g tissue to about 100 mg/g tissue, or about 100 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which is less than about 200 ng/ml, 100 ng/ml, 50 ng/ml 25 ng/ml, or 10 ng/ml. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which is smaller than a median maximum serum concentration (C_{max}) of the direct factor IIa inhibitor generated by systemic delivery of the direct factor IIa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which does not exceed a median maximum serum concentration (C_{max}) of the direct factor IIa inhibitor generated by systemic delivery of the direct factor IIa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease for more than about 6 hours to about 3 days. IN some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of the direct factor IIa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year. In some examples, the weight compositional ratio of the direct factor Xa inhibitor to the direct factor IIa inhibitor in the therapeutic composition is within a range of about 3:1 to about 1:3. For example, the weight compositional ratio of the direct factor Xa inhibitor to the direct factor IIa inhibitor in the therapeutic composition may be about 1:1.

[0037] In some examples, the therapeutic composition comprises one or more anticoagulant agents that has an IC₅₀ to inhibit factor Xa and factor II at a dose ranging from 0.0001nM to 1000nM, preferably at a dose ranging from 0.0001nM to 100nM, more preferably

at a dose ranging from 0.0001nM to 10nM, and most preferably at a dose ranging from 0.0001nM to 1nM.

[0038] In some examples, the therapeutic composition comprising a factor Xa inhibitor, a factor IIa inhibitor, or a combination of a factor Xa inhibitor and a factor IIa inhibitor, administered in combination with one or more additional pharmaceutical agents. In some examples, the one or more additional pharmaceutical agents comprises one or more anti-platelet agent, anti-fibrotic agents, anti-inflammatory agents, anti-proliferative agents, anti-glaucoma agents, immune suppressive agents, anti-diabetic agents, anti-viral agents, anti-angiogenic agents, anti-VEGF agents, serine protease, complement activation inhibitor, anti-histamine agents, or combinations thereof.

[0039] In some examples, the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration sufficient to generate a blood concentration of pirfenidone of less than about 20 µg/ml, 12 µg/ml, 7 µg/ml, or 6.5 µg/ml. In some examples, the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pirfenidone of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pirfenidone of within a range of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pirfenidone of within a range of about 10 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of pirfenidone of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0040] In some examples, the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a blood concentration of nintedanib of less than about 100 ng/ml, 50 ng/ml, 40 ng/ml, or 30 ng/ml. In some examples, the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of nintedanib of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of nintedanib of within a range of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of nintedanib of within a range of about 10 ng/mg tissue to about 100 mg/g tissue. In some

examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of nintedanib of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year. In some examples, the anti-fibrotic agent comprises nintedanib and the therapeutically effective dose comprises nintedanib within a range of about 0.1 mg to about 5 mg.

[0041] In some examples, the anti-viral agent or anti-diabetic agent comprises metformin or its salt and is present in the therapeutic composition at a concentration sufficient to generate a blood concentration of metformin or its salt of less than about 1800 µg/ml, 900 µg/ml, 500 µg/ml, 50 µg/ml, 10 µg/ml, 1.3 µg/ml, 1 µg/ml, or 0.8 µg/ml. In some examples, the anti-viral agent or anti-diabetic agent comprises metformin or its salt and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of metformin or its salt of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-viral agent or anti-diabetic agent comprises metformin or its salt and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of metformin or its salt of within a range of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of metformin or its salt of about 0.1 ng/mg tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0042] In some examples, the anti-angiogenic agent comprises an anti-VEGF agent. In some examples, the anti-VEGF agent comprises ranibizumab, aflibercept, pegaptanib sodium, bevacizumab, brolucizumab, lapatinib, sorafenib, axitinib, pazopanib, sunitinib, cabozantinib, lenvatinib, ponatinib, ramucirumab, regorafenib, vandetanib, cediranib, thiazolidinediones, abicipar pegol, faricimab, conbercept, or RGX-314, or other direct or indirect anti-VEGF agents.

[0043] In some examples, the anti-VEGF agent comprises ranibizumab and is present in the therapeutic composition at a concentration within a range of about 6 mg/ml to about 40 mg/ml. In some examples, the anti-VEGF agent comprises ranibizumab and the therapeutically effective dose comprises ranibizumab within a range of about 0.3 mg to about 2 mg. In some examples, the anti-VEGF agent comprises ranibizumab and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of ranibizumab of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue or about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of ranibizumab within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0044] In some examples, the anti-VEGF agent comprises aflibercept and is present in the therapeutic composition at a concentration within a range of about 10 mg/ml to about 100 mg/ml. In some examples, the anti-VEGF agent comprises aflibercept and the therapeutically effective dose comprises aflibercept within a range of about 0.5 mg to about 5 mg. In some examples, the anti-VEGF agent comprises aflibercept and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of aflibercept of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue or about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of aflibercept within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0045] In some examples, the anti-VEGF agent comprises pegaptanib and is present in the therapeutic composition at a concentration within a range of about 1 mg/ml to about 10 mg/ml. In some examples, the anti-VEGF agent comprises pegaptanib and the therapeutically effective dose comprises pegaptanib within a range of about 0.1 mg to about 1 mg. In some examples, the anti-VEGF agent comprises pegaptanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pegaptanib of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue or about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of pegaptanib within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0046] In some examples, the anti-proliferative agent comprises an mTOR inhibitor. In some examples, the mTOR inhibitor comprises rapamycin, everolimus, biolimus, temsirolimus, ridaforolimus, zotarolimus, myolimus, or novolimus, or analogues, metabolites, or salts thereof.

[0047] In some examples, the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration within a range of 1 mg/ml to about 250 mg/ml. In some examples, the anti-proliferative agent comprises sirolimus and the therapeutically effective dose comprises sirolimus within a range of about 50 µg to about 5 mg, about 0.1 mg to about 5 mg, or about 0.5 mg to about 3 mg. In some examples, the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of sirolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of sirolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 1 day. In some examples, the anti-proliferative agent

comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of sirolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 72 days. In some examples, the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to maintain a tissue concentration of sirolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0048] In some examples, the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration within a range of 1 mg/ml to about 250 mg/ml. In some examples, the anti-proliferative agent comprises novolimus and the therapeutically effective dose comprises novolimus within a range of about 50 µg to about 5 mg, about 0.1 mg to about 5 mg, or about 0.5 mg to about 3 mg. In some examples, the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of novolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of novolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 1 day. In some examples, the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of novolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 72 days. In some examples, the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient to maintain a tissue concentration of novolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0049] In some examples, the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration within a range of 1 mg/ml to about 250 mg/ml. In some examples, the anti-proliferative agent comprises myolimus and the therapeutically effective dose comprises myolimus within a range of about 50 µg to about 5 mg, about 0.1 mg to about 5 mg, or about 0.5 mg to about 3 mg. In some examples, the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of myolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of myolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to

about 100 mg/g tissue within about 1 day. In some examples, the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of myolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 72 days. In some examples, the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to maintain a tissue concentration of myolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0050] In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the anti-proliferative agent which is smaller than a median maximum serum concentration ($C_{\max}$) of the anti-proliferative agent generated by systemic delivery of the anti-proliferative agent to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the anti-proliferative agent which does not exceed a median maximum serum concentration ($C_{\max}$) of the anti-proliferative agent generated by systemic delivery of the anti-proliferative agent to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease for more than about 6 hours to about 7 days. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the anti-proliferative agent which is less than about 10 ng/ml.

[0051] In some examples, the serine protease agent comprises tissue plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA), Streptokinase or plasmin and is present in the therapeutic composition at a concentration within a range of 1 mg/ml to about 250 mg/ml. In some examples, the serine protease agent comprises t-PA and the therapeutically effective dose comprises a range of about 50 μ g to about 5 mg, about 25 μ g to about 5 mg, or about 0.3 μ g to about 3 mg.

[0052] In some examples, the thrombolytic drugs comprise complement inhibitors target the modulation of complement proteins C3, C5, factor B, factor D, and properdin. Complement activation inhibitors include but not limit to C3 inhibitor such as APL-2, Compstatin and AMY106, Anti-factor D such as Lampalizumab, Anti-Properdin such as CLG561, Anti-C5 such as Eculizumab, Ravulizumab, ARC1905, and LFG316, CD59 such as AAVCAGsCD59, analogues and the like. In some examples, the thrombolytic drugs comprise complement activation inhibitors and the therapeutically effective dose comprises a range of about 20 μ g to about 2000 mg, about 10 μ g to about 1500 mg, or about 5 μ g to about 1200 mg.

[0053] In some examples, the therapeutic composition comprises one or more of a pharmaceutically acceptable carrier, a diluent, an adjuvant, an excipient, a vehicle, a preserving

agent, a filler, a polymer, a disintegrating agent, a glidant, a wetting agent, an emulsifying agent, a suspending agent, a sweetening agent, a flavoring agent, a perfuming agent, a lubricating agent, an acidifying agent, and a dispensing agent.

[0054] In some examples, the therapeutically effective dose of the therapeutic composition is effective to inhibit thrombosis, inhibit clot formation, or inhibit fibrin formation in the patient's eye.

[0055] In another aspect, a therapeutic composition for inhibiting an inflammatory ophthalmic condition or disease in a patient is provided. The composition comprises a direct factor Xa inhibitor formulated for delivery to an eye of the patient by any one of injection, implant (such as an ocular shunt or stent), eye drop, emulsion, suspension, ointment, nanocarrier, nanomicelle, nanoparticle, nanosuspension, liposome, in-situ gel, contact lens, reservoir (implanted insider or outside the eye) or microneedle.

[0056] In some examples, delivery of a therapeutically effective dose of the therapeutic composition is effective to suppress or prevent initiation, progression, or relapses of disease, including the progression of established disease.

[0057] In some examples, the inflammatory ophthalmic condition or disease comprises one or more of angiogenesis, leaking blood vessel, protein deposition, fibrosis, extracellular matrix (ECM) deposition, thrombin generation, clot formation, glaucoma, or fibrin formation.

[0058] In some examples, a site of the inflammatory ophthalmic condition or disease is selected from the group consisting of an eyelid, a meibomian gland, a trabecular meshwork, Schlemm's canal, a cornea, a sclera, a vitreous, a choroid, a uvea, and a retina.

[0059] In some examples, the inflammatory ophthalmic condition or disease is selected from the group consisting of wet age-related macular degeneration (AMD), dry AMD, diabetic retinopathy, glaucoma, uveitis, cataracts, conjunctivitis, glaucoma, and dry eye disease.

[0060] In some examples, the direct factor Xa inhibitor is selected from the group consisting of apixaban, betrixaban, edoxaban, otamixaban, razaxaban, rivaroxaban, (r)-n-(2-(4-(1-methylpiperidin-4-yl)piperazin-1-yl)-2-oxo-1-phenylethyl)-1h-indole-6-carboxamide (LY-517717), daraxaban (YM-150), 2-[(7-carbamimidoylnaphthalen-2-yl)methyl-4-(1-ethanimidoylpiperidin-4-yl)oxyphenyl]sulfamoyl]acetic acid (YM-466 or YM-60828), or eribaxaban (PD 0348292), 2-(5-carbamimidoyl-2-hydroxy-phenyl) 4-[5-(2,6-dimethylpiperidin-1-yl)-pentyl]-3-oxo-3, 4-dihydro-quinoxaline-6-carboxylic acid (PD0313052), (S)-3-(7-carbamimidoylnaphthalen-2-yl)-2-(4-(((S)-1-(1-iminoethyl)pyrrolidin-3-yl)oxy)phenyl)propanoic acid hydrochloride pentahydrate (DX9065a), letaxaban (TAK-442), GW813893, JTV-803, KFA-144, DPC-423, RPR-209685, MCM-09, or antistasin. For example, the direct factor Xa inhibitor may comprise rivaroxaban, or a salt, isomer, solvate, analog,

derivative, metabolite, or prodrugs thereof. Alternatively, or in combination, the direct factor Xa inhibitor may comprise apixaban, or a salt, isomer, solvate, analog, derivative, metabolite, or prodrugs thereof.

[0061] In some examples, an IC_{50} of the direct factor Xa inhibitor is within a range of about 0.01 nM to about 1000 nM. For example, the IC_{50} of the direct factor Xa inhibitor is within a range of about 0.1 nM to about 1000 nM, about 1 nM to about 1000 nM, or about 10 nM to about 1000 nM.

[0062] In some examples, a therapeutically effective dose of the direct factor Xa inhibitor is within a range of about 50 micrograms to about 10 mg. For example, the therapeutically effective dose of the direct factor Xa inhibitor may be within a range of about 0.1 mg to about 10 mg or about 1 mg to about 5 mg.

[0063] In some examples, a therapeutically effective dose of the direct factor Xa inhibitor is sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue. For example, the therapeutically effective dose may be sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 0.2 ng/g tissue to about 100 mg/g tissue, about 0.5 ng/g tissue to about 100 mg/g tissue, about 1 ng/g tissue to about 100 mg/g tissue, about 10 ng/g tissue to about 100 mg/g tissue, or about 100 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor Xa inhibitor which is less than about 200 ng/ml, 100 ng/ml, 50 ng/ml, 25 ng/ml, or 10 ng/ml. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor Xa inhibitor which is less than a systemic therapeutic concentration of the direct factor Xa inhibitor for any systemic indication. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor Xa inhibitor which is smaller than a median maximum serum concentration (C_{max}) of the direct factor Xa inhibitor generated by systemic delivery of the direct factor Xa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor Xa inhibitor which does not exceed a median maximum serum concentration (C_{max}) of the direct factor Xa inhibitor generated by systemic delivery of the direct factor Xa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease for more than about 6 hours to about 3 days.

[0064] In some examples, a therapeutically effective dose of the direct factor Xa inhibitor is sufficient to maintain a tissue concentration of the direct factor Xa inhibitor of about 0.1 ng/g

tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0065] In some examples, the therapeutic composition further comprises at least one additional therapeutically active substance. In some examples, the at least one additional therapeutically active substance comprises a direct factor IIa inhibitor selected from the group consisting of argatroban, dabigatran, ximelagatran, melagatran, efegatran, inogatran, atecegatran metoxil (AZD-0837), hirudin, hirudin analogs, bivalirudin, desirudin, or lepirudin. In some examples, the direct factor IIa inhibitor comprises argatroban, or a salt, isomer, solvate, analog, derivative, metabolite, or prodrugs thereof. In some examples, the direct factor Xa inhibitor comprises apixaban and the direct factor IIa inhibitor comprises argatroban. In some examples, a therapeutically effective dose of the direct factor IIa inhibitor is within a range of about 50 micrograms to about 10 mg. In some examples, the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue. For example, the therapeutically effective dose may be sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 0.2 ng/g tissue to about 100 mg/g tissue, about 0.5 ng/g tissue to about 100 mg/g tissue, about 1 ng/g tissue to about 100 mg/g tissue, about 10 ng/g tissue to about 100 mg/g tissue, or about 100 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which is less than about 200 ng/ml, 100 ng/ml, 50 ng/ml, 25 ng/ml, or 10 ng/ml. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which is smaller than a median maximum serum concentration ($C_{\max}$) of the direct factor IIa inhibitor generated by systemic delivery of the direct factor IIa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which does not exceed a median maximum serum concentration ($C_{\max}$) of the direct factor IIa inhibitor generated by systemic delivery of the direct factor IIa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease for more than about 6 hours to about 3 days. In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of the direct factor IIa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year. In some examples, the weight compositional ratio of the direct factor Xa inhibitor to the direct factor IIa inhibitor in the therapeutic composition is within a range of about 3:1 to about 1:3. For example, the weight

compositional ratio of the direct factor Xa inhibitor to the direct factor IIa inhibitor in the therapeutic composition may be about 1:1.

[0066] In some examples, the therapeutic composition is administered in combination with one or more additional pharmaceutical agents. In some examples, the one or more additional pharmaceutical agents comprises one or more anti-fibrotic agents, anti-inflammatory agents, anti-histamine agents, anti-proliferative agents, anti-diabetic agents, anti-viral agents, metformin, anti-angiogenic agents, or combinations thereof.

[0067] In some examples, the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration sufficient to generate a blood concentration of pirfenidone of less than about 20 µg/ml, 12 µg/ml, 7 µg/ml, or 6.5 µg/ml. In some examples, the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pirfenidone of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pirfenidone of within a range of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pirfenidone of within a range of about 10 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of pirfenidone of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0068] In some examples, the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a blood concentration of nintedanib of less than about 100 ng/ml, 50 ng/ml, 40 ng/ml, or 30 ng/ml. In some examples, the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of nintedanib of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of nintedanib of within a range of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of nintedanib of within a range of about 10 ng/mg tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of nintedanib of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year. In some examples,

the anti-fibrotic agent comprises nintedanib and the therapeutically effective dose comprises nintedanib within a range of about 0.1 mg to about 5 mg.

[0069] In some examples, the anti-viral agent or anti-diabetic agent comprises metformin or its salt and is present in the therapeutic composition at a concentration sufficient to generate a blood concentration of metformin or its salt of less than about 1800 µg/ml, 900 µg/ml, 500 µg/ml, 50 µg/ml, 10 µg/ml, 1.3 µg/ml, 1 µg/ml, or 0.8 µg/ml. In some examples, the anti-viral agent or anti-diabetic agent comprises metformin or its salt and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of metformin or its salt of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-viral agent or anti-diabetic agent comprises metformin or its salt and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of metformin or its salt of within a range of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of metformin or its salt of about 0.1 ng/mg tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0070] In some examples, the anti-angiogenic agent comprises an anti-VEGF agent. In some examples, the anti-VEGF agent comprises ranibizumab, aflibercept, pegaptanib sodium, bevacizumab, brolocizumab, lapatinib, sorafenib, axitinib, pazopanib, sunitinib, cabozantinib, lenvatinib, ponatinib, ramucirumab, regorafenib, vandetanib, cediranib, thiazolidinediones or RGX-314.

[0071] In some examples, the anti-VEGF agent comprises ranibizumab and is present in the therapeutic composition at a concentration within a range of about 6 mg/ml to about 40 mg/ml. In some examples, the anti-VEGF agent comprises ranibizumab and the therapeutically effective dose comprises ranibizumab within a range of about 0.3 mg to about 2 mg. In some examples, the anti-VEGF agent comprises ranibizumab and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of ranibizumab of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue or about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of ranibizumab within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0072] In some examples, the anti-VEGF agent comprises brolocizumab and is present in the therapeutic composition at a concentration within a range of about 50 mg/ml to about 500 mg/ml or more. In some examples, the anti-VEGF agent comprises brolocizumab and the therapeutically effective dose comprises brolocizumab within a range of about 0.3mg to about

30mg. In some examples, the anti-VEGF agent comprises brolucizumab and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of brolucizumab of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue or about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of brolucizumab within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0073] In some examples, the anti-VEGF agent comprises aflibercept and is present in the therapeutic composition at a concentration within a range of about 10 mg/ml to about 100 mg/ml. In some examples, the anti-VEGF agent comprises aflibercept and the therapeutically effective dose comprises aflibercept within a range of about 0.5 mg to about 5 mg. In some examples, the anti-VEGF agent comprises aflibercept and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of aflibercept of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue or about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of aflibercept within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0074] In some examples, the anti-VEGF agent comprises pegaptanib and is present in the therapeutic composition at a concentration within a range of about 1 mg/ml to about 10 mg/ml. In some examples, the anti-VEGF agent comprises pegaptanib and the therapeutically effective dose comprises pegaptanib within a range of about 0.1 mg to about 1 mg. In some examples, the anti-VEGF agent comprises pegaptanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pegaptanib of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue or about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of pegaptanib within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0075] In some examples, the anti-proliferative agent comprises an mTOR inhibitor. In some examples, the mTOR inhibitor comprises rapamycin, everolimus, biolimus, temsirolimus, ridaforolimus, zotarolimus, myolimus, or novolimus.

[0076] In some examples, the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration within a range of 1 mg/ml to about 250 mg/ml. In some examples, the anti-proliferative agent comprises sirolimus and the therapeutically effective dose comprises sirolimus within a range of about 50 µg to about 5 mg, about 0.1 mg to

about 5 mg, or about 0.5 mg to about 3 mg. In some examples, the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of sirolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of sirolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 1 day. In some examples, the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of sirolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 72 days. In some examples, the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to maintain a tissue concentration of sirolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0077] In some examples, the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration within a range of 1 mg/ml to about 250 mg/ml. In some examples, the anti-proliferative agent comprises novolimus and the therapeutically effective dose comprises novolimus within a range of about 50 µg to about 5 mg, about 0.1 mg to about 5 mg, or about 0.5 mg to about 3 mg. In some examples, the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of novolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of novolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 1 day. In some examples, the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of novolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 72 days. In some examples, the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient to maintain a tissue concentration of novolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0078] In some examples, the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration within a range of 1 mg/ml to about 250 mg/ml. In some examples, the anti-proliferative agent comprises myolimus and the therapeutically

effective dose comprises myolimus within a range of about 50 µg to about 5 mg, about 0.1 mg to about 5 mg, or about 0.5 mg to about 3 mg. In some examples, the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of myolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue. In some examples, the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of myolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 1 day. In some examples, the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of myolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 72 days. In some examples, the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to maintain a tissue concentration of myolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0079] In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the anti-proliferative agent which is smaller than a median maximum serum concentration (C_{max}) of the anti-proliferative agent generated by systemic delivery of the anti-proliferative agent to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the anti-proliferative agent which does not exceed a median maximum serum concentration (C_{max}) of the anti-proliferative agent generated by systemic delivery of the anti-proliferative agent to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease for more than about 6 hours to about 7 days. In some examples, the therapeutically effective dose is sufficient to generate a blood concentration of the anti-proliferative agent which is less than about 10 ng/ml.

[0080] In some examples, the therapeutic composition comprises one or more of a pharmaceutically acceptable carrier, a diluent, an adjuvant, an excipient, a vehicle, a preserving agent, a filler, a polymer, a disintegrating agent, a glidant, a wetting agent, an emulsifying agent, a suspending agent, a sweetening agent, a flavoring agent, a perfuming agent, a lubricating agent, an acidifying agent, and a dispensing agent.

[0081] In some examples, delivery of a therapeutically effective dose of the therapeutic composition is effective to inhibit thrombosis, inhibit clot formation, or inhibit fibrin formation in the patient's eye.

[0082] In another aspect, a method for inhibiting clot formation or fibrin formation in an eye of a patient is provided. The method comprises providing a therapeutic composition comprising a direct factor Xa inhibitor; and delivering a therapeutically effective dose of the therapeutic composition to a site of the clot formation or fibrin formation in the patient's eye.

[0083] The illustrative examples described are not meant to be limiting. Other examples may be utilized, and other changes may be made, or combined in whole or in part, without departing from the scope of the subject matter presented herein. It will be readily understood that the aspects of the present disclosure, as generally described herein, and illustrated in the figures, and detailed description, and in the examples, can be arranged, substituted, combined, separated, and designed in a wide variety of different configurations, all of which are explicitly contemplated herein.

[0084] These and other embodiments are described in further detail in the following description related to the appended drawing figures.

INCORPORATION BY REFERENCE

[0085] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0086] The novel features of the present disclosure are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present disclosure will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the disclosure are utilized, and the accompanying drawings of which:

[0087] FIG. 1A shows a plot of HAoSMC cell proliferation in the presence of rapamycin and varying concentrations of Apixaban, in accordance with examples;

[0088] FIG. 1B shows a plot of HAoSMC cell proliferation in the presence of rapamycin and varying concentrations of Argatroban, in accordance with examples;

[0089] FIG. 1C shows a plot of HAoSMC cell proliferation in the presence of rapamycin and varying concentrations of Apixaban and Argatroban, in accordance with examples;

[0090] FIG. 1D shows a plot of HAoSMC cell proliferation in the presence of difference concentrations of Apixaban, in accordance with examples;

[0091] FIG. 1E shows a plot of HAoSMC cell proliferation in the presence of difference concentrations of Argatroban, in accordance with examples;

[0092] FIG. 2A shows a plot of activated clotting time (ACT) versus drug concentration, in accordance with examples;

[0093] FIG. 2B shows a plot of activated clotting time (ACT) versus drug concentration, in accordance with examples;

[0094] FIG. 2C shows a plot of activated clotting time (ACT) versus drug concentration, showing the synergistic effects of Apixaban in combination with Argatroban, in accordance with examples;

[0095] FIG. 2D shows a plot of various synergistic effects of drug combination ratios between Apixaban and Argatroban, in accordance with examples;

[0096] FIG. 3 shows a reaction scheme of Argatroban with poly N-(2-Hydroxypropyl) methacrylamide, in accordance with examples.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0097] In the following detailed description, reference is made to the accompanying figures, which form a part hereof. In the figures, similar symbols typically identify similar components, unless context dictates otherwise. The illustrative examples described in the detailed description, figures, and claims are not meant to be limiting. Other examples may be utilized, and other changes may be made, or combined in whole or in part, without departing from the scope of the subject matter presented herein. It will be readily understood that the aspects of the present disclosure, as generally described herein, and illustrated in the figures, can be arranged, substituted, combined, separated, and designed in a wide variety of different configurations, all of which are explicitly contemplated herein.

[0098] Although certain examples and examples are disclosed below, inventive subject matter extends beyond the specifically disclosed examples to other alternative examples and/or uses, and to modifications and equivalents thereof. Thus, the scope of the claims appended hereto is not limited by any of the particular examples described below. For example, in any method or process disclosed herein, the acts or operations of the method or process may be performed in any suitable sequence and are not necessarily limited to any particular disclosed sequence. Various operations may be described as multiple discrete operations in turn, in a manner that may be helpful in understanding certain examples, however, the order of description should not be construed to imply that these operations are order dependent. Additionally, the structures, systems, and/or devices described herein may be embodied as integrated components or as separate components.

[0099] For purposes of comparing various examples, certain aspects and advantages of these examples are described. Not necessarily all such aspects or advantages are achieved by any

particular embodiment. Thus, for example, various examples may be carried out in a manner that achieves or optimizes one advantage or group of advantages as taught herein without necessarily achieving other aspects or advantages as may also be taught or suggested herein.

[0100] Every embodiment of the present disclosure may optionally be combined with any one or more of the other examples described herein. Every patent literature, and every non-patent literature, cited herein is incorporated herein by reference in its entirety.

[0101] The present disclosure is described in relation to treating ophthalmic disease or conditions (e.g., inflammatory ophthalmic condition or disease) in a patient via ocular delivery of a therapeutic composition. However, one of skill in the art will appreciate that this is not intended to be limiting and the devices and methods disclosed herein may be used in other anatomical areas and in other therapeutic procedures.

[0102] As used herein, the term coagulation comprises one or more of thrombin formation, fibrin formation, platelet activation, platelet aggregation, and/or thrombus/clot formation. Coagulation typically arises in response to a body part injury and/or to a foreign body such as a device, and/or an infection from a virus or bacteria, and/or caused by irritants.

[0103] As used herein, the term anti-coagulant refers to an agent that inhibits one or more of immune activation or reaction, thrombin formation, fibrin formation, platelet activation, platelet aggregation, thrombus (clot) formation, thrombin dissolution, fibrin dissolution, or thrombus dissolution, thereby inhibiting one or more of inflammation, thrombin, clot, immune activation or reaction, glaucoma, or fibrin. .

[0104] The terms anti-thrombin, thrombin inhibitor, and thrombin formation inhibitor are used interchangeably herein. Also, the terms anti-fibrin, fibrin inhibitor, and fibrin formation inhibitor are used interchangeably herein.

[0105] The terms therapeutically active substance and pharmaceutical agent are used interchangeably herein and refer to any bioactive agent. It will be understood by one of ordinary skill in the art that the devices and methods described herein may be used in combination with one or more additional bioactive agents. Such substances and/or agents optionally include anti-coagulants, anti-mitotic agents, anti-proliferative agents, cytostatic agents, anti-migratory agents, anti-fibrotic agents, immunomodulators, immunosuppressants, anti-inflammatory agents, anti-ischemia agents, anti-hypertensive agents, vasodilators, anti-hyperlipidemia agents, anti-diabetic agents, metformin, anti-cancer agents, anti-tumor agents, anti-angiogenic agents, anti-histamine agents, angiogenic agents, anti-chemokine agents, healing-promoting agents, anti-viral agents, anti-bacterial agents, anti-fungal agents, steroids, interferons, and combinations thereof. It is understood that a bioactive agent may exert more than one biological effect.

[0106] As used herein, a direct factor Xa inhibitor refers to a direct, selective inhibitor of factor Xa that acts directly on factor Xa without using antithrombin as a mediator. The term “direct factor Xa inhibitor” is used herein interchangeably with the term “factor Xa inhibitor” or “anti-factor Xa”. Direct factor Xa inhibitors inhibit thrombin formation and/or fibrin formation, thereby inhibiting clot formation. Direct factor Xa inhibitors include, but are not limited to, apixaban, betrixaban, edoxaban, otamixaban, razaxaban, rivaroxaban, (r)-n-(2-(4-(1-methylpiperidin-4-yl)piperazin-1-yl)-2-oxo-1-phenylethyl)-1h-indole-6-carboxamide (LY-517717), daraxaban (YM-150), or 2-[(7-carbamimidoylnaphthalen-2-yl)methyl]-4-(1-ethanimidoylpiperidin-4-yl)oxyphenyl]sulfamoyl]acetic acid (YM-466 or YM-60828), and eribaxaban (PD 0348292), or 2-(5-carbamimidoyl-2-hydroxy-phenyl) 4-[5-(2,6-dimethylpiperidin-1-yl)-pentyl]-3-oxo-3, 4-dihydro-quinoxaline-6-carboxylic acid (PD0313052), (S)-3-(7-carbamimidoylnaphthalen-2-yl)-2-(4-(((S)-1-(1-iminoethyl)pyrrolidin-3-yl)oxy)phenyl)propanoic acid hydrochloride pentahydrate (DX9065a), letaxaban (TAK-442), GW813893, JTV-803, KFA-144, DPC-423, RPR-209685, MCM-09, and antistasin, and salt, isomer, solvate, analog, derivative, metabolite, or prodrugs thereof. Preferred direct Xa inhibitors include apixaban and rivaroxaban.

[0107] As used herein, a direct factor IIa inhibitor refers to a direct, selective inhibitor of factor IIa (also referred to herein as thrombin) which acts directly on factor IIa/thrombin. The term “direct factor IIa inhibitor” is used herein interchangeably with the term “factor IIa inhibitor” or “anti-factor IIa”. Direct factor IIa inhibitors inhibit thrombin formation and/or fibrin formation, thereby inhibiting clot formation. Direct thrombin/factor IIa inhibitors include, but are not limited to, argatroban, dabigatran, ximelagatran, melagatran, efegatran, inogatran, atecagatran metoxil (AZD-0837), hirudin, hirudin analogs, bivalirudin, desirudin, and lepirudin, and salt, isomer, solvate, analog, derivative, metabolite, or prodrugs thereof. Preferred direct factor IIa inhibitors include argatroban.

[0108] As used herein, an anti-fibrotic agent refers to an agent which acts to reduce or eliminate tissue fibrosis. Anti-fibrotic agents include, but are not limited to, nintedanib, pirfenidone, and salt, isomer, solvate, analog, derivative, metabolite, or prodrugs thereof.

[0109] As used herein, an anti-proliferative agent refers to anti-proliferative agents, anti-mitotic agents, cytostatic agents and anti-migratory agents which suppress cell growth, proliferation, and/or metabolism. Examples of anti-proliferative agents include without limitation inhibitors of mammalian target of rapamycin (mTOR), rapamycin (also called sirolimus), deuterated rapamycin, rapamycin prodrug Tafa93, 40-O-alkyl-rapamycin derivatives, 40-O-hydroxyalkyl-rapamycin derivatives, everolimus {40-O-(2-hydroxyethyl)-rapamycin}, 40-O-(3-hydroxy)propyl-rapamycin, 40-O-[2-(2-hydroxy)ethoxy]ethyl-rapamycin,

40-O-alkoxyalkyl-rapamycin derivatives, biolimus {40-O-(2-ethoxyethyl)-rapamycin}, 40-O-acyl-rapamycin derivatives, temsirolimus {40-(3-hydroxy-2-hydroxymethyl-2-methylpropanoate)-rapamycin, or CCI-779 (temsirolimus), 40-O-phospho-containing rapamycin derivatives, ridaforolimus (40-dimethylphosphinate-rapamycin, or AP23573 (ridaforolimus, formerly known as deforolimus), 40(R or S)-heterocyclyl- or heteroaryl-containing rapamycin derivatives, zotarolimus {40-epi-(N1-tetrazolyl)-rapamycin, or ABT-578 (zotarolimus), 40-epi-(N2-tetrazolyl)-rapamycin, 32(R or S)-hydroxy-rapamycin, myolimus (32-deoxo-rapamycin), novolimus (16-O-desmethyl-rapamycin), taxanes, paclitaxel, docetaxel, cytochalasins, cytochalasins A through J, latrunculins, and salts, isomers, solvates, analogs (including deuterated analogs), derivatives, metabolites, and prodrugs thereof. The IUPAC numbering system for rapamycin is used herein. Preferred anti-proliferative agents include mTOR inhibitors and/or taxanes, or salts, isomers, solvates, analogs, derivatives, metabolites, or prodrugs thereof.

[0110] Table 1 provides non-limiting examples of derivatives of each of rapamycin, everolimus, biolimus, temsirolimus, ridaforolimus, zotarolimus, myolimus and novolimus.

[0111] Table 1. Derivatives of rapamycin-type compounds

Derivatives of Each of Rapamycin, Everolimus, Biolimus, Temsirolimus, Ridaforolimus, Zotarolimus, Myolimus and Novolimus
N7-oxide
2-hydroxy
3-hydroxy
4-hydroxy
5-hydroxy
6-hydroxy
11-hydroxy
12-hydroxy
13-hydroxy
14-hydroxy
23-hydroxy
24-hydroxy
25-hydroxy

31-hydroxy
35-hydroxy
43-hydroxy (11-hydroxymethyl)
44-hydroxy (17-hydroxymethyl)
45-hydroxy (23-hydroxymethyl)
46-hydroxy (25-hydroxymethyl)
47-hydroxy (29-hydroxymethyl)
48-hydroxy (31-hydroxymethyl)
49-hydroxy (35-hydroxymethyl)
17,18-dihydroxy
19,20-dihydroxy
21,22-dihydroxy
29,30-dihydroxy
10-phosphate
28-phosphate
40-phosphate
16-O-desmethyl
27-O-desmethyl
39-O-desmethyl
16,27-bis(O-desmethyl)
16,39-bis(O-desmethyl)
27,39-bis(O-desmethyl)
16,27,39-tris(O-desmethyl)
16-desmethoxy
27-desmethoxy
39-O-desmethyl-14-hydroxy
17,18-epoxide
19,20-epoxide

21,22-epoxide
29,30-epoxide
17,18-29,30-bis-epoxide
17,18-19,20-21,22-tris-epoxide
19,20-21,22-29,30-tris-epoxide
16-O-desmethyl-17,18-19,20-bis-epoxide
16-O-desmethyl-17,18-29,30-bis-epoxide
16-O-desmethyl-17,18-19,20-21,22-tris-epoxide
16-O-desmethyl-19,20-21,22-29,30-tris-epoxide
27-O-desmethyl-17,18-19,20-21,22-tris-epoxide
39-O-desmethyl-17,18-19,20-21,22-tris-epoxide
16,27-bis(O-desmethyl)-17,18-19,20-21,22-tris-epoxide
16-O-desmethyl-24-hydroxy-17,18-19,20-bis-epoxide
16-O-desmethyl-24-hydroxy-17,18-29,30-bis-epoxide
12-hydroxy and opened hemiketal ring

[0112] As used herein, an anti-angiogenic agent refers to an agent which reduces or inhibits angiogenesis directly or indirectly. Anti-angiogenic agents include, but are not limited to, anti-VEGF agents, anti-ANG agents, anti-FGF agents, anti-TGF agents, anti-PDGF agents, anti-IGF agents, anti-IL-8 agents, anti-MMP agents, anti-CSF1 agents, anti-PGE2 agents, anti-S1P agents, anti-heparinase agents, anti-NO agents, anti-peroxynitrite agents, anti-serotonin agents, anti-histamine agents, TSP, ANG2, angiostatin, endostatin, vasostatin, calreticulin, CXCL4, TIMP, CDAI, Meth-1, Meth-2, IFN- α , IFN- β , IFN- γ , CXCL10, IL-4, IL-12, IL-18, prolactin, VEGI, SPARC, osteopontin, maspin, canstatin, proliferin-related protein, and combinations thereof.

[0113] As used herein, an anti-VEGF agent refers to an agent which selectively or non-selectively inhibit the VEGF pathway including agents which target VEGF-A, VEGF-B, VEGF-C, VEGF-D, PlGF, VEGFR1, VEGFR2, VEGFR3. Anti-VEGF agents include, but are not limited to, ranibizumab, aflibercept, pegaptanib sodium, bevacizumab, brolucizumab, lapatinib, sorafenib, axitinib, pazopanib, sunitinib, cabozantinib, lenvatinib, ponatinib, ramucirumab, regorafenib, vandetanib, cediranib, thiazolidinediones or RGX-314. Preferred anti-VEGF agents include brolucizumab, ranibizumab, aflibercept, and pegaptanib.

[0114] As used herein, an anti-platelet agent refers to any substance which inhibits the aggregation of platelets, including platelet aggregation inhibitors and thrombocyte aggregation inhibitors, and/or platelet adhesion inhibitors. Anti-platelet agents include, but are not limited to, acetylsalicylic acid (or aspirin), P2Y₁₂ antagonists such as ticlopidine, clopidogrel, prasugrel, ticagrelor, cangrelor, and elinogrel, PAR-1 antagonists such as vorapaxar, PAR-4 antagonists, EP3 antagonists such as DG041, GPVI antagonists such as soluble dimeric glycoprotein VI-Fc fusion protein, GPIIb antagonists, and anti-VWF agents such as caplacizumab, and other antiplatelet drug such as Abciximab, Eptifibatide, Orbofiban, Roxifiban, Sibrafiban, Tirofiban, Beraprost, Iloprost, Prostacyclin, Treprostinil, Acetylsalicylic acid/Aspirin, AloxiPrin, Carbasalate calcium, Indobufen, Triflusal, Dipyridamole/ aspirin, Picotamide, Terbogrel, Terutroban, Cilostazol, Dipyridamole, Triflusal, Cloricromen, Ditazole, Vorapaxar, Ticlopidine, or others; and/or thrombolytic drugs/fibrinolytics drug such as Plasminogen activators r-tPA, Alteplase, Reteplase, Tenecteplase, Desmoteplase, Saruplase, Urokinase, Anistreplase, Montepulse, Streptokinase, Ancrod, Brinase, Fibrinolysin, or others; and/or Citrate, EDTA, Oxalate; and/or an inhibitor for intrinsic pathway of coagulation and thrombosis such as FXIa inhibitor, protein Z-dependent protease inhibitor, Vitamin K antagonist such as Acenocoumarol, Coumatetralyl, Dicoumarol, Ethyl biscoumacetate, Phenprocoumon, Warfarin, Clorindione, Diphenadione, Phenindione, Tiocloamarol, or others; and/or other anti-coagulant drug such as Antithrombin III, Defibrotide, Protein C (Drotrecogin alfa), Ramatroban, REG1, or others.

[0115] As used here, other therapeutic agents that can be incorporated into the formulations in the invention include but not limit to ranibizumab, aflibercept, pegaptanib sodium, bevacizumab, brolucizumab, lapatinib, sorafenib, axitinib, pazopanib, sunitinib, cabozantinib, lenvatinib, ponatinib, ramucirumab, regorafenib, vandetanib, verteporfin, cediranib, thiazolidinediones, RGX-314, bimatoprost, latanoprost, tafluprost, travoprost, bethamethasone, dexamethasone, methylprednisolone, cortisone, hydrocortisone, triamcinolone, prednisolone, chloramphenicol, sulfacetamide sodium, moxifloxacin, gatifloxacin, erythromycin, polymyxin/trimethoprim, bacitracin, ocumycin, polytracin, nepafenac, bromfenac, salicylate, indomethacin, ibuprofen, diclofenac, flurbiprofen, naproxen, piroxicam, nabumetone, sulfonamides, sulfacetamide, sulfamethizole, sulfisoxazole; nitrofurazone, sodium propionate, antazoline, methapyriline, chlorpheniramine, pyrilamine, propenpyridamine, pilocarpine, eserine salicylate, carbachol, di-isopropyl fluorophosphate, phospholine iodine, demecarium bromide, atropine sulfate, cyclopentolate, homatropine, scopolamine, tropicamide, eucatropine, hydroxyamphetamine, and the like or a salt, isomer, solvate, analog, derivative, metabolite, or prodrugs thereof.

[0116] Described herein are devices, compositions, and methods for inhibiting clot formation, fibrin, fibrosis, and/or inflammation and/or glaucoma, and/or AMD of ophthalmic disease or condition in a patient. A therapeutic composition may be provided as described herein. A therapeutically effective dose of the therapeutic composition may be delivered to the site of the disease or condition in the patient's eye(s). The therapeutically effective dose of the therapeutic composition may be effective to suppress or prevent initiation, progression, or relapses of disease, including the progression of established disease. In some examples, the ophthalmic disease or condition comprises one or more of angiogenesis, leaking blood vessel, protein deposition, fibrosis, glaucoma, extracellular matrix (ECM) deposition, thrombin generation, clot formation, or fibrin formation. For example, the ophthalmic disease or condition may be wet age-related macular degeneration (AMD), dry AMD, diabetic retinopathy, glaucoma, uveitis, cataracts, conjunctivitis, or dry eye disease. In some examples, the patient may be diagnosed as having the ophthalmic disease prior to delivering the therapeutically effective dose of the therapeutic composition.

[0117] Tissue plasminogen activator (tPA) is one of the essential components of the dissolution of blood clots. Its primary function includes catalyzing the conversion of plasminogen to plasmin, the primary enzyme involved in dissolving blood clots. Since fibrin clots can occur in several sites of the body, including the eye, the tPA therapy could be effective for the rapid dissolution of fibrin clots in the anterior chamber of the human eye, as well as for lysis of fibrin clots after vitrectomy and failed blebs after glaucoma filtering surgery.

[0118] Tissue plasminogen activator (tPA) can dissolve any fibrin material that forms in the anterior chamber or vitreous humor. Ocular tissues that contain tPA include the conjunctiva, cornea, trabecular meshwork, lens, vitreous, and retina. In normal human eyes, the aqueous humour contains a significant amount of tPA; however, for disease eyes related to tPA deficiency, medication tPA could be considered.

[0119] Since fibrin clots can occur in several sites of the body, including the eye, the tPA therapy could be effective for the rapid dissolution of fibrin clots in the anterior chamber of the human eye, as well as for lysis of fibrin clots after vitrectomy and failed blebs after glaucoma filtering surgery. The thrombolytic drugs comprise tissue plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA), Streptokinase or plasmin.

[0120] The serine protease agent comprises one of t-PA, U-PA, or plasmin, or other agents that has similar mechanism of action. The tPA therapeutic agent can be, for example, tPA, a tPA derivative, a small molecule direct or indirect tPA agonist, or a gene therapy vector. The tPA eye drops administered topically to patients with postoperative hyphema could give rapid clearance of the clot. Intracameral tPA injection is a safe and effective way to help lyse fibrin

and dissolve blood clots to reopen occluded glaucoma drainage devices. tPA is a nonsurgical eye injection that's often used to treat patients suffering from wet age-related macular degeneration (AMD). It's also used to treat a common side effect of AMD, submacular hemorrhages. The tPA eye drops or injections can benefit eye disease such as vitreous opacity, wet age-related macular degeneration (AMD), glaucoma and anterior chamber fibrinoid syndrome after cataract extraction etc. tPA use could have prevented the need for additional glaucoma surgery. One or more injections of tPA may have been used to clear or prevent tube blockage by blood and/or fibrin to result in a successful ocular outcome.

[0121] In a preferred example the agent is present in the therapeutic composition at a concentration within a range of 1 mg/ml to about 250 mg/ml. In some examples, the serine protease agent comprises t-PA and the therapeutically effective dose comprises a range of about 50 µg to about 5 mg, about 25 µg to about 5 mg, or about 0.3 µg to about 3mg.

[0122] Ocular inflammation is a sight threatening disease and its dysregulation can catalyze and or propagate ocular neurodegenerative maladies such as age-related macular degeneration (AMD). The complement system, an intrinsic component of the innate immunity, has an integral role in maintaining immune-surveillance and homeostasis in the ocular microenvironment; however, overstimulation can drive ocular inflammatory diseases. The incidence of advanced AMD, both geographic atrophy and neovascular AMD, increases exponentially with age and while there are therapies for wet AMD, Geographic atrophy (GA) currently has no approved treatment options. The aim of most current clinical trials is to reduce the progression of GA lesion enlargement.

[0123] Complement is the main driver of disease for AMD, Uveitis, Glaucoma. Local inhibition of complement activation has been considered a promising approach for treating these diseases. Complement pathway has three pathways which involves various factors, B, D, H & I, which interact with each other, and with C3b, to form a C3 convertase, C3bBb, that can activate more C3, hence the pathway is sometimes called 'the amplification loop'. C3 is the converging point for all 3 complement pathways. Complement inhibitors target the modulation of complement proteins C3, C5, factor B, factor D, and properdin.

[0124] In some examples, the complement-mediated therapeutics include but not limit to C3 inhibitor such as APL-2, Compstatin and AMY106, Anti-factor D such as Lampalizumab, Anti-Properdin such as CLG561, Anti-C5 such as Eculizumab, Ravulizumab, ARC1905, and LFG316, CD59 such as AAVCAGsCD59, analogues and the like.

[0125] In some examples, the complement inhibitor is Compstatin or its analogue. Compstatin is a 13 amino acid cyclic peptide that inhibits complement activation by binding C3 and interfering with convertase formation and C3 cleavage (and the subsequent C3b opsonization,

amplification, and generation of effectors). Compstatin could be a potent inhibitor, blocking all three complement systems.

[0126] In some examples, the complement inhibitor is Anti-C5 such as Eculizumab and ravulizumab. Eculizumab or Ravulizumab specifically binds to the terminal complement component 5, or C5, which acts at a late stage in the complement cascade. When activated, C5 is involved in activating host cells, thereby attracting pro-inflammatory immune cells, while also destroying cells by triggering pore formation. By inhibiting the complement cascade at this point, the normal, disease-preventing functions of proximal complement system are largely preserved, while the properties of C5 that promote inflammation and cell destruction are impeded.

[0127] In some examples, the eye injections comprise complement inhibitor Compstatin or Eculizumab or Ravulizumab or its analogue, which can benefit eye disease such as AMD, Uveitis, Glaucoma, etc.

[0128] In some examples, the eye injections comprise complement inhibitor Compstatin or Eculizumab or Ravulizumab or its analogue with combination of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban. This combination therapy will have a big impact on ocular inflammation and may be efficient to eye disease such as AMD, Uveitis and Glaucoma. The rate of progression of atrophy in the eyes of patients with dry AMD might be slowed down or stopped with combination of Eculizumab or Ravulizumab and direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban, which otherwise has no treatment available.

[0129] Inflammation, fibrosis, and/or clot formation of the ocular tissue caused by the ophthalmic disease or condition may lead to fluid build-up, thrombin generation, fibrosis, and/or fibrin formation in the eye, which can damage tissue in the eye. Ocular tissues include, but are not limited to, an eyelid, a meibomian gland, a trabecular meshwork, Schlemm's canal, a cornea, a sclera, a vitreous, a choroid, a uvea, and/or a retina.

[0130] In some examples, the therapeutic composition is locally delivered to the eye. In some examples, the therapeutic composition is delivered to ocular tissue including an eyelid, a meibomian gland, a trabecular meshwork, Schlemm's canal, a cornea, a sclera, a vitreous, a choroid, a uvea, and/or a retina. The therapeutic composition may be delivered to the eye by any method or device for ocular delivery. For example, the therapeutic composition may be delivered with an injection, an implant, an eye drop, an emulsion, a suspension, an ointment, a nanocarrier, a nanomicelle, a nanoparticle, a nanosuspension, a liposome, an in-situ gel, a contact lens, or a microneedle.

[0131] When an implant is utilized, the implant can be implanted intraocularly or intravitreally by an intervention procedure. Such implants can be non-biodegradable, biodegradable, removable or permanent. In other examples, implants can be a shunt or stent placed in the duct, such as the tear duct. In still other examples, implants can be placed adjacent to the ocular body, or intraocularly, adjacent to the vitreal body or intravitreally. One of ordinary skill in the art will appreciate that other locations are useful in the present disclosure.

[0132] In some examples, the therapeutic composition may be delivered to the eye(s) by one or more methods or devices. For example, a first portion (e.g., one or more bioactive agents) of the therapeutic composition may be delivered to the eye with a first device (e.g., an implant) and a second portion (e.g., one or more additional pharmaceutical agents) of the therapeutic composition may be delivered to the eye with a second device (e.g., a second implant, a contact lens, etc.) before or after delivery of the first portion of the therapeutic composition. Alternatively, all components (e.g., all bioactive agents) of the therapeutic composition may be delivered with the same device (e.g., an implant). It will be understood by one of ordinary skill in the art that the therapeutic composition may comprise one or many components which may be delivered simultaneously or sequentially in any order or combination as desired with any combination of methods or devices desired.

[0133] In some examples, local delivery of the therapeutic composition may be preferable to systemic delivery of the therapeutic composition in at least some instances. For example, local delivery of the therapeutic composition may reduce off-target effects by reducing systemic concentrations and/or increase the tissue concentration of the therapeutic composition at the target site compared to what can be achieved with systemic delivery safety maximums.

[0134] In some examples, the therapeutic composition may comprise one or more of a pharmaceutically acceptable carrier, a diluent, an adjuvant, an excipient, a vehicle, a preserving agent, a filler, a polymer, a disintegrating agent, a glidant, a wetting agent, an emulsifying agent, a suspending agent, a sweetening agent, a flavoring agent, a perfuming agent, a lubricating agent, an acidifying agent, and a dispensing agent.

[0135] In some examples, the therapeutic composition may comprise or be co-administered with one or more additional bioactive agents which address or treat the underlying disease or infection in order to improve therapeutic outcome.

[0136] While many of the examples described herein depict the therapeutic composition being in the form of an eye drop for local delivery, it will be understood by one of ordinary skill in the art that local delivery may be achieved through any other means desired. For examples, the therapeutic composition may be coated, dipped, printed, deposited, painted, brushed, loaded, or otherwise disposed on one or more surfaces of a device for local delivery. In some examples,

the therapeutic composition may be locally delivered via direct injection using a device (e.g., a needle, a microneedle, etc.) comprising or coupled to a drug reservoir.

[0137] It is an objective of this application to show that ophthalmic delivery of the direct factor Xa inhibitor and/or additional therapeutically active substance (e.g., an anti-coagulant such as a direct factor IIa inhibitor), and optionally in combination with one or more additional pharmaceutical agent, inhibits one or more of inflammation, thrombin formation, fibrin formation, clot formation, smooth muscle proliferation, fibrosis, and/or adverse clinical events.

[0138] It is an objective of this application to show that ophthalmic delivery of a pharmaceutical agent to inhibits one or more of inflammation, fibrosis, and/or adverse clinical events.

[0139] Described herein are devices and methods for locally delivering a therapeutic composition to a patient. The therapeutic composition includes one or more anti-coagulant agents which inhibit thrombin, fibrin, and/or thrombus formation or promote thrombin, fibrin, and/or thrombus dissolution. In preferred examples, the therapeutic composition includes a direct Xa inhibitor. In another preferred example, the therapeutic composition includes a direct Xa inhibitor and/or a direct IIa inhibitor. In another preferred example, one or more additional pharmaceutical agents may be added to the therapeutic composition of the direct Xa inhibitor and/or the direct IIa inhibitor. The additional pharmaceutical agents may, for example, include one or more anti-fibrotic agents, metformin, anti-angiogenic agents, anti-VEGF, anti-proliferative agents, or combinations thereof.

[0140] In some examples, the pharmaceutical agents may, for example, include one or more anti-fibrotic agents, metformin, metformin hydroxychloride (HCl), anti-angiogenic agents, anti-proliferative agents, or combinations thereof.

[0141] In some examples, the pharmaceutical agent may, for example, include one or more anti-viral/anti-diabetic agents such as metformin or its salt metformin HCl. Metformin was originally introduced as an anti-influenza drug and was found to have glucose-lowering side effects, making it effective as both an anti-viral and an anti-diabetic agent. Metformin activates AMP-activated protein kinase (AMPK) which, among other things, phosphorylates and upregulates angiotensin-converting enzyme 2 (ACE2) and inhibits the mTOR signaling cascade. The anti-viral activity of metformin may, in at least some instances, be related to these activities. Metformin can also be used for treatment of ophthalmic disorders. For example, metformin may be used for treatment of diabetic retinopathy and/or macular edema. In at least some instances, metformin may play a protective role in microvascular ocular complications, including retinopathy, glaucoma, and/or age-related macular degeneration (AMD) in patients with type-2 diabetes. For example, metformin inhibits development of diabetic retinopathy by inducing

microRNA-497a-5p, a VEGF-A-inhibiting microRNA, resulting in reduced retina neovascularization.

[0142] In some examples, the therapeutic composition comprises a direct factor Xa inhibitor, a additional therapeutically active substance (e.g., an anti-coagulant), and/or one or more additional pharmaceutical agent(s) (e.g., an anti-fibrotic agent, an anti-viral agent, an anti-diabetic agent, an anti-angiogenic agent, and/or an anti-proliferative agent).

[0143] In some examples, the therapeutic composition comprises a direct factor Xa inhibitor, a direct factor IIa inhibitor, and/or an anti-fibrotic agent. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor, a direct factor IIa inhibitor, and an anti-fibrotic agent. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor and a direct factor IIa inhibitor but no anti-fibrotic agent. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor and an anti-fibrotic agent but no direct factor IIa inhibitor. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor but no direct factor IIa inhibitor or anti-fibrotic agent.

[0144] In some examples, the direct factor Xa inhibitor, direct factor IIa inhibitor, and/or anti-fibrotic agent may be delivered simultaneously or sequentially in any order or combination as desired with any combination of methods or devices desired. For example, the same eye drop may be used to deliver the direct factor Xa inhibitor, the direct factor IIa inhibitor, and the anti-fibrotic agent. Alternatively, a first eye dropper may be used to deliver the direct factor Xa inhibitor and a second eye dropper may be used to deliver the direct factor IIa inhibitor and the anti-fibrotic agent. Alternatively, a first eye dropper may be used to deliver the direct factor Xa inhibitor and the direct factor IIa inhibitor and a second eye dropper may be used to deliver the anti-fibrotic agent. Alternatively, each agent may be delivered by its own dedicated eye dropper.

[0145] In some examples, the therapeutic composition comprises a direct factor Xa inhibitor, a direct factor IIa inhibitor, and/or an anti-viral/anti-diabetic agent. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor, a direct factor IIa inhibitor, and anti-viral/anti-diabetic agent. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor and a direct factor IIa inhibitor but no anti-viral/anti-diabetic agent. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor and an anti-viral/anti-diabetic agent but no direct factor IIa inhibitor. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor but no direct factor IIa inhibitor or anti-viral/anti-diabetic agent.

[0146] In some examples, the direct factor Xa inhibitor, direct factor IIa inhibitor, and/or anti-viral/anti-diabetic agent may be delivered simultaneously or sequentially in any order or combination as desired with any combination of methods or devices desired. For example, the

same eye dropper may be used to deliver the direct factor Xa inhibitor, the direct factor IIa inhibitor, and the anti-viral/anti-diabetic agent. Alternatively, a first eye dropper may be used to deliver the direct factor Xa inhibitor and a second eye dropper may be used to deliver the direct factor IIa inhibitor and the anti-viral/anti-diabetic agent. Alternatively, a first eye dropper may be used to deliver the direct factor Xa inhibitor and the direct factor IIa inhibitor and a second eye dropper may be used to deliver the anti-viral/anti-diabetic agent. Alternatively, each agent may be delivered by its own dedicated eye dropper.

[0147] In some examples, the therapeutic composition comprises a direct factor Xa inhibitor, a direct factor IIa inhibitor, and/or an anti-proliferative agent. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor, a direct factor IIa inhibitor, and an anti-proliferative agent. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor and a direct factor IIa inhibitor but no anti-proliferative agent. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor and an anti-proliferative agent but no direct factor IIa inhibitor. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor but no direct factor IIa inhibitor or anti-proliferative agent.

[0148] In some examples, the direct factor Xa inhibitor, direct factor IIa inhibitor, and/or anti-proliferative agent may be delivered simultaneously or sequentially in any order or combination as desired with any combination of methods or devices desired. For example, the same eye dropper may be used to deliver the direct factor Xa inhibitor, the direct factor IIa inhibitor, and the anti-proliferative agent. Alternatively, a first eye dropper may be used to deliver the direct factor Xa inhibitor and a second eye dropper may be used to deliver the direct factor IIa inhibitor and the anti-proliferative agent. Alternatively, a first eye dropper may be used to deliver the direct factor Xa inhibitor and the direct factor IIa inhibitor and a second eye dropper may be used to deliver the anti-proliferative agent. Alternatively, each agent may be delivered by its own dedicated eye dropper.

[0149] In some examples, the therapeutic composition comprises a direct factor Xa inhibitor, a direct factor IIa inhibitor, and/or an anti-angiogenic agent. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor, a direct factor IIa inhibitor, and an anti-angiogenic agent. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor and a direct factor IIa inhibitor but no anti-angiogenic agent. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor and an anti-angiogenic agent but no direct factor IIa inhibitor. In some examples, the therapeutic composition may comprise a direct factor Xa inhibitor but no direct factor IIa inhibitor or anti-angiogenic agent.

[0150] In some examples, the direct factor Xa inhibitor, direct factor IIa inhibitor, and/or anti-angiogenic agent may be delivered simultaneously or sequentially in any order or combination as desired with any combination of methods or devices desired. For example, the same eye dropper may be used to deliver the direct factor Xa inhibitor, the direct factor IIa inhibitor, and the anti-angiogenic agent. Alternatively, a first eye dropper may be used to deliver the direct factor Xa inhibitor and a second eye dropper may be used to deliver the direct factor IIa inhibitor and the anti-angiogenic agent. Alternatively, a first eye dropper may be used to deliver the direct factor Xa inhibitor and the direct factor IIa inhibitor and a second eye dropper may be used to deliver the anti-angiogenic agent. Alternatively, each agent may be delivered by its own dedicated eye dropper.

[0151] When the therapeutic composition comprises a direct factor Xa inhibitor and a direct factor IIa inhibitor, the weight compositional ratio of the direct factor Xa inhibitor to the direct factor IIa inhibitor in the therapeutic composition may be within a range of about 3:1 to about 1:3. For example, the weight compositional ratio of the direct factor Xa inhibitor to the direct factor IIa inhibitor in the therapeutic composition may be about 1:1.

[0152] In some examples, the direct factor Xa inhibitor may have an IC₅₀ inhibition potency for factor Xa ranging from about 0.001 nM to about 50 nM.

[0153] In some examples, the direct factor IIa inhibitor may have an IC₅₀ inhibition potency for factor IIa ranging from about 0.001 nM to about 100 nM.

[0154] In some examples, the direct factor Xa inhibitor may have a half maximal inhibitory concentration (IC₅₀) within a range of about 0.01 nM to about 1000 nM, about 0.1 nM to about 1000 nM, about 1 nM to about 1000 nM, 10 nM to about 1000 nM, or about 100 nM to about 1000 nM.

[0155] In some examples, the direct factor IIa inhibitor may have an inhibition potency for factor IIa ranging from about 0.001 nM to about 100 nM.

[0156] In some examples, the direct factor IIa inhibitor may have a half maximal inhibitory concentration (IC₅₀) within a range of about 0.01 nM to about 1000 nM, about 0.1 nM to about 1000 nM, about 1 nM to about 1000 nM, 10 nM to about 1000 nM, or about 100 nM to about 1000 nM.

[0157] In some examples, the direct factor IIa inhibitor may comprise argatroban, or a salt, isomer, solvate, analog (including deuterated analog), derivative, metabolite, or prodrugs thereof.

[0158] In some examples, the direct factor Xa inhibitor may comprise apixaban, or a salt, isomer, solvate, analog (including deuterated analog), derivative, metabolite, or prodrugs thereof.

[0159] In some examples, the direct factor Xa inhibitor may comprise rivaroxaban, or a salt, isomer, solvate, analog (including deuterated analog), derivative, metabolite, or prodrugs thereof.

[0160] In some examples, the anti-fibrotic agent may comprise nintedanib, or a salt, isomer, solvate, analog (including deuterated analog), derivative, metabolite, or prodrugs thereof.

[0161] In some examples, the anti-fibrotic agent may comprise pirfenidone, or a salt, isomer, solvate, analog (including deuterated analog), derivative, metabolite, or prodrugs thereof.

[0162] In some examples, the anti-viral/anti-diabetic agent may comprise metformin, or a salt, isomer, solvate, analog (including deuterated analog), derivative, metabolite, or prodrugs thereof.

[0163] In some examples, the anti-proliferative agent may comprise sirolimus, or a salt, isomer, solvate, analog (including deuterated analog), derivative, metabolite, or prodrugs thereof.

[0164] In some examples, the anti-proliferative agent may comprise novolimus, or a salt, isomer, solvate, analog (including deuterated analog), derivative, metabolite, or prodrugs thereof.

[0165] In some examples, the anti-proliferative agent may comprise myolimus, or a salt, isomer, solvate, analog (including deuterated analog), derivative, metabolite, or prodrugs thereof.

[0166] In some examples, the anti-angiogenic agent may comprise ranibizumab, or a salt, isomer, solvate, analog (including deuterated analog), derivative, metabolite, or prodrugs thereof.

[0167] In some examples, the anti-angiogenic agent may comprise brotuzumab, or a salt, isomer, solvate, analog (including deuterated analog), derivative, metabolite, or prodrugs thereof.

[0168] In some examples, the anti-angiogenic agent may comprise aflibercept, or a salt, isomer, solvate, analog (including deuterated analog), derivative, metabolite, or prodrugs thereof.

[0169] In some examples, the anti-angiogenic agent may comprise pegaptanib, or a salt, isomer, solvate, analog (including deuterated analog), derivative, metabolite, or prodrugs thereof.

[0170] In some examples, the direct factor Xa inhibitor may comprise apixaban and the direct factor IIa inhibitor may comprise argatroban.

[0171] In some examples, the direct factor Xa inhibitor may comprise rivaroxaban and the direct factor IIa inhibitor may comprise argatroban.

[0172] In some examples, the direct factor Xa inhibitor may comprise apixaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-fibrotic agent may comprise nintedanib.

[0173] In some examples, the direct factor Xa inhibitor may comprise apixaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-fibrotic agent may comprise pirfenidone.

[0174] In some examples, the direct factor Xa inhibitor may comprise rivaroxaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-fibrotic agent may comprise nintedanib.

[0175] In some examples, the direct factor Xa inhibitor may comprise rivaroxaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-fibrotic agent may comprise pirfenidone.

[0176] In some examples, the direct factor Xa inhibitor may comprise apixaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-diabetic agent may comprise metformin or its salt.

[0177] In some examples, the direct factor Xa inhibitor may comprise rivaroxaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-diabetic agent may comprise metformin or its salt.

[0178] In some examples, the direct factor Xa inhibitor may comprise apixaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-proliferative agent may comprise sirolimus or its isomer, solvate, analog, derivative, metabolite, or prodrugs.

[0179] In some examples, the direct factor Xa inhibitor may comprise apixaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-proliferative agent may comprise novolimus or its isomer, solvate, analog, derivative, metabolite, or prodrugs.

[0180] In some examples, the direct factor Xa inhibitor may comprise apixaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-proliferative agent may comprise myolimus or its isomer, solvate, analog, derivative, metabolite, or prodrugs.

[0181] In some examples, the direct factor Xa inhibitor may comprise rivaroxaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-proliferative agent may comprise sirolimus or its isomer, solvate, analog, derivative, metabolite, or prodrugs.

[0182] In some examples, the direct factor Xa inhibitor may comprise rivaroxaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-proliferative agent may comprise novolimus or its isomer, solvate, analog, derivative, metabolite, or prodrugs.

[0183] In some examples, the direct factor Xa inhibitor may comprise rivaroxaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-proliferative agent may comprise myolimus or its isomer, solvate, analog, derivative, metabolite, or prodrugs.

[0184] In some examples, the direct factor Xa inhibitor may comprise rivaroxaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-angiogenic agent may comprise ranibizumab.

[0185] In some examples, the direct factor Xa inhibitor may comprise rivaroxaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-angiogenic agent may comprise brolucizumab.

[0186] In some examples, the direct factor Xa inhibitor may comprise rivaroxaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-angiogenic agent may comprise aflibercept.

[0187] In some examples, the direct factor Xa inhibitor may comprise rivaroxaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-angiogenic agent may comprise pegaptanib.

[0188] In some examples, the direct factor Xa inhibitor may comprise apixaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-angiogenic agent may comprise ranibizumab.

[0189] In some examples, the direct factor Xa inhibitor may comprise apixaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-angiogenic agent may comprise brolucizumab.

[0190] In some examples, the direct factor Xa inhibitor may comprise apixaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-angiogenic agent may comprise aflibercept.

[0191] In some examples, the direct factor Xa inhibitor may comprise apixaban, the direct factor IIa inhibitor may comprise argatroban, and the anti-angiogenic agent may comprise pegaptanib.

[0192] In some examples, the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration within a range of about 1 mg/ml to about 250 mg/ml, 10 mg/ml to about 250 mg/ml, or 100 mg/ml to about 250 mg/ml.

[0193] In some examples, the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration within a range of about 1 mg/ml to about 250 mg/ml, 10 mg/ml to about 250 mg/ml, or 100 mg/ml to about 250 mg/ml.

[0194] In some examples, the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration within a range of about 1 mg/ml to about 250 mg/ml, 10 mg/ml to about 250 mg/ml, or 100 mg/ml to about 250 mg/ml.

[0195] In some examples, the anti-angiogenic agent comprises brotuzumab and is present in the therapeutic composition at a concentration within a range of about 50 mg/ml to about 500 mg/ml.

[0196] In some examples, the anti-angiogenic agent comprises ranibizumab and is present in the therapeutic composition at a concentration within a range of about 6 mg/ml to about 40 mg/ml.

[0197] In some examples, the anti-angiogenic agent comprises aflibercept and is present in the therapeutic composition at a concentration within a range of about 10 mg/ml to about 100 mg/ml.

[0198] In some examples, the anti-angiogenic agent comprises pegaptanib and is present in the therapeutic composition at a concentration within a range of about 1 mg/ml to about 10 mg/ml.

[0199] In some examples, a therapeutically effective dose of the direct factor Xa inhibitor is within a range of about 50 micrograms to about 10 mg. In some examples, the therapeutically effective dose of the direct factor Xa inhibitor is within a range of about 0.1 mg to about 10 mg. In some examples, the therapeutically effective dose of the direct factor Xa inhibitor is within a range of about 1 mg to about 5 mg.

[0200] In some examples, a therapeutically effective dose of the direct factor IIa inhibitor is within a range of about 50 micrograms to about 10 mg. In some examples, the therapeutically effective dose of the direct factor IIa inhibitor is within a range of about 0.1 mg to about 10 mg. In some examples, the therapeutically effective dose of the direct factor IIa inhibitor is within a range of about 1 mg to about 5 mg.

[0201] In some examples, the anti-fibrotic agent comprises nintedanib and a therapeutically effective dose of nintedanib is within a range of about 0.1 mg to about 5 mg.

[0202] In some examples, the anti-proliferative agent comprises sirolimus and a therapeutically effective dose of sirolimus is within a range of about 50 µg to about 5 mg, about 0.1 mg to about 5 mg, or about 0.5 mg to about 3 mg.

[0203] In some examples, the anti-proliferative agent comprises novolimus and a therapeutically effective dose of novolimus is within a range of about 50 µg to about 5 mg, about 0.1 mg to about 5 mg, or about 0.5 mg to about 3 mg.

[0204] In some examples, the anti-proliferative agent comprises myolimus and a therapeutically effective dose of myolimus is within a range of about 50 µg to about 5 mg, about 0.1 mg to about 5 mg, or about 0.5 mg to about 3 mg.

[0205] In some examples, the anti-angiogenic agent comprises brolucizumab and a therapeutically effective dose of brolucizumab is within a range of about 0.3 mg to about 30 mg.

[0206] In some examples, the anti-angiogenic agent comprises ranibizumab and a therapeutically effective dose of ranibizumab is within a range of about 0.3 mg to about 2 mg.

[0207] In some examples, the anti-angiogenic agent comprises aflibercept a therapeutically effective dose of aflibercept is within a range of about 0.5 mg to about 5 mg.

[0208] In some examples, the anti-angiogenic agent comprises pegaptanib and a therapeutically effective dose of pegaptanib is within a range of about 0.1 mg to about 1 mg.

[0209] In some examples, the direct factor Xa inhibitor is present in the therapeutic composition at a concentration effective to achieve a tissue concentration of the direct factor Xa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue when a therapeutically effective dose of the therapeutic composition is delivered to the tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 0.2 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the direct factor Xa inhibitor of 0.5 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 10 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 100 ng/g tissue to about 100 mg/g tissue.

[0210] In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of the direct factor Xa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0211] In some examples, the direct factor IIa inhibitor is present in the therapeutic composition at a concentration effective to achieve a tissue concentration of the direct factor IIa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue when a therapeutically effective dose of the therapeutic composition is delivered to the tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 0.2 ng/g tissue to about 100 mg/g tissue. In some examples, the

therapeutically effective dose may be sufficient to generate a tissue concentration of the direct factor IIa inhibitor of 0.5 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 10 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 100 ng/g tissue to about 100 mg/g tissue.

[0212] In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of the direct factor IIa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0213] In some examples, the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration effective to achieve a tissue concentration of the anti-fibrotic agent of about 0.1 ng/g tissue to about 100 mg/g tissue when a therapeutically effective dose of the therapeutic composition is delivered to the tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-fibrotic agent of about 0.2 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-fibrotic agent of about 0.5 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-fibrotic agent of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-fibrotic agent of about 100 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-fibrotic agent of about 100 ng/g tissue to about 100 mg/g tissue.

[0214] In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of nintedanib of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0215] In some examples, the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration effective to achieve a tissue concentration of the anti-fibrotic agent of about 0.1 ng/g tissue to about 100 mg/g tissue when a therapeutically effective dose of the therapeutic composition is delivered to the tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-

fibrotic agent of about 0.2 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-fibrotic agent of 0.5 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-fibrotic agent of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-fibrotic agent of about 10 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-fibrotic agent of about 100 ng/g tissue to about 100 mg/g tissue.

[0216] In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of pirfenidone of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0217] In some examples, the anti-viral/anti-diabetic agent comprises metformin or its salt and is present in the therapeutic composition at a concentration effective to achieve a tissue concentration of the anti-viral/anti-diabetic agent of about 0.1 ng/g tissue to about 100 mg/g tissue when a therapeutically effective dose of the therapeutic composition is delivered to the tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-viral/anti-diabetic agent of about 0.2 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-viral/anti-diabetic agent of 0.5 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-viral/anti-diabetic agent of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-viral/anti-diabetic agent of about 10 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-viral/anti-diabetic agent of about 100 ng/g tissue to about 100 mg/g tissue.

[0218] In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of metformin or its salt of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0219] In some examples, the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration effective to achieve a tissue concentration of the anti-proliferative agent of about 0.1 ng/g tissue to about 100 mg/g tissue when a therapeutically

effective dose of the therapeutic composition is delivered to the tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 0.2 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of 0.5 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 10 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 100 ng/g tissue to about 100 mg/g tissue.

[0220] In some examples, the therapeutic composition comprises sirolimus at a concentration sufficient to generate a tissue concentration of sirolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 1 day. In some examples, the therapeutic composition comprises sirolimus at a concentration sufficient to generate a tissue concentration of sirolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 72 days.

[0221] In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of sirolimus of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0222] In some examples, the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration effective to achieve a tissue concentration of the anti-proliferative agent of about 0.1 ng/g tissue to about 100 mg/g tissue when a therapeutically effective dose of the therapeutic composition is delivered to the tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 0.2 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of 0.5 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 10 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 100 ng/g tissue to about 100 mg/g tissue.

[0223] In some examples, the therapeutic composition comprises novolimus at a concentration sufficient to generate a tissue concentration of sirolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 1 day. In some examples, the therapeutic composition comprises novolimus at a concentration sufficient to generate a tissue concentration of sirolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 72 days.

[0224] In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of novolimus of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0225] In some examples, the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration effective to achieve a tissue concentration of the anti-proliferative agent of about 0.1 ng/g tissue to about 100 mg/g tissue when a therapeutically effective dose of the therapeutic composition is delivered to the tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 0.2 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of 0.5 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 10 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 100 ng/g tissue to about 100 mg/g tissue.

[0226] In some examples, the therapeutic composition comprises myolimus at a concentration sufficient to generate a tissue concentration of sirolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 1 day. In some examples, the therapeutic composition comprises myolimus at a concentration sufficient to generate a tissue concentration of sirolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 72 days.

[0227] In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of myolimus of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0228] In some examples, the anti-angiogenic agent comprises ranibizumab and is present in the therapeutic composition at a concentration effective to achieve a tissue concentration of the anti-angiogenic agent of about 0.1 ng/g tissue to about 100 mg/g tissue when a therapeutically effective dose of the therapeutic composition is delivered to the tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 0.2 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of 0.5 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 10 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 100 ng/g tissue to about 100 mg/g tissue.

[0229] In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of ranibizumab of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0230] In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of brolicizumab within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year. In some examples, the anti-angiogenic agent comprises aflibercept and is present in the therapeutic composition at a concentration effective to achieve a tissue concentration of the anti-angiogenic agent of about 0.1 ng/g tissue to about 100 mg/g tissue when a therapeutically effective dose of the therapeutic composition is delivered to the tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 0.2 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of 0.5 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 10 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 100 ng/g tissue to about 100 mg/g tissue.

[0231] In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of aflibercept of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0232] In some examples, the anti-angiogenic agent comprises pegaptanib and is present in the therapeutic composition at a concentration effective to achieve a tissue concentration of the anti-angiogenic agent of about 0.1 ng/g tissue to about 100 mg/g tissue when a therapeutically effective dose of the therapeutic composition is delivered to the tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 0.2 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of 0.5 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 1 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 10 ng/g tissue to about 100 mg/g tissue. In some examples, the therapeutically effective dose may be sufficient to generate a tissue concentration of the anti-proliferative agent of about 100 ng/g tissue to about 100 mg/g tissue.

[0233] In some examples, the therapeutically effective dose is sufficient to maintain a tissue concentration of pegaptanib of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

[0234] In some examples, a therapeutically effective dose of the therapeutic composition may be delivered to the target tissue to generate a therapeutically effective concentration in the target tissue. The therapeutic composition may be formulated such that the therapeutically effective concentration may be substantially higher than a systemic blood concentration of each agent in order to treat effectively the disease or condition of the lung. Preferably, the tissue concentration is at least about 1 times a median maximum serum concentration (C_{max}) blood concentration, at least 1.5 times, 2 times, or 5 times higher than the systemic C_{max} blood concentration or about 10 times higher than the systemic dose.

[0235] In some examples, formulations of the present invention for treating dry eye may include drugs to reduce eyelid inflammation, antibiotics; substances to control cornea inflammation, for example containing immune-suppressing medication such as cyclosporine, corticosteroids, and the like, tear-stimulating drugs such as cholinergic agents (e.g., pilocarpine, cevimeline), and the like.

[0236] In some examples, formulations for glaucoma treatment may include prostaglandin analogs, such as bimatoprost, latanoprost, tafluprost, travoprost and the like; beta blockers, such as betaxolol, timolol and the like; alpha-adrenergic agonists, such as apraclonidine, brimonidine and the like; carbonic anhydrase inhibitors, such as brinzolamide, dorzolamide, cholinergic agents, such as pilocarpine and cevimeline; and the like, as well as combinations, such as timolol and dorzolamide, brimonidine and timolol, brimonidine and brinzolamide, and the like.

[0237] In some examples, a therapeutically effective concentration of the therapeutic composition may be delivered to the target tissue. The therapeutic composition may be formulated such that the therapeutically effective concentration may be substantially higher than a systemic therapeutic blood concentration of each agent, preferably higher than a systemic therapeutic blood $C_{\max}$ dose of each agent, while the agent blood $C_{\max}$ (from agent diffusing into the systemic circulation) remains under systemic dose of the agent blood $C_{\max}$. For example, tissue concentration of the agent Apixaban in the ocular tissue (e.g., the scleral tissue) may range from 0.1 ng/g tissue while the blood concentration in the systemic circulation remains below 100 ng/ml of blood.

[0238] For example, typical blood concentrations for systemically delivered apixaban ranges from about 10 ng/ml to about 40 ng/ml in the blood and the $C_{\max}$ depending on the doses ranges from about 50ng/ml to about 100ng/ml (sometimes up to about 200ng/ml). If we assume density of the tissue (being mostly water) is about 1 g/ml, these ranges can be converted from ng/ml to ng/g, with 10ng/ml being equivalent to about 10 ng/g tissue and 200 ng/ml being equivalent to about 200 ng/g tissue, which means $C_{\max}$ is at or below 200 ng/g of tissue.

[0239] In some examples, the therapeutically effective dose in the ocular tissue may be sufficient to generate a blood concentration of the direct factor Xa inhibitor which is less than about 200 ng/ml, 100 ng/ml, 50 ng/ml, 40 ng/ml, or 10 ng/ml.

[0240] In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of the direct factor Xa inhibitor which is smaller than a median maximum serum concentration ($C_{\max}$) of the direct factor Xa inhibitor generated by systemic delivery of the direct factor Xa inhibitor to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of the direct factor Xa inhibitor which does not exceed a median maximum serum concentration ($C_{\max}$) of the direct factor Xa inhibitor generated by systemic delivery of the direct factor Xa inhibitor to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition for more than about 6 hours to about 3 days. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a

smallest oral dose of the direct factor Xa inhibitor. In some examples, the $C_{\max}$ is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median $C_{\max}$ is 80ng/ml, or 123ng/ml, or 171ng/ml, or 321ng/ml, or 480ng/ml of blood.

[0241] In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of the direct factor Xa inhibitor which is less than a systemic therapeutic concentration of the direct factor Xa inhibitor for any systemic indication.

[0242] In some examples, the therapeutic composition is formulated to release a dose of the direct factor Xa inhibitor sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of the direct factor Xa inhibitor generated by systemic delivery of the direct factor Xa inhibitor to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In other examples, the therapeutic composition is formulated to release a dose of the direct factor Xa inhibitor sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of the direct factor Xa inhibitor generated by systemic delivery of the direct factor Xa inhibitor when taking one or more oral dose of said factor Xa inhibitor. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of the direct factor Xa inhibitor. In some examples, the (AUC (0-24) or AUC (0- ∞)) is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median (AUC (0-24) or AUC (0- ∞)) is 724 ng.h/ml, or 1437 ng.h/ml, or 2000 ng.h/ml, or 4000 ng.h/ml.

[0243] In some examples, the therapeutically effective dose in ocular tissue may be sufficient to generate a blood concentration of the direct factor IIa inhibitor which is less than about 200 ng/ml, 100 ng/ml, 50 ng/ml, 40 ng/ml, or 10 ng/ml.

[0244] In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of the direct factor IIa inhibitor which is smaller than a median maximum serum concentration ($C_{\max}$) of the direct factor IIa inhibitor generated by systemic delivery of the direct factor IIa inhibitor to achieve the same tissue concentration at the site of the the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of the direct factor IIa inhibitor which does not exceed a median maximum serum concentration ($C_{\max}$) of the direct factor IIa inhibitor generated by systemic delivery of the direct factor IIa inhibitor to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition for more than about 6 hours to about 3 days. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a

smallest oral dose of the direct factor IIa inhibitor. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of the direct factor IIa inhibitor. In some examples, the $C_{\max}$ is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median $C_{\max}$ is 80ng/ml, or 123ng/ml, or 171ng/ml, or 321ng/ml, or 480ng/ml of blood.

[0245] In some examples, the therapeutic composition is formulated to release a dose of the direct factor IIa inhibitor sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of the direct factor IIa inhibitor generated by systemic delivery of the direct factor IIa inhibitor to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In other examples, the therapeutic composition is formulated to release a dose of the direct factor IIa inhibitor sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of the direct factor IIa inhibitor generated by systemic delivery of the direct factor IIa inhibitor when taking one or more oral dose of said factor IIa inhibitor. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of the direct factor IIa inhibitor. In some examples, the (AUC (0-24) or AUC (0- ∞)) is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median (AUC (0-24) or AUC (0- ∞)) is 724 ng.h/ml, or 1437 ng.h/ml, or 2000 ng.h/ml, or 4000 ng.h/ml.

[0246] In some examples, the therapeutically effective dose in ocular tissue may be sufficient to generate a blood concentration of the anti-fibrotic agent comprising nintedanib which is less than about 100 ng/ml, 50 ng/ml, 40 ng/ml, or 30 ng/ml.

[0247] In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of nintedanib which is smaller than a median maximum serum concentration ($C_{\max}$) of nintedanib generated by systemic delivery of nintedanib to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of nintedanib which does not exceed a median maximum serum concentration ($C_{\max}$) of nintedanib generated by systemic delivery of nintedanib to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition for more than about 6 hours to about 12 hours. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of nintedanib. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of nintedanib. In some examples, the

$C_{\max}$ is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median $C_{\max}$ is 31.8 ng/ml or 43.2 ng/ml of blood.

[0248] In some examples, the therapeutic composition is formulated to release a dose of nintedanib sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of nintedanib generated by systemic delivery of nintedanib to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In other examples, the therapeutic composition is formulated to release a dose of nintedanib sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of nintedanib generated by systemic delivery of nintedanib when taking one or more oral dose of nintedanib. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of nintedanib. In some examples, the (AUC (0-24) or AUC (0- ∞)) is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median (AUC (0-24) or AUC (0- ∞)) is 266 ng.h/ml.

[0249] In some examples, the therapeutically effective dose in ocular tissue may be sufficient to generate a blood concentration of the anti-fibrotic agent comprising pirfenidone which is less than about 20000 ng/ml, 12000 ng/ml, 7000 ng/ml, or 6500 ng/ml.

[0250] In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of pirfenidone which is smaller than a median maximum serum concentration ($C_{\max}$) of pirfenidone generated by systemic delivery of pirfenidone to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of pirfenidone which does not exceed a median maximum serum concentration ($C_{\max}$) of pirfenidone generated by systemic delivery of pirfenidone to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition for more than about 6 hours to about 3 days. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of pirfenidone. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of pirfenidone. In some examples, the $C_{\max}$ is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median $C_{\max}$ is 6560 ng/ml, or 7640 ng/ml, or 12300 ng/ml, or 12500 ng/ml, or 12600 ng/ml, or 19800 ng/ml of blood.

[0251] In some examples, the therapeutic composition is formulated to release a dose of pirfenidone sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC

(0-∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0-∞)) in ng.h/ml of pirfenidone generated by systemic delivery of pirfenidone to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In other examples, the therapeutic composition is formulated to release a dose of pirfenidone sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0-∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0-∞)) in ng.h/ml of pirfenidone generated by systemic delivery of pirfenidone when taking one or more oral dose of pirfenidone. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of pirfenidone. In some examples, the (AUC (0-24) or AUC (0-∞)) is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median (AUC (0-24) or AUC (0-∞)) is 39800 ng.h/ml, or 40900 ng.h/ml, or 49400 ng.h/ml, or 49700 ng.h/ml, or 55900 ng.h/ml, or 92900 ng.h/ml.

[0252] In some examples, the therapeutically effective dose in ocular tissue may be sufficient to generate a blood concentration of the anti-viral/anti-diabetic agent comprising metformin which is less than about 1800 µg/ml, 900 µg/ml, 500 µg/ml, 50 µg/ml, 1.3 µg/ml, 1 µg/ml, or 0.8 µg/ml.

[0253] In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of metformin which is smaller than a median maximum serum concentration (C_{max}) of metformin generated by systemic delivery of metformin to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of metformin which does not exceed a median maximum serum concentration (C_{max}) of metformin generated by systemic delivery of metformin to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition for more than about 6 hours to about 3 days. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of metformin. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of metformin. In some examples, the C_{max} is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median C_{max} is 811.9 ng/ml, or 959.1 ng/ml, or 1301.4 ng/ml of blood.

[0254] In some examples, the therapeutic composition is formulated to release a dose of metformin sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0-∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0-∞)) in ng.h/ml of metformin generated by systemic delivery of metformin to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease

or condition. In other examples, the therapeutic composition is formulated to release a dose of metformin sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0-∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0-∞)) in ng.h/ml of metformin generated by systemic delivery of metformin when taking one or more oral dose of metformin. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of metformin. In some examples, the (AUC (0-24) or AUC (0-∞)) is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median (AUC (0-24) or AUC (0-∞)) is 14182 ng.h/ml, or 15260 ng.h/ml, or 15342 ng.h/ml.

[0255] In some examples, the therapeutically effective dose in ocular tissue may be sufficient to generate a blood concentration of the anti-proliferative agent comprising sirolimus which is less than about 200 ng/ml.

[0256] In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of sirolimus which is smaller than a median maximum serum concentration ($C_{\max}$) of sirolimus generated by systemic delivery of sirolimus to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of sirolimus which does not exceed a median maximum serum concentration ($C_{\max}$) of sirolimus generated by systemic delivery of sirolimus to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition for more than about 6 hours to about 7 days. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of sirolimus. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of sirolimus. In some examples, the $C_{\max}$ is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median $C_{\max}$ is less than about 144 ng/ml.

[0257] In some examples, the therapeutic composition is formulated to release a dose of sirolimus sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0-∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0-∞)) in ng.h/ml of sirolimus generated by systemic delivery of sirolimus to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In other examples, the therapeutic composition is formulated to release a dose of sirolimus sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0-∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0-∞)) in ng.h/ml of sirolimus generated by systemic delivery of sirolimus when taking one or more oral dose of sirolimus. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a

smallest oral dose of sirolimus. In some examples, the (AUC (0-24) or AUC (0-∞)) is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median (AUC (0-24) or AUC (0-∞)) is 160 ng.h/ml, or 200 ng.h/ml, or 230 ng.h/ml, or 400 ng.h/ml, or 5432 ng.h/ml, or 34920 ng.h/ml.

[0258] In some examples, the therapeutically effective dose in ocular tissue may be sufficient to generate a blood concentration of the anti-proliferative agent comprising novolimus which is less than about 100 ng/ml.

[0259] In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of novolimus which is smaller than a median maximum serum concentration ($C_{\max}$) of novolimus generated by systemic delivery of novolimus to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of novolimus which does not exceed a median maximum serum concentration ($C_{\max}$) of novolimus generated by systemic delivery of novolimus to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition for more than about 6 hours to about 7 days. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of novolimus. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of novolimus. In some examples, the $C_{\max}$ is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median $C_{\max}$ is less than about 100 ng/ml.

[0260] In some examples, the therapeutic composition is formulated to release a dose of novolimus sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0-∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0-∞)) in ng.h/ml of novolimus generated by systemic delivery of novolimus to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In other examples, the therapeutic composition is formulated to release a dose of novolimus sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0-∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0-∞)) in ng.h/ml of novolimus generated by systemic delivery of novolimus when taking one or more oral dose of novolimus. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of novolimus. In some examples, the (AUC (0-24) or AUC (0-∞)) is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median (AUC (0-24) or AUC (0-∞)) is 61 ng.h/ml, or 489 ng.h/ml to 1032 ng.h/ml, or within a range of about 3060 ng.h/ml to about 6660ng.h/ml.

[0261] In some examples, the therapeutically effective dose in ocular tissue may be sufficient to generate a blood concentration of the anti-proliferative agent comprising myolimus which is less than about 200 ng/ml.

[0262] In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of myolimus which is smaller than a median maximum serum concentration ($C_{\max}$) of myolimus generated by systemic delivery of myolimus to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of myolimus which does not exceed a median maximum serum concentration ($C_{\max}$) of myolimus generated by systemic delivery of myolimus to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition for more than about 6 hours to about 7 days. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of myolimus. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of myolimus. In some examples, the $C_{\max}$ is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median $C_{\max}$ is less than about 200 ng/ml.

[0263] In some examples, the therapeutic composition is formulated to release a dose of myolimus sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of myolimus generated by systemic delivery of myolimus to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In other examples, the therapeutic composition is formulated to release a dose of myolimus sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of myolimus generated by systemic delivery of myolimus when taking one or more oral dose of myolimus. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of myolimus. In some examples, the (AUC (0-24) or AUC (0- ∞)) is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median (AUC (0-24) or AUC (0- ∞)) is 100 ng.h/ml, or 200 ng.h/ml, or 300 ng.h/ml, or 400 ng.h/ml, 1000 ng.h/ml or 5000 ng.h/ml, or 50000 ng.h/ml.

[0264] In some examples, the therapeutically effective dose in ocular tissue may be sufficient to generate a blood concentration of the anti-angiogenic agent comprising ranibizumab which is less than about 1000 μ g/ml, 500 μ g/ml, 250 μ g/ml, 100 μ g/ml or 50 μ g/ml.,

[0265] In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of ranibizumab which is smaller than a median maximum serum concentration ($C_{\max}$) of ranibizumab generated by systemic delivery of ranibizumab to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of ranibizumab which does not exceed a median maximum serum concentration ($C_{\max}$) of ranibizumab generated by systemic delivery of ranibizumab to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition for more than about 6 hours to about 3 days. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of ranibizumab. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of ranibizumab. In some examples, the $C_{\max}$ is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median $C_{\max}$ is 620 $\mu\text{g/ml}$, or 500 $\mu\text{g/ml}$, or 230 $\mu\text{g/ml}$, or 170 $\mu\text{g/ml}$ or 120 $\mu\text{g/ml}$.

[0266] In some examples, the therapeutic composition is formulated to release a dose of ranibizumab sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of ranibizumab generated by systemic delivery of ranibizumab to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In other examples, the therapeutic composition is formulated to release a dose of ranibizumab sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of ranibizumab generated by systemic delivery of ranibizumab when taking one or more oral dose of ranibizumab. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of ranibizumab. In some examples, the (AUC (0-24) or AUC (0- ∞)) is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median (AUC (0-24) or AUC (0- ∞)) is 0.2 $\mu\text{g.d/ml}$, or 1 $\mu\text{g.d/ml}$, or 10 $\mu\text{g.d/ml}$, or 150 $\mu\text{g.d/ml}$, or 220 $\mu\text{g.d/ml}$, or 315 $\mu\text{g.d/ml}$, or 433 $\mu\text{g.d/ml}$, or 586 $\mu\text{g.d/ml}$, or 687 $\mu\text{g.d/ml}$, or 1500 $\mu\text{g.d/ml}$, or 3230 $\mu\text{g.d/ml}$.

[0267] In some examples, the therapeutically effective dose in ocular tissue may be sufficient to generate a blood concentration of the anti-angiogenic agent comprising brolucizumab which is less than about 100 $\mu\text{g/ml}$.

[0268] In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of brolucizumab which is smaller than a median maximum serum concentration ($C_{\max}$) of brolucizumab generated by systemic delivery of brolucizumab to

achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of brolocizumab which does not exceed a median maximum serum concentration ($C_{\max}$) of brolocizumab generated by systemic delivery of brolocizumab to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition for more than about 6 hours to about 3 days. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of brolocizumab. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of brolocizumab. In some examples, the $C_{\max}$ is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median $C_{\max}$ is 100 $\mu\text{g/ml}$, or 50 $\mu\text{g/ml}$, or 10 $\mu\text{g/ml}$, or 1 $\mu\text{g/ml}$ or 0.1 $\mu\text{g/ml}$.

[0269] In some examples, the therapeutic composition is formulated to release a dose of brolocizumab sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of brolocizumab generated by systemic delivery of brolocizumab to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In other examples, the therapeutic composition is formulated to release a dose of brolocizumab sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of brolocizumab generated by systemic delivery of brolocizumab when taking one or more oral dose of brolocizumab. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of brolocizumab. In some examples, the (AUC (0-24) or AUC (0- ∞)) is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median (AUC (0-24) or AUC (0- ∞)) is 0.2 $\mu\text{g.d/ml}$, or 1 $\mu\text{g.d/ml}$, or 10 $\mu\text{g.d/ml}$, or 30 $\mu\text{g.d/ml}$, or 60 $\mu\text{g.d/ml}$, or 100 $\mu\text{g.d/ml}$, or 200 $\mu\text{g.d/ml}$, or 300 $\mu\text{g.d/ml}$, or 400 $\mu\text{g.d/ml}$, or 500 $\mu\text{g.d/ml}$, or 700 $\mu\text{g.d/ml}$, or 1500 $\mu\text{g.d/ml}$.

[0270] In some examples, the therapeutically effective dose in ocular tissue may be sufficient to generate a blood concentration of the anti-angiogenic agent comprising aflibercept which is less than about 400 $\mu\text{g/ml}$.

[0271] In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of aflibercept which is smaller than a median maximum serum concentration ($C_{\max}$) of aflibercept generated by systemic delivery of aflibercept to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In some examples, the therapeutically effective dose may be

sufficient to generate a blood concentration of aflibercept which does not exceed a median maximum serum concentration ($C_{\max}$) of aflibercept generated by systemic delivery of aflibercept to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition for more than about 6 hours to about 3 days. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of aflibercept. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of aflibercept. In some examples, the $C_{\max}$ is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median $C_{\max}$ is 68 $\mu\text{g/ml}$ or 74 $\mu\text{g/ml}$.

[0272] In some examples, the therapeutic composition is formulated to release a dose of aflibercept sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of aflibercept generated by systemic delivery of aflibercept to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In other examples, the therapeutic composition is formulated to release a dose of aflibercept sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of aflibercept generated by systemic delivery of aflibercept when taking one or more oral dose of aflibercept. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of aflibercept. In some examples, the (AUC (0-24) or AUC (0- ∞)) is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median (AUC (0-24) or AUC (0- ∞)) is 124 $\mu\text{g.d/ml}$ or 174 $\mu\text{g.d/ml}$.

[0273] In some examples, the therapeutically effective dose in ocular tissue may be sufficient to generate a blood concentration of the anti-angiogenic agent comprising pegaptanib which is less than about 1000 $\mu\text{g/ml}$.

[0274] In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of pegaptanib which is smaller than a median maximum serum concentration ($C_{\max}$) of pegaptanib generated by systemic delivery of pegaptanib to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In some examples, the therapeutically effective dose may be sufficient to generate a blood concentration of pegaptanib which does not exceed a median maximum serum concentration ($C_{\max}$) of pegaptanib generated by systemic delivery of pegaptanib to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition for more than about 6 hours to about 3 days. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or

a smallest oral dose of pegaptanib In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of pegaptanib. In some examples, the $C_{\max}$ is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median $C_{\max}$ is 0.1 $\mu\text{g/ml}$ to 2 $\mu\text{g/ml}$.

[0275] In some examples, the therapeutic composition is formulated to release a dose of pegaptanib sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of pegaptanib generated by systemic delivery of pegaptanib to achieve the same tissue concentration at the site of the inflammatory, fibrotic, and/or clot formation ophthalmic disease or condition. In other examples, the therapeutic composition is formulated to release a dose of pegaptanib sufficient to generate a plasma drug level area under the curve (AUC (0-24) or AUC (0- ∞)) in ng.h/ml which is smaller than a median (AUC (0-24) or AUC (0- ∞)) in ng.h/ml of pegaptanib generated by systemic delivery of pegaptanib when taking one or more oral dose of pegaptanib. In some examples, the systemic delivery comprises a single oral dose, a daily oral dose, or a smallest oral dose of pegaptanib. In some examples, the (AUC (0-24) or AUC (0- ∞)) is measured using one of plasma blood, serum blood, or whole blood. In other examples, the median (AUC (0-24) or AUC (0- ∞)) is 2 $\mu\text{g.h/ml}$, or 8 $\mu\text{g.h/ml}$ to 9 $\mu\text{g.h/ml}$, or 26 $\mu\text{g.h/ml}$ to 29 $\mu\text{g.h/ml}$.

[0276] In some examples, measurements of blood or tissue described herein comprise one or more of mammalian blood or tissue, porcine blood or tissue, human blood or tissue, rabbit blood or tissue, rat blood or tissue, mouse blood or tissue, or the like.

[0277] In some examples, measurements of dose described herein are of human and adjustment to dose to account for total blood and/or fluid in other animal species compared to human may be necessary to equate to human dose.

[0278] In some examples, one or more agents in the therapeutic composition may have low solubility. For example, one or more of the direct factor Xa inhibitor, the direct factor IIa inhibitor, the anti-fibrotic agent, the anti-angiogenic agent, the anti-proliferative agent, the anti-viral/anti-diabetic agent, etc. may have low solubility.

[0279] In some examples, two or more active substances (e.g., direct factor Xa inhibitor, direct factor IIa inhibitor, anti-proliferative agent, anti-viral agent, etc.) may be present in a polymeric material. In some examples, the polymeric material may comprise a material selected from the group consisting of polyacrylates, polymethacrylates, poly(n-butyl methacrylate), poly(hydroxyethylmethacrylate), poly(ethylene glycol), polyethylene oxide (PEO), polydimethylsiloxane, polyvinylpyrrolidone, ethylene-vinyl acetate, phosphorylcholine-containing polymers, poly(2-methacryloyloxyethylphosphorylcholine), poly(2-

methacryloyloxyethylphosphorylcholine-co-butyl methacrylate), and copolymers and combinations thereof.

[0280] In some examples, implants or vehicles for ocular drug delivery includes but are not limited to ocular inserts, microneedles with hollow for injection, drug coated microneedles with biodegradable or erode polymers, drug coated contact lens, intraocular lens implant with drug, a Port Delivery System (PDS) implant, and the like.

[0281] In some examples, an implant or vehicles delivery one or more drugs locally, wherein locally comprises delivery of said one or more drugs to one or more of site-specific ocular location.

[0282] In some examples, the therapeutic composition is formulated to release substantially all of the one or more active substances and the combination had synergistic effects, the effects that was better than either alone. In some examples, the therapeutic composition may be delivered simultaneously or sequentially in any order or combination as desired with any combination of methods or devices desired. For example, the microneedle can be configured to deposit a drug formulation within the cornea or sclera to release substantially all of the one or more active substances to one or more ocular tissues surrounding the depot to generate a tissue concentration of each of the agents at the treated site within about certain time of the ocular tissue contact. This release could be extended or sustained than that obtained by eye drops or other topical applications. The microneedle can be configured to delivery liquid formulation or can be configured as an implant with a degradable polymer and drugs.

[0283] One or more microneedles can be employed to delivery one or more drugs to one or more of site-specific ocular location from a formulation identical to or different from one another of drug formulation (drug type, volume, amount, and ingredient etc., or a combination of these parameters). For example, two or more microneedles can be inserted into the ocular tissue, each microneedle may form an independent pocket in the ocular tissue into or through which a drug formulation may be deposited.

[0284] Further, the first microneedles can be inserted into the ocular tissue with formulation to generate a drug to ocular tissue with a burst release rate and the second microneedles can be inserted into the ocular tissue with formulation to generate a drug to ocular tissue with a sustained or extended-release rate. Each microneedle may form an independent pocket in the ocular tissue into or through which a drug formulation may be deposited. Alternatively, a first microneedle may be used to deliver the anti-VEGF agent and a second microneedle may be used to deliver the direct factor Xa inhibitor and the direct factor IIa inhibitor.

[0285] The microneedle can be formed an implant from a drug encapsulated within a biodegradable/bioerodable polymer, which can be implanted into the ocular tissue for drug release upon degradation/dissolution of the microneedle. The microneedle can also be formed of drug coated metal and delivers the drug by dissolution/diffusion upon insertion into the ocular tissue.

[0286] In some examples, a drug delivery system comprised a drug coated contact lens. The drug can be nano dispersed and diffuse into or migrate through contact lens and into the post-lens tear film when contact lens is placed on the eye.

[0287] In some examples, a drug delivery system comprised Port Delivery System (PDS). The PDS is designed to be a permanent, reusable drug reservoir as a refillable eye implant as the size of a grain of rice to continuously deliver a drug over a period of months, potentially reducing the treatment burden associated with frequent eye injections. It is initially placed as an implant, and subsequent refills with a specialized needle that flushes out the contents of the device while at the same time refilling it with fresh drug in a doctor office. At the distal end of the septum of the needle, there is a semipermeable titanium membrane that permits continuous passive diffusion of the drug from the higher concentration in the reservoir into the vitreous. The reservoir capacity is from about 0.005 cc to about 0.2 cc. The length of the device is no more than about 6 mm as the size of a grain of rice from the sclera into the vitreous has a minimal effect on patient vision. Extended or sustained release is achieved with the therapeutic agent released from the therapeutic device PDS after implantation. Upon release at the vitreous, the therapeutic agent is stable in the device for at least 30 days. In some cases, the therapeutic agent is released from the device for at least 6 months.

[0288] Controlled delivery implants may be formed from biodegradable/erodible polymer or water-soluble polymers matrix. The suitable polymer include but not limited to polyesters, polylactide, polyglycolide, poly(ϵ -caprolactone), polydioxanone, poly(hydroxyalkanoates), poly(L-lactide-co-D-lactide), poly(L-lactide-co-D,L-lactide), poly(D-lactide-co-D,L-lactide), poly(lactide-co-glycolide) (including 70:30 to 99:1 PLA-co-PGA, such as 85:15 PLA-co-PGA), poly(lactide-co- ϵ -caprolactone) (including 70:30 to 99:1 PLA-co-PCL, such as 90:10 PLA-co-PCL), poly(glycolide-co- ϵ -caprolactone), poly(lactide-co-dioxanone), poly(glycolide-co-dioxanone), poly(lactide-co-trimethylene carbonate), poly(glycolide-co-trimethylene carbonate), poly(lactide-co-ethylene carbonate), and copolymers and combinations thereof, wherein lactide includes L-lactide, D-lactide and D,L-lactide, block copolymer of ethylene oxide and propylene oxide, di-block copolymer of polyethylene oxide and polypropylene oxide, tri-block copolymer of polyethylene oxide and polypropylene oxide, and copolymers and combinations thereof.

EXPERIMENTAL EXAMPLES

[0289] Examples 1 through 29 describe coating implants in the form of stents, such as vascular stents and drug delivery balloons. The methods for implant fabrication and the successful elution of drugs described under vascular conditions for these implants would apply as well to the fabrication of ocular implants, such as shunts, drug coated microneedles, and ocular stents.

Example 1: Preparation of Anticoagulant (Rivaroxaban, Argatroban, and dalteparin) Eluting Implants

[0290] Poly(n-butyl methacrylate) polymer was dissolved into dichloromethane (tetrahydrofuran (THF) was used for Dalteparin) at room temperature and vortexed until the polymer had uniformly dissolved/dispersed. Rivaroxaban was dissolved into dichloromethane at room temperature and vortexed until the drug was uniformly dissolved/dispersed. Argatroban (and Argatroban in combination with Rivaroxaban) was dissolved in Methanol and dichloromethane and vortexed at room temperature until the drug was uniformly dispersed/dissolved. Dalteparin was dissolved in water and THF until fully dissolved.

[0291] Each polymer solution and each drug solution were combined together (Rivaroxaban to poly(n-butyl methacrylate) by weight ratio was 6:1), (Argatroban to poly(n-butyl methacrylate) by weight ratio was 3:4), (Dalteparin to poly(n-butyl methacrylate) by weight ratio was 2:3), and (Rivaroxaban in combination with Argatroban to Poly (n-butyl methacrylate) weight ratio was 3:2:2) according to the target drug dose of 150 µg for each drug (and 100 µg each for the Rivaroxaban and Argatroban combination).

[0292] A microprocessor-controlled ultrasonic sprayer was used to coat each of the implant (a stent but these methods would work as well for ocular shunts and other ocular implants) over 14 mm length uniformly with each of the drug/polymer matrix solutions. After coating, the implants were placed in a vacuum chamber to remove the solvent. The stent implants were then mounted on balloon catheters and crimped. The catheters were then inserted in coils and packaged. The pouches were sterilized. The bare metal control stents were the same as the other stents without a drug or polymer coating.

Example 2: In vivo testing of drug eluting stent with different drugs

[0293] The drug eluting stent systems containing different anticoagulants prepared as described in Example 1 were evaluated at 3 hours, 6 hours, 1 day, 3 days, 6 days, 7 days, or 28 days following implantation in a porcine coronary artery model.

[0294] The porcine model was chosen as this model has been used extensively for stent and angioplasty studies resulting in a large volume of data on the pulmonary response properties

and its correlation to human pulmonary response (Schwartz et al, Circulation. 2002; 106:1867-1873). The animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals as established by the National Research Council.

[0295] After induction of anesthesia, the left or right femoral artery was accessed using standard techniques and an arterial sheath was introduced and advanced into the artery. Vessel angiography was performed under fluoroscopic guidance, a 7 Fr. guide catheter was inserted through the sheath and advanced to the appropriate location where intracoronary nitroglycerin was administered. An appropriate implantation segment of coronary artery was randomly selected and a 0.014" guidewire inserted. Quantitative Coronary Angiography (QCA) was performed to document the results. The appropriately-sized stent was advanced to the deployment site. The balloon was inflated at a steady rate to a pressure sufficient to achieve a balloon to artery ratio of approximately 1.1 to 1.0 but less than 1.2:1.

[0296] Follow up angiography was performed at the designated timepoint for each of the animals. Late lumen loss (LLL) can be expressed as:

$$LLL = \text{Post-stent minimum lumen diameter} - \text{Final minimum lumen diameter}$$

The LLL is an indicator of the amount smooth muscle cell (SMC) proliferation or inhibition. It is used to measure efficacy between drugs for SMC proliferation inhibition. The smaller the LLL, the better the efficacy of the drug.

[0297] Stented portions of coronary arteries were embedded in methyl methacrylate (MMA), then divided into a target of at least three blocks of approximately similar lengths for histology evaluation. Quantitative histopathological evaluation of stented artery sections was then performed and scored as indicated. For Fibrin formation, scores ranged from 0 to 3, with a score of 0 indicating absent or rare minimal spotting around struts of the stent, a score of 1 indicating the presence of fibrin in small amounts localized only around the struts, a score of 2 indicating the moderately abundant or denser presence of fibrin around and extending beyond the struts, and a score of 3 indicating the presence of abundant and dense fibrin and/or bridging of the fibrin between the struts. The mean score was calculated and reported. The mean of each section was then averaged to provide a mean fibrin score per stent. The smaller the fibrin score, the better efficacy.

[0298] The percentage and/or amount of each anticoagulant drug for or by each time point indicated were analyzed for each stent from the different devices in the example and the average drug tissue concentration reported.

[0299] Tissue concentrations and the amount of drug released from the stents were measured using stents implanted in porcine arteries for the drugs as indicated. The arteries at the designated time point were excised and a length of stented artery spanning from 5 mm proximal

to the stented segment to 5 mm distal to the stented segment was cut. The stented artery was cut longitudinally with surgical scissors. The stents were separated from the tissue. The tissue content of each drug was analyzed using liquid chromatography mass spectroscopy (LCMS) and reported as a mean for each of the timepoint indicated. For drug remaining on each stent, each drug was extracted from the stent, measured using HPLC, and reported as a mean for each of the timepoint indicated as drug released or drug remaining on a stent (where drug remaining is equal to 100% minus the percentage of drug released).

Table 1

Histopathology Scores, Quantitative Coronary Angiography data and PK data of Rivaroxaban, Argatroban, and Dalteparin (low molecular weight heparin) released from 14mm stents at day 7.

Stent coating information (n=3 for each arm)	Fibrin score at day 7	Cumulative Percent Release of drug by day 7	Tissue concentration at day 7 (ng/mg)	LLL at day 7	Injury	Inflammation	Diameter Stenosis from Coronary (%)
150µg Rivaroxaban & 25 µg Poly(n-butyl methacrylate) matrix coated stent	0.72±0.12	99.6%	3.9±0.6	0.31±0.20	0.03±0.04	1.15±0.34	11.4±2.5
150µg Argatroban & 200 µg Poly(n-butyl methacrylate) matrix coated stent	1.25±0.87	47.7%	5.7±1.8	0.34±0.20	0.08 ± 0.08	0.81 ± 0.46	2.4±2.3
100µg Argatroban and 100µg Rivaroxaban in 150 µg Poly(n-butyl methacrylate) matrix coated stent	0.80±0.39	70.9% for Rivaroxaban 96.5% for Argatroban	48.1±43.6 for Rivaroxaban 8.9±6.3 for Argatroban	0.04±0.06	0.06 ± 0.07	1.11 ± 0.35	0.5±0.9
150µg Dalteparin(low molecular weight heparin) in 225 µg Poly(n-butyl methacrylate) matrix coated stent	1.50±0.82	98.3%	Not tested	0.23±0.20	0.05±0.06	0.65±0.30	3.7±5.2
Bare metal control stent (BMS)	1.02±0.35	N/A	N/A	0.17±0.22	0.05 ± 0.08	0.77 ± 0.45	3.3±1.7

[0300] As shown in Table 1, Rivaroxaban composition released from stents was more effective at inhibiting fibrin formation compared to bare metal control stents at 7 days, while Argatroban composition released from stents or Dalteparin composition released from stents were not more effective at inhibiting fibrin formation compared to bare metal control stents at 7 days.

[0301] As shown in Table 1, Rivaroxaban, Argatroban, or Dalteparin compositions released from stents as single agents had larger LLLs compared to control and thus were not more effective at inhibiting smooth muscle cell proliferation compared to bare metal control stents at 7 days.

[0302] As shown in Table 1, the combination of Rivaroxaban and Argatroban composition released from stents had a smaller LLL compared to control and thus was more effective at inhibiting smooth muscle cell proliferation compared to bare metal control stents at 7 days. Furthermore, the combination of Rivaroxaban and Argatroban composition released from stents was more effective at inhibiting fibrin formation compared to bare metal control stents.

[0303] As shown in Table 1, Rivaroxaban composition comprising fast released from stents at a dose of about 150 μg within 7 days from implant (or from vessel injury) was more effective at inhibiting fibrin formation at or within 7 days.

[0304] As shown in Table 1, Rivaroxaban composition comprising fast released from stents at a dose of about 1.8 $\mu\text{g}/\text{mm}^2$ within 7 days from implant (or from vessel injury) was more effective at inhibiting fibrin formation at or within 7 days.

[0305] As shown in Table 1, Rivaroxaban composition comprising fast released from stents at a dose of about 10.7 $\mu\text{g}/\text{mm}$ of stent length within 7 days from implant (or from vessel injury) was more effective at inhibiting fibrin formation.

[0306] As shown in Table 1, Rivaroxaban composition comprising a dose of about 150 μg , and/or of about 1.8 $\mu\text{g}/\text{mm}^2$, and/or of about 10.7 $\mu\text{g}/\text{mm}$ of device length, released from a stents device at a release rate comprising of about 99.5% within 7 days from implant (or from time of injury) was more effective at inhibiting fibrin formation.

[0307] As shown in Table 1, Rivaroxaban composition released from stents at a release rate comprising of about 70.9% within 7 days when combined with Argatroban composition at a release rate comprising of about 96.9% within 7 days from implant (or from time of injury) was more effective at inhibiting fibrin formation.

[0308] Table 1 shows Rivaroxaban composition released from a stent at a dose of about 100 μg , or at a dose comprising of about 1.2 $\mu\text{g}/\text{mm}^2$, and/or at a dose of about 7.14 $\mu\text{g}/\text{mm}$ of stent length, at a release rate comprising of about 70.9% within 7 days when combined with Argatroban composition released from a stent at a dose comprising of about 100 μg , and/or at a dose of about 1.2 $\mu\text{g}/\text{mm}^2$, and/or at a dose of about 7.14 $\mu\text{g}/\text{mm}$ of stent length, at a release rate comprising of about 96.5% within 7 days from implant (or from time of injury) was more effective at inhibiting fibrin formation.

Example 3: Preparation of Rivaroxaban and m-TOR inhibitor releasing implant

[0309] Base coat of Novolimus (m-TOR inhibitor) and Poly (n-butyl methacrylate) matrix: Poly(n-butyl methacrylate) polymer was dissolved into dichloromethane at room temperature and vortex until the polymer had uniformly dissolved/dispersed. Novolimus was placed in another vial and dissolved in dichloromethane at room temperature until uniformly dissolved or dispersed, The polymer solution and drug solutions were mixed together and coated as a matrix (the drug to polymer weight ratio was 2:3 by weight).

[0310] Top layer or coat of Rivaroxaban and poly (n-butyl methacrylate) matrix: Poly(n-butyl methacrylate) polymer was dissolved in dichloromethane at room temperature and vortex until the polymer had uniformly dissolved/dispersed. Rivaroxaban was dissolved into dichloromethane at room temperature and vortex until the drug was uniformly dissolved/dispersed. Each polymer solution and each drug solutions were mixed together as a matrix (Rivaroxaban to poly(n-butyl methacrylate) by weight ratio was 6:1 for the Rivaroxaban fast formulation without m-TOR). Rivaroxaban to poly (n-butyl methacrylate) ratio was 4:1 for the fast release formulation with m-TOR base coat matrix, and 2:1 for the slow release formulation with m-TOR base coat matrix according to the target drug dose of 100 µg Rivaroxaban and 25 µg poly(n-butyl methacrylate) for fast release formulation, and 100 µg Rivaroxaban and 50 µg poly(n-butyl methacrylate) for the slow release formulation.

[0311] A microprocessor-controlled ultrasonic sprayer was used to coat each of the the implants (the stents but these methods would work as well for ocular shunts and other ocular implants) ' 14mm length uniformly with each of the drug/polymer matrix solution with the base coat matrix first, placing the stents in vacuum chamber to remove the solvent, followed by the top layer or coat matrix. The stents were placed in a vacuum chamber again to remove the solvents. The stents were then mounted on balloon catheters and crimped. The catheters were then inserted in coils and packaged. The pouches were sterilized. The Novolimus (m-TOR inhibitor) stents controls (DES) consisted of only the base coat drug/polymer matrix, without the top layer or coat drug/polymer matrix, otherwise being the same as the other stents. The bare metal control stents (BMS) were the same as the other stents without a drug or polymer coating.

Table 2

Histopathology Scores, Quantitative Coronary Angiography data and PK of Rivaroxaban releasing 14mm stents at day 7 and at day 28.

Time point	Stent coating information n=5 for each arm for each time point	Fibrin score	Cumulative percent release of drug	Tissue concentration (ng)	Late lumen loss (mm)	Injury score	Inflammation score	Diameter stenosis (%)
Day 7 (n=5)	25 µg Poly(n-butyl methacrylate) & 150 µg Rivaroxaban matrix	0.79 ± 0.14	99.7%	3.95 ± 1.57	0.26±0.24	0.07 ± 0.05	1.19 ± 0.26	8.5±5.2
	66 µg Novolimus (m-TOR inhibitor) and 100 µg Poly(n-butyl methacrylate) matrix as a base coat then 100 µg Rivaroxaban and 25 µg Poly(n-butyl methacrylate) matrix as a top coat	1.16 ± 0.60	88.9%	6.18 ± 5.37	0.21±0.26	0.17 ± 0.09	1.32 ± 0.22	5.5±5.9
	66 µg Novolimus (m-TOR inhibitor) and 100 µg Poly(n-butyl methacrylate) matrix as a base coat then 100 µg Rivaroxaban and 50 µg Poly(n-butyl methacrylate) matrix as a top coat	1.18 ± 0.53	68.1%	8.56 ± 3.56	0.23±0.22	0.26 ± 0.19	1.48 ± 0.13	4.1±3.6
	66 µg Novolimus (m-TOR inhibitor) and 100 µg Poly(n-butyl methacrylate) matrix (DES) control	1.51 ± 0.71	--	--	0.13±0.13	0.20 ± 0.13	1.32 ± 0.51	9.0±7.3
	Bare metal control (BMS)	1.35 ± 0.41	--	--	0.05±0.09	0.13 ± 0.07	1.22 ± 0.30	5.4±3.4
Day 28 (n=5)	25 µg Poly(n-butyl methacrylate) & 150 µg Rivaroxaban matrix	0.07 ± 0.05	100%	3.93 ± 2.43	0.62±0.21	0.25 ± 0.16	0.73 ± 0.24	22.7±8.7
	66 µg Novolimus (m-TOR inhibitor) and 100 µg Poly(n-butyl methacrylate) matrix as a base coat then 100 µg Rivaroxaban and 25 µg Poly(n-butyl methacrylate) matrix as a topcoat	1.06 ± 0.46	92.5%	14.61 ± 17.68	0.88±0.96	0.68 ± 0.57	1.04 ± 0.70	38.9±36.0
	66 µg Novolimus (m-TOR inhibitor) and 100 µg Poly(n-butyl methacrylate) matrix as a base coat then 100 µg Rivaroxaban and 50 µg Poly(n-butyl methacrylate) matrix as a topcoat	1.49 ± 0.37	72.7%	15.23 ± 8.12	0.84±0.73	0.53 ± 0.38	0.72 ± 0.86	22.4±20.8
	66 µg Novolimus (m-TOR inhibitor) and 100 µg Poly(n-butyl methacrylate) matrix (DES) control	1.37 ± 0.44	--	--	0.88±0.21	0.55 ± 0.33	0.79 ± 0.80	24.8±13.9

[0312] As shown in Table 2, Rivaroxaban composition comprising fast released formulation from a stent was more effective at inhibiting fibrin formation compared to control at 7 days and/or at 28 days when it was released at a faster rate formulation.

[0313] As shown in Table 2, Rivaroxaban composition released from a stent was more effective at inhibiting fibrin formation compared to control at 7 days and/or at 28 days when it was released at a rate comprising of about 88.9% within 7 days and/or when a dose of about

88.9 μg , and/or a dose of about 1.2 $\mu\text{g}/\text{mm}^2$, and/or a dose of about 7.14 $\mu\text{g}/\text{mm}$ of stent length, was released within 7 days from vessel injury (or from implantation).

[0314] As shown in Table 2, Rivaroxaban formulation released from a stent was more effective at inhibiting fibrin formation compared to control at 7 days and/or at 28 days when it was released at a rate comprising of about 92.5% within 28 days and/or when a dose of about 92.5 μg , and/or a dose of about 1.1 $\mu\text{g}/\text{mm}^2$, or a dose of about 6.6 $\mu\text{g}/\text{mm}$ of stent length, was released at or within 28 days from vessel injury (or from implantation).

[0315] As shown in Table 2, Rivaroxaban composition released from a stent was more effective at inhibiting fibrin formation compared to control at 7 days and/or at 28 days when it was released at a rate comprising of 88.9% within 7 days and/or when a dose of 88.9 μg , and/or a dose of 1.2 $\mu\text{g}/\text{mm}^2$, and/or a dose of 7.14 $\mu\text{g}/\text{mm}$ of stent length, was released within 7 days after vessel injury (or from implantation), and/or at a rate comprising of about 92.5% within 28 days and/or when a dose of about 92.5 μg , and/or a dose of about 1.1 $\mu\text{g}/\text{mm}^2$, and/or a dose of about 6.6 $\mu\text{g}/\text{mm}$ of stent length, was released within 28 days after vessel injury (or from implantation).

[0316] As shown in Table 2, Rivaroxaban composition released in combination with an m-TOR inhibitor from a stent was more effective at inhibiting fibrin formation compared to control at 7 days and/or at 28 days when it was released in a faster formulation rate in accordance with the experiment.

[0317] As shown in Table 2, Rivaroxaban composition released in combination with an m-TOR formulation inhibitor from a stent was more effective at inhibiting fibrin formation compared to control at 7 days and/or at 28 days when Rivaroxaban composition was released at a rate comprising of about 88.9 μg , or a dose of about 1.2 $\mu\text{g}/\text{mm}^2$, or a dose of about 7.14 $\mu\text{g}/\text{mm}$ of stent length, within 7 days, and/or released at a rate comprising of about 92.5 μg , and/or a dose of about 1.1 $\mu\text{g}/\text{mm}^2$, and/or a dose of about 6.6 $\mu\text{g}/\text{mm}$ of stent length, within 28 days.

[0318] As shown in Table 2, Rivaroxaban composition released in combination with an m-TOR inhibitor formulation from a stent was more effective at inhibiting fibrin formation compared to control at 7 days when Rivaroxaban composition was released at a rate comprising of about 68.1 μg , or at rate comprising of 0.84 $\mu\text{g}/\text{mm}^2$, and/or at a rate comprising of about 4.86 $\mu\text{g}/\text{mm}$ of stent length, within 7 days.

[0319] As shown in Table 2, Rivaroxaban tissue concentration ranges from at least 3.96 ng/mg of tissue adjacent to the stented segment to at least 15 ng/mg of tissue adjacent to the stented segment, within or at 7 days, or within or 28 days from implant (or tissue injury)

[0320] It was reported that Rivaroxaban IC₅₀ for factor Xa inhibition to be about 21nM or 0.0092ng/mg. As shown in Table 2, the tissue concentration for Rivaroxaban was at least 426 times Rivaroxaban IC₅₀ for factor Xa inhibition.

Table 3

Tissue concentration of Rivaroxaban and Argatroban at 7 days show multiple folds higher (or times higher) than IC₅₀ for Anti-factor Xa/IIa and antiplatelet for the respective drugs.

Stent coated with combination of Rivaroxaban and Argatroban	Rivaroxaban*		Argatroban**	
	Tissue concentration at day 7 in folds higher than IC ₅₀ of anti-Factor Xa	Tissue concentration at day 7 in folds higher than IC ₅₀ of anti-platelet	Tissue concentration at day 7 in folds higher than IC ₅₀ of anti-Factor IIa	Tissue concentration at day 7 in folds higher than IC ₅₀ of anti-platelet
100µg Argatroban and 100µg Rivaroxaban in Poly(n-butyl methacrylate) matrix	5253	354	835	1223

* Rivaroxaban IC₅₀ for Anti-Factor Xa is 21nM or 0.00916ng/mg

*Rivaroxaban IC₅₀ for Tissue factor generated antiplatelet is 312nM or 0.136ng/mg

**Argatroban IC₅₀ for Anti-Factor IIa is 21nM or 0.0107ng/mg

**Argatroban IC₅₀ for Tissue factor generated antiplatelet is 79nM or 0.04ng/mg

[0321] Table 3 shows the tissue PK data for Rivaroxaban and Argatroban at or by or within 7 days from implants of stented vessels. It shows Rivaroxaban and Argatroban has therapeutic tissue concentrations in the tissue segment up to 7 days. Table 3 is Rivaroxaban and Argatroban concentration (ng/mg) in the tissue of treated area of the implanted device fold higher than IC₅₀ for anti-Factor Xa or Anti-Factor IIa and anti-platelet. It shows that Rivaroxaban and Argatroban in tissue concentrations have several order of magnitudes, has from 2 to 4 orders of magnitude of tissue concentration for each of the drugs compared to their IC₅₀, in the treated tissue segments up to 7 days, therefore inhibiting or enhancing dissolution of one or more of cell proliferation, fibrin formation, or clot formation on the device surfaces, the stented segment tissue, and/or the tissue adjacent to the stented segment.

Example 4: Preparation of Anticoagulant1/Anticoagulant2/mTOR Eluting implants

[0322] Poly(L-lactide acid-co-glycolic acid) polymer was dissolved into dichloromethane at room temperature and vortex until the polymer had uniformly dissolved/dispersed. Sirolimus and anticoagulants (Apixaban or Rivaroxaban and Argatroban) were placed in a vial and

dissolved in dichloromethane or dichloromethane/Methanol at room temperature and vortex until all the drug was uniformly dissolved/dispersed.

[0323] Each polymer solution and each drug (or combined drugs) solutions were combined together (SS7 arm anticoagulant (Apixaban to Argatroban was 1:1) to poly(L- lactide acid-co-glycolic acid) matrix by weight ratio was 3:1 as a base coat and Sirolimus to poly(L- lactide acid-co-glycolic acid) matrix by weight ratio was 2:3 and coated as a top coat), (SS9 arm Sirolimus and anticoagulant Apixaban and Argatroban was (1:1:1) to poly(L- lactide acid-co-glycolic acid) by weight ratio was 5:2 on matrix), (SS15 arm Sirolimus and Apixaban and Argatroban were combined in a ratio of (1:1:1) with poly(L- lactide acid-co- glycolic acid) by weight ratio which was (1:2)(by weight of 23 µg Sirolimus, 23 µg Apixaban and 23 µg Argatroban combined with 138 µg poly(L- lactide acid-co- glycolic acid)) and mixed together, and coated as a base coat (drug/polymer matrix as a base coat). In addition, Sirolimus and Apixaban and Argatroban were combined in the ratio of (3:4:4) with poly(L- lactide acid-co-glycolic acid) by weight ratio which was (5:3) and coated as a top layer or coat (drug/polymer matrix top layer or coat), (by weight of 71 µg Sirolimus, 94 µg Apixaban and 94 µg Argatroban and combined with 155 µg poly(L- lactide acid-co- glycolic acid) and coated as a top layer or coat, for cumulative total target drug dose of 117 µg for each anticoagulant and 94 µg for Sirolimus for a 14mm stent length, (Slider II Arm1 (SS16) Sirolimus and Rivaroxaban and Argatroban were combined together in the ratio of (1:1:1) and were combined with poly(L- lactide acid-co- glycolic acid) by weight ratio which was (1:2) and coated as a base coat (drug/polymer matrix as base coat). In addition, Sirolimus and Rivaroxaban and Argatroban were combined in the ratio of (3:4:4) and combined with poly(L- lactide acid-co- glycolic acid) by weight ratio was (5:3) and coated as a top layer or coat (drug/polymer matrix as top layer or coat), (by weight of 23 µg Sirolimus, 23 µg Rivaroxaban and 23 µg Argatroban and 138 µg poly(L- lactide acid-co- glycolic acid) mixed together and coated as base coat; and by weight of 71 µg Sirolimus, 94 µg Rivaroxaban and 94 µg Argatroban and 155 µg poly(L- lactide acid-co-glycolic acid) mix together in a matrix and coated as top layer or coat, for a total target drug dose of 117 µg for each anticoagulant and 94 µg for Sirolimus for a 14mm stent length , (Slider II Arm2 (SS17) Sirolimus and Rivaroxaban and Argatroban were combined in a ratio of (4:1:1) and combined with poly(L- lactide acid-co- glycolic acid) by weight ratio which was (1:1) and coated as a base coat (drug/polymer matrix as base coat). In addition, Rivaroxaban and Argatroban were combined in a ratio of (1:1) and combined with poly(L- lactide acid-co-glycolic acid) by weight ratio which was (5:3) and coated as a top layer or coat on the stent (drug/polymer matrix as a top layer or coat), (by weight of 94 µg Sirolimus, 23 µg Rivaroxaban and 23 µg Argatroban and 140 µg poly(L- lactide acid-co- glycolic acid) mixed together and

coated as base coat; and by weight of 94 µg Rivaroxaban and 94 µg Argatroban and 113 µg poly(L- lactide acid-co- glycolic acid) were mixed together and coated as top layer or coat, for a total target drug dose of 117 µg for each anticoagulant and 94 µg for Sirolimus for a 14mm stent length. The preceding doses for SS7, SS9, SS15, SS16, and SS17 were for 14mm stent lengths. Drug and polymer doses are adjusted accordingly for each stent length. Control was 14mm stent length eluting 65 µg Novolimus (m-TOR inhibitor). A microprocessor controlled ultrasonic sprayer was used to coat each of the implants' (the stents but these methods would work as well for ocular shunts and other ocular implants) 14mm length uniformly with each of the drug/polymer matrix solution. After coating, the stents were placed in a 70°C oven for about 2 hours to remove the solvent. The stents were then mounted on balloon catheters and crimped. The catheters were then inserted in coils and packaged. The pouches were sterilized.

[0324] The following tables 4A-4K describe results from in vivo testing for the following arms of SS7, SS9, SS15, SS16, and SS17 from example 4.

Table 4A

In-vivo cumulative percent drug release profile of Rapamycin, Apixaban/ Rivaroxaban and Argatroban in stented segments.

Sample Matrix	Sample Size	Time period	1H	3H	24H	6D	7D	28D	90D
SS7 Argatroban/Apixaban /Sirolimus	n=1	Apixaban,%	N/A	30	91	98	99	99	N/A
		Argatroban,%	N/A	91	99	98	99	99	N/A
		Sirolimus,%	N/A	50	70	69	73	77	N/A
SS9 Argatroban/Apixaban /Sirolimus	n=1	Apixaban,%	N/A	68	97	98	98	99	N/A
		Argatroban,%	N/A	79	98	98	98	99	N/A
		Sirolimus,%	N/A	60	91	93	94	97	N/A
SS15 Argatroban/Apixaban /Sirolimus	n=5	Apixaban,%	49	61	77	N/A	80	84(n=3)	87(n=3)
		Argatroban,%	51	63	77	N/A	80	84(n=3)	86(n=3)
		Sirolimus,%	44	55	71	N/A	81	90(n=3)	97(n=3)
SS16 Argatroban/Rivaroxaban /Sirolimus	n=5	Rivaroxaban,%	36	47	83	N/A	86	89	N/A
		Argatroban,%	35	49	82	N/A	85	87	N/A
		Sirolimus,%	29	44	73	N/A	87	94	N/A
SS17 Argatroban/Rivaroxaban /Sirolimus	n=5	Rivaroxaban,%	65	71	86	N/A	92	94	N/A
		Argatroban,%	78	80	86	N/A	91	93	N/A
		Sirolimus,%	8	18	63	N/A	77	87	N/A

N/A: Not available

[0325] Table 4A: SS7 and SS9 provide a therapeutic composition where about 90% of the factor Xa and factor IIa inhibitors are released from the stent within 24 hours. It also shows that these agents are released substantially completely within about 28 days.

[0326] Table 4A: SS15, SS16, and SS17 provide therapeutic compositions where each composition providing a bolus drug release from time of injury and/or implant, and an extended

drug release from time of injury and/or implant for each of Apixaban, Rivaroxaban, and Argatroban.

[0327] Table 4A: SS15 provides a therapeutic composition providing a bolus drug release phase (or formulation) from time of injury and/or implant and an extended drug release phase (or formulation) from time of injury and/or implant for the combination of Apixaban and Argatroban, wherein the bolus drug release occurs within an hour, within 3 hours, or within 24 hours, from time of injury and/or implant; and the extended drug release extends beyond 7 day, extends beyond 28 days, or extends beyond 90 days from time of injury and/or implant.

[0328] Table 4A: SS15 provides a therapeutic composition a bolus drug release phase (or formulation) from time of injury and/or implant and an extended drug release phase (or formulation) from time of injury and/or implant for the combination of Apixaban, Argatroban, and Sirolimus, wherein the bolus drug release occurs within an hour to within 24 hours from time of injury and/or implant and the extended drug release extends beyond 7 day, extends beyond 28 days, or extends beyond 90 days from time of injury and/or implant.

[0329] Table 4A: SS15 provides a therapeutic composition providing a bolus drug release phase (or formulation) from time of injury and/or implant and an extended drug release phase (or formulation) from time of injury and/or implant for the combination of Apixaban and Argatroban, wherein the bolus drug release occurs within an hour from time of injury and/or implant and wherein Apixaban bolus release is about 49% within an hour and wherein Argatroban bolus release is about 51% within an hour and the extended drug release of each of the drugs is about 80% within 7 days, about 84% within 28 days, and about 86% within 90 days from time of injury and/or implant. In this arm, the drugs are released or commence release substantially about the same time.

[0330] Table 4A: SS16 and SS17 provide therapeutic composition providing a bolus drug release phase (or formulation) from time of injury and/or implant and an extended drug release phase (or formulation) from time of injury and/or implant for the combination of Rivaroxaban and Argatroban, wherein the bolus drug release occurs within an hour to within 24 hours from time of injury and/or implant and the extended drug release extends beyond 7 day, or extends beyond 28 days from time of injury and/or implant.

[0331] Table 4A: SS16 provides a therapeutic composition providing a bolus drug release phase (or formulation) from time of injury and/or implant and an extended drug release phase (or formulation) from time of injury and/or implant for the combination of Rivaroxaban, Argatroban, and Sirolimus, wherein the bolus drug release occurs within an hour to within 24 hours from time of injury and/or implant and the extended drug release extends beyond 7 day, or

extends beyond 28 days from time of injury and/or implant. In this arm, the drugs are released or commence release substantially about the same time.

[0332] Table 4A: SS16 and SS17 provide therapeutic compositions providing a bolus drug release phase (or formulation) from time of injury and/or implant and an extended drug release phase (or formulation) from time of injury and/or implant for the combination of Rivaroxaban and Argatroban, wherein the bolus drug release occurs within an hour to within 24 hours from time of injury and/or implant and wherein Rivaroxaban bolus release ranges from 36% to 68% within an hour and wherein Argatroban bolus release ranges from 35% to 78% within an hour and the extended drug release of each of the drugs ranges from 85% to 92% for Rivaroxaban within 7 days, 86%-91% for Argatroban within 7 days, ranges from 89%-94% within 28 days for Rivaroxaban and 87%-93% for Argatroban from time of injury and/or implant to within 28 days.

[0333] Table 4A: SS16 and SS17 formulations each has one formulation providing a bolus drug release and another formulation providing an extended drug release for the combination of Rivaroxaban and Argatroban, wherein the bolus drug release occurs within an hour to within 24 hours of injury or implantation and the extended drug release extends beyond 7 day, or extends beyond 28 days.

[0334] Table 4A: SS16 and SS17 shows multiple formulations providing a bolus drug release formulation and an extended drug release formulation for the combination of each of Rivaroxaban and Argatroban, wherein the extended release extends beyond 7 day, or extends beyond 28 days.

[0335] Table 4A: SS15, SS16, and SS17 Provides therapeutic compositions comprising two drugs/polymer formulations each, wherein each formulation contains at least two drugs: a factor Xa inhibitor and a factor IIa inhibitor. A third drug being an M-tor inhibitor is present in each of the formulations except in SS17 where it is present in only one formulation (base formulation) configured to delay the release of M-tor in SS17 providing a smaller bolus within the first hour for M-tor. All formulations provide an extended release of the drugs beyond 7 days, or beyond 28 days. Arm SS17 factor IIa inhibitor and factor Xa inhibitor commence release prior to the anti-proliferative which was intended/configured to delay commence of its release compared to the other two drugs.

Table 4B

Tissue drug concentration (ng/mg) of Apixaban, Rivaroxaban, Argatroban and Rapamycin in the stented segment tissue at the indicated time points following implantation.

Sample Matrix	Sample Size	Time period	1H	3H	24H	6D	7D	28D	90D
SS7 Argatroban /Apixaban /Sirolimus	n=1	Apixaban	N/A	102.9	16.5	0.07	0.03	0.12	N/A
		Argatroban	N/A	54	0.75	0.09	0.05	0.15	N/A
		Sirolimus	N/A	7.34	4.46	1.38	0.73	1.79	N/A
SS9 Argatroban /Apixaban /Sirolimus	n=1	Apixaban	N/A	91.8	4.17	0.28	2.65	1.69	N/A
		Argatroban	N/A	123.1	0.18	0.33	2.54	1.76	N/A
		Sirolimus	N/A	40.57	0.93	1.61	3.24	2.48	N/A
SS15 Argatroban /Apixaban /Sirolimus	n=5	Apixaban	66.94 ± 27.33	25.31 ± 11.21	13.25 ± 10.17	NA	1.15 ± 0.52	1.28 ± 0.47(n=3)	3.05 ± 1.77(n=3)
		Argatroban	71.37 ± 31.32	27.65 ± 15.00	15.64 ± 12.08	NA	1.41 ± 0.69	1.69 ± 0.64(n=3)	3.74 ± 1.89(n=3)
		Sirolimus	43.22 ± 14.73	23.37 ± 6.88	29.17 ± 18.65	NA	1.54 ± 0.35	1.67 ± 0.22(n=3)	1.28 ± 0.07(n=3)
SS16 Argatroban /Rivaroxaban /Sirolimus	n=5	Rivaroxaban	48.75 ± 25.52	21.48 ± 5.80	3.67 ± 5.59	N/A	0.31 ± 0.24	0.34 ± 0.27	N/A
		Argatroban	61.87 ± 24.60	32.81 ± 10.96	3.80 ± 4.87	N/A	0.42 ± 0.32	0.52 ± 0.37	N/A
		Sirolimus	45.10 ± 14.77	38.30 ± 9.29	9.18 ± 5.69	N/A	1.46 ± 0.38	0.94 ± 0.19	N/A
SS17 Argatroban /Rivaroxaban /Sirolimus	n=5	Rivaroxaban	38.31 ± 16.08	26.23 ± 23.50	1.31 ± 0.28	N/A	1.07 ± 1.88	0.52 ± 0.63	N/A
		Argatroban	11.80 ± 2.69	8.06 ± 3.48	1.19 ± 0.46	N/A	1.35 ± 2.41	0.67 ± 0.87	N/A
		Sirolimus	21.34 ± 7.51	27.32 ± 6.86	10.85 ± 3.55	N/A	3.80 ± 4.86	1.73 ± 1.52	N/A

[0336] Table 4B shows drug concentration in tissue adjacent to the stented segment for each of the drugs: Apixaban of about 67ng/mg within one hour, of about 25ng/mg tissue within 3 hours, of about 1.15ng/mg tissue within 7 days, 1.28ng/mg tissue within 28 days, and of about 3ng/mg tissue within 90 days from time of injury and/or implant; Rivaroxaban of about 38ng/mg, or of about 49ng/mg within one hour, of about 21ng/mg, or of about 26ng/mg tissue within 3 hours, of about 0.3ng/mg, or of about 1.1ng/mg tissue within 7 days, of about 0.34ng/mg, or of about 0.52ng/mg tissue within 28 days, from time of injury and/or implant; Argatroban of about 12ng/mg, of about 62ng/mg tissue, or of about 71ng/mg tissue within 1 hour, of about 8ng/mg tissue, of about 33ng/mg tissue, or of about 27ng/mg tissue within 3 hours, of about 0.42ng/mg tissue, of about 1.35ng/mg tissue, or of about 1.41ng/mg tissue within 7 days, of about 0.52ng/mg tissue, of about 0.67ng/mg tissue, or of about 1.69ng/mg tissue within 28 days, and of about 3.74ng/mg tissue within 90 days from time of injury and/or implant; and Sirolimus of about 21ng/mg tissue, of about 45ng/mg tissue, or of about 43ng/mg tissue within one hour, of about 27ng/mg tissue, or about 38ng/mg tissue, or of about 24ng/mg

tissue within 3 hours, of about 1.46ng/mg tissue, of about 3.8ng/mg tissue, or of about 1.54ng/mg tissue within 7 days, of about 0.94ng/mg tissue, of about 1.73ng/mg tissue, or of about 1.67ng/mg tissue within 28 days, and of about 1.28ng/mg tissue within 90 days, from time of tissue injury and/or implant.

Table 4C

In Vivo drug remaining on stent (μg) and average cumulative percentage releases (%) of Apixaban, Rivaroxaban, Argatroban and Sirolimus in the stent at the indicated time points following implantation.

Sample matrix* (n=5)	SS15 Argatroban/Apixaban /Sirolimus in base coat and in topcoat			SS16 Argatroban/Rivaroxaban /Sirolimus in base coat and in topcoat			SS17 Argatroban/Rivaroxaban /Sirolimus in base coat and in topcoat		
Drug remaining on stent, μg & percentage released (%)	Apixaban	Argatroban	Sirolimus	Rivaroxaban	Argatroban	Sirolimus	Rivaroxaban	Argatroban	Sirolimus
Time (hrs)									
0	119	123	96	120	119	90	121	121	96
1H	61 $\pm$ 4.8 (49%)	60 $\pm$ 5.5 (51%)	54 $\pm$ 2.8 (44%)	76 $\pm$ 7.1 (36%)	77 $\pm$ 59.2 (35%)	63 $\pm$ 4.7 (29%)	43 $\pm$ 3.5 (65%)	26 $\pm$ 0.8 (78%)	88 $\pm$ 1.5 (8%)
3H	46 $\pm$ 5.2 (61%)	46 $\pm$ 5.1 (63%)	43 $\pm$ 3.9 (55%)	64 $\pm$ 9.5 (47%)	61 $\pm$ 13.9 (49%)	50 $\pm$ 6.1 (44%)	34 $\pm$ 5.1 (71%)	24 $\pm$ 1.9 (80%)	79 $\pm$ 3.5 (18%)
24H	27 $\pm$ 3.6 (77%)	28 $\pm$ 1.4 (77%)	28 $\pm$ 0.8 (71%)	21 $\pm$ 4.8 (83%)	21 $\pm$ 1.6 (82%)	24 $\pm$ 7.1 (73%)	17 $\pm$ 5.9 (86%)	17 $\pm$ 3.8 (86%)	36 $\pm$ 7.3 (63%)
7D	24 $\pm$ 1.4 (80%)	25 $\pm$ 1.0 (80%)	18 $\pm$ 0.3 (81%)	16 $\pm$ 2.5 (86%)	18 $\pm$ 1.7 (85%)	12 $\pm$ 1.2 (87%)	9 $\pm$ 0.3 (92%)	11 $\pm$ 0.4 (91%)	22 $\pm$ 0.5 (77%)
28D	19 $\pm$ 0.9 (84%)	20 $\pm$ 6.1 (84%)	9 $\pm$ 0.3 (90%)	14 $\pm$ 1.0 (89%)	15 $\pm$ 0.8 (87%)	5 $\pm$ 0.4 (94%)	7 $\pm$ 0.4 (94%)	9 $\pm$ 0.7 (93%)	13 $\pm$ 0.7 (87%)
90D	16 $\pm$ 1.6 (87%)	17 $\pm$ 0.6 (86%)	3 $\pm$ 0.1 (97%)	N/A	N/A	N/A	N/A	N/A	N/A

N/A: Not available

*SS15 28D and 90D (n=3)

Table 4D

In Vivo drug concentration (ng/mg) in the tissue within 5mm proximal and 5mm distal (tissue adjacent to the stented segment) to the stented segment.

Sample Matrix (Sample Size)	Drug	1 Hour		3 Hour		1 Day		6 Day		7 Day		28 Day		98 Day	
		Proximal	Distal	Proximal	Distal	Proximal	Distal	Proximal	Distal	Proximal	Distal	Proximal	Distal	Proximal	Distal
SS15 (n=5 except 2SD & 50D n=3)	Amiroxaban, ng/mg	7.26 ± 3.01	3.53 ± 1.95	5.15 ± 1.59	6.77 ± 2.27	6.14 ± 0.98	6.36 ± 0.29	N/A	N/A	0.01 ± 0.01	0.01 ± 0.01	0.04 (n=1) BQL (n=2)	0.02 (n=1) BQL (n=2)	0.0005 ± 0.0002 (n=2) BQL (n=1)	0.0002 ± 0.0001 (n=2)
	Argatroban, ng/mg	0.57 ± 3.06	3.84 ± 2.42	0.45 ± 2.67	6.46 ± 3.14	0.05 ± 0.03	0.17 ± 0.09	N/A	N/A	0.01 ± 0.01	0.01 ± 0.003	0.04 (n=1) BQL (n=2)	0.02 (n=1) BQL (n=2)	0.0006 ± 0.0004 (n=2) BQL (n=1)	0.0004 ± 0.0004 (n=2) BQL (n=1)
	Sarelinus, ng/mg	2.82 ± 1.15	1.66 ± 0.78	3.52 ± 0.51	3.51 ± 0.61	0.12 ± 0.11	3.18 ± 3.01	N/A	N/A	0.02 ± 0.01	0.27 ± 0.11	0.02 ± 0.01	0.14 ± 0.04	0.01 ± 0.01	0.02 ± 0.01
SS16 (Slider II Arm 1 (n=5)) except 3H n=4, 28d n=5)	Rivaroxaban, ng/mg	2.63 ± 1.14	6.51 ± 2.45	1.72 ± 0.53	2.16 ± 0.83	0.09 ± 0.03	0.09 ± 0.02	N/A	N/A	BQL	BQL	0.02 (n=1); BQL (n=2)	0.02 (n=1); BQL (n=2)	N/A	N/A
	Argatroban, ng/mg	3.74 ± 1.93	7.37 ± 3.04	2.48 ± 0.36	2.63 ± 0.87	0.07 ± 0.03	0.09 ± 0.03	N/A	N/A	BQL	BQL	BQL	BQL	N/A	N/A
	Sarelinus, ng/mg	2.39 ± 1.22	5.25 ± 1.62	2.62 ± 0.87	3.78 ± 0.81	0.18 ± 0.06	1.10 ± 0.44	N/A	N/A	0.04 ± 0.03	0.50 ± 0.22	0.05 ± 0.05 (n=4); BQL (n=2)	0.11 ± 0.05 (n=2)	N/A	N/A
SS17 (Slider II Arm 2 (n=5 except 3H n=4), 28d n=6)	Rivaroxaban, ng/mg	1.52 ± 0.70	2.61 ± 1.38	3.09 ± 1.50	2.72 ± 1.66	0.09 ± 0.05	0.07 ± 0.02	N/A	N/A	0.01 (n=1); BQL (n=4)	0.01 (n=1); BQL (n=4)	0.02 ± 0.001 (n=2); BQL (n=4)	BQL	N/A	N/A
	Argatroban	0.65 ± 0.23	0.91 ± 0.51	0.68 ± 0.28	0.57 ± 0.52	0.04 ± 0.01	0.03 ± 0.01	N/A	N/A	BQL	BQL	BQL	BQL	N/A	N/A
	Sarelinus, ng/mg	1.39 ± 0.83	1.31 ± 0.83	2.49 ± 1.02	3.95 ± 3.09	0.26 ± 0.27	0.86 ± 0.48	N/A	N/A	0.01 (n=3); BQL (n=2)	0.34 ± 0.21	0.03 ± 0.02 (n=4); BQL (n=2)	0.12 ± 0.06 (n=2)	N/A	N/A

BQL: Below Quantification Limit

N/A: Not Available

Table 4E

In Vivo Apixaban concentration (ng/mg) in the stented segment tissue and adjacent segments of 5mm proximal and 5mm distal to the implanted device.

Apixaban in treated area tissue content, ng/mg							
Stent (Sample Size)	1 hour	3 hours	1 day	6 days	7 days	28 days	90 days
SS15(n=5)	66.94 ± 27.33	25.31 ± 11.21	13.28 ± 10.17	N/A	1.15 ± 0.52	1.28 ± 0.47(n=3)	3.05 ± 1.77(n=3)
Apixaban in Proximal tissue content, ng/mg							
Stent (Sample Size)	1 hour	3 hours	1 day	6 days	7 days	28 days	90 days
SS15 (n=5)	7.86 ± 3.01	5.15 ± 1.59	0.14 ± 0.08	N/A	0.01 ± 0.01	0.04(n=1) BQL(n=2)	0.0003 ± 0.0007(n=2) BQL (n=1)
Apixaban in Distal tissue content, ng/mg							
Stent (Sample Size)	1 hour	3 hours	1 day	6 days	7 days	28 days	90 days
SS15(n=5)	3.53±1.95	6.77±2.22	0.36 ± 0.29	N/A	0.01 ± 0.01	0.02(n=1) BQL(n=2)	0.0002 ± 0.0001(n=3)

BQL: Below Quantification Limit

Table 4F

In Vivo Rivaroxaban concentration (ng/mg) in the stented segment tissue and adjacent segments of 5mm proximal and 5mm distal to the implanted device.

Rivaroxaban in treated area tissue content, ng/mg					
Stent (Sample Size)	1 hour	3 hours	1 day	7 days	28 days
SS16: Slider II Arm1 (n=5 except 3H n=4, 28d n=6)	48.75 ± 25.52	21.48± 5.80	3.67± 5.59	0.31 ± 0.24	0.34 ± 0.27
SS17: Slider II Arm2 (n=5 except 3H n=4, 28d n=6)	38.31± 16.08	26.23 ± 3.50	1.31 ± 0.28	1.07 ± 1.88	0.52 ± 0.63
Rivaroxaban in Proximal tissue content, ng/mg					
Stent (Sample Size)	1 hour	3 hours	1 day	7 days	28 days
SS16: Slider II Arm1 (n=5 except 3H n=4, 28d n=6)	2.63 ± 1.14	1.72 ± 0.53	0.09 ± 0.03	BQL	0.02 (n=1); BQL(n=5)
SS17: Slider II Arm2 (n=5 except 3H n=4, 28d n=6)	1.52 ± 0.70	3.00 ± 1.50	0.09 ± 0.05	0.01(n=1); BQL(n=4)	0.02 ± 0.001 (n=2); BQL(n=4)
Rivaroxaban in Distal tissue content, ng/mg					
Stent (Sample Size)	1 hour	3 hours	1 day	7 days	28 days
SS16: Slider II Arm1 (n=5 except 3H n=4, 28d n=6)	6.51 ± 2.45	2.16± 0.83	0.09± 0.02	BQL	0.02(n=1); BQL(n=5)
SS17: Slider II Arm2 (n=5 except 3H n=4, 28d n=6)	2.61 ± 1.38	2.72± 1.66	0.07± 0.02	0.01(n=1); BQL (n=4)	BQL

BQL: Below Quantification Limit

N/A: Not Available

Table 4G

In Vivo Argatroban concentration (ng/mg) in the stented segment tissue and adjacent tissue segment of 5mm proximal and 5mm distal to the implanted device.

Argatroban in treated tissue content, ng/mg							
Stent (Sample Size)	1 hour	3 hours	1 day	6 days	7 days	28 days	90 days
SS7 (n=1)	N/A	54.00	0.75	0.09	0.05	0.15	N/A
SS9 (n=1)	N/A	123.10	0.18	0.33	2.54	1.76	N/A
SS15 (n=5)	71.37 ± 31.32	27.65 ± 15.00	15.64 ± 12.08	N/A	1.41 ± 0.69	1.69 ± 0.64(n=3)	3.74 ± 1.89(n=3)
SS16: Slider II Arm1 (n=5 except 3H n=4, 28d n=6)	61.87 ± 24.60	32.81 ± 10.96	3.80 ± 4.87	N/A	0.42 ± 0.32	0.52 ± 0.37	N/A
SS17: Slider II Arm2 (n=5 except 3H n=4, 28d n=6)	11.80 ± 2.69	8.06 ± 3.48	1.19 ± 0.46	N/A	1.35 ± 2.41	0.67 ± 0.87	N/A
Argatroban in Proximal tissue content, ng/mg							
Stent (Sample Size)	1 hour	3 hours	1 day	6 days	7 days	28 days	90 days
SS7 (n=1)	N/A	13.39	BQL	BQL	BQL	BQL	N/A
SS9 (n=1)	N/A	1.27	BQL	BQL	BQL	BQL	N/A
SS15(n=5)	8.57 ± 3.66	5.43 ± 2.67	0.05 ± 0.03	N/A	0.01 ± 0.01	0.04(n=1) BQL(n=2)	0.0008 (n=1) BQL (n=2)
SS16: Slider II Arm1 (n=5 except 3H n=4, 28d n=6)	3.74 ± 1.93	2.48 ± 0.36	0.07 ± 0.03	N/A	BQL	BQL	N/A
SS17: Slider II Arm2 (n=5 except 3H n=4, 28d n=6)	0.65 ± 0.23	0.68 ± 0.28	0.04 ± 0.01	N/A	BQL	BQL	N/A
Argatroban in Distal tissue content, ng/mg							
Stent (Sample Size)	1 hour	3 hours	1 day	6 days	7 days	28 days	90 days
SS7 (n=1)	N/A	5.20	BQL	BQL	BQL	BQL	N/A
SS9 (n=1)	N/A	11.33	BQL	N/A	BQL	BQL	N/A
SS15 (n=5)	3.84 ± 2.42	6.46 ± 3.14	0.17 ± 0.09	N/A	0.01 ± 0.003	0.02(n=1) BQL(n=2)	0.0006 ± 0.0004(n=2) BQL (n=1)
SS16: Slider II Arm1 (n=5 except 3H n=4, 28d n=6)	7.87 ± 3.04	2.63 ± 0.87	0.09 ± 0.03	N/A	BQL	BQL	N/A
SS17: Slider II Arm2 (n=5 except 3H n=4, 28d n=6)	0.91 ± 0.51	0.87 ± 0.52	0.03 ± 0.01	N/A	BQL	BQL	N/A

BQL: Below Quantification Limit

N/A: Not available

Table 4H

In Vivo Rapamycin concentration (ng/mg) in the tissue of treated segment (stented segment) and adjacent tissue segment of 5mm proximal and 5mm distal to the implanted device.

Sirolimus in treated tissue content, ng/mg							
Stent (Sample Size)	1 hour	3 hours	1 day	6 days	7 days	28 days	90 days
SS7 (n=1)	N/A	7.34	4.46	1.38	0.73	1.79	N/A
SS9 (n=1)	N/A	40.57	0.93	1.61	3.24	2.48	N/A
SS15 (n=5)	43.22 ± 14.73	23.37 ± 6.88	29.17 ± 18.65	N/A	1.54 ± 0.35	1.67 ± 0.22(n=3)	1.28 ± 0.07(n=3)
SS16: Slider II Arm1 (n=5 except 3H n=4, 28d n=6)	45.10 ± 14.77	38.30 ± 9.29	9.18 ± 5.69	N/A	1.46 ± 0.38	0.94 ± 0.19	N/A
SS17: Slider II Arm2 (n=5 except 3H n=4, 28d n=6)	21.34 ± 7.51	27.32 ± 6.86	10.85 ± 3.55	N/A	3.80 ± 4.86	1.73 ± 1.52	N/A
Sirolimus in Proximal tissue content, ng/mg							
Stent (Sample Size)	1 hour	3 hours	1 day	6 days	7 days	28 days	90 days
SS7 (n=1)	N/A	3.21	BQL	0.14	BQL	0.01	N/A
SS9 (n=1)	N/A	0.38	0.58	BQL	0.15	0.1	N/A
SS15 (n=5)	2.82 ± 1.15	3.52 ± 0.51	0.12 ± 0.11	N/A	0.02 ± 0.01	0.02 ± 0.01(n=3)	0.01 ± 0.01(n=3)
SS16: Slider II Arm1 (n=5 except 3H n=4, 28d n=6)	2.30 ± 1.22	2.62 ± 0.82	0.18 ± 0.06	N/A	0.04 ± 0.03	0.05 ± 0.05 (n=4); BQL(n=2)	N/A
SS17: Slider II Arm2 (n=5 except 3H n=4, 28d n=6)	1.39 ± 0.83	2.49 ± 1.02	0.26 ± 0.27	N/A	0.01(n=3); BQL(n=2)	0.03 ± 0.02 (n=4); BQL(n=2)	N/A
Sirolimus in Distal tissue content, ng/mg							
Stent (Sample Size)	1 hour	3 hours	1 day	6 days	7 days	28 days	90 days
SS7 (n=1)	N/A	1.86	0.99	0.69	0.46	0.14	N/A
SS9 (n=1)	N/A	6.56	1.88	0.39	0.61	0.21	N/A
SS15 (n=5)	1.66 ± 0.78	3.81 ± 0.61	3.18 ± 3.01	N/A	0.27 ± 0.11	0.14 ± 0.04(n=3)	0.02 ± 0.01(n=3)
SS16: Slider II Arm1 (n=5 except 3H n=4, 28d n=6)	5.25 ± 1.62	3.78 ± 0.81	1.10 ± 0.44	N/A	0.55 ± 0.22	0.11 ± 0.05	N/A
SS17: Slider II Arm2 (n=5 except 3H n=4, 28d n=6)	1.81 ± 0.83	3.95 ± 3.09	0.86 ± 0.48	N/A	0.34 ± 0.21	0.13 ± 0.06	N/A

BQL: Below Quantification Limit

N/A: Not available

Table 4I

Tissue concentration of Rivaroxaban, Argatroban and Sirolimus number of orders of magnitude higher than IC50 for Anti-factor Xa/IIa or anti- cell proliferation for the respective drugs in the tissue of treated segment and adjacent tissue segment of 5mm proximal and 5mm distal to the implanted device for SS15.

Ratio of Tissue content to IC50	1 Hour			3 Hours			1 Day			7 Days			28 days			3 month		
	Proximal	Treated	Distal	Proximal	Treated	Distal	Proximal	Treated	Distal	Proximal	Treated	Distal	Proximal	Treated	Distal	Proximal	Treated	Distal
Apixaban	215142	1821895	95316	141612	688998	185185	3813	362200	9804	272	31318	272	1089	34858	545	8	83061	5
Argatroban	806	6693	356	506	2587	609	5	1462	16	1	132	1	4	158	2	0	351	0
Sirolimus	28000	432000	17000	35000	234000	38000	1200	292000	31800	200	15400	2700	200	16700	1400	100	12800	200

*IC50 of Apixaban for anti-Xa 0.08nM or 0.00004ng/mg

*IC50 of Argatroban for anti-IIa 21nM or 0.01ng/mg

*IC50 of Sirolimus for cell proliferation 0.1nM or 0.0001ng/mg

Table 4J

Tissue concentration of Rivaroxaban, Argatroban and Sirolimus number of orders of magnitude higher than IC50 for Anti-factor Xa/IIa or anti- cell proliferation for the respective drugs in the tissue of treated segment and adjacent tissue segment of 5mm proximal and 5mm distal to the implanted device for Slider II Arm1.

Ratio of Tissue content to IC50	1 Hour			3 Hours			1 Day			7 Days			28 days		
	Proximal	Treated	Distal	Proximal	Treated	Distal	Proximal	Treated	Distal	Proximal	Treated	Distal	Proximal	Treated	Distal
Rivaroxaban	286	5299	708	187	2335	235	10	399	10	N/A	34	N/A	2	37	2
Argatroban	351	5800	738	232	3076	247	7	356	8	N/A	39	N/A	N/A	49	N/A
Rapamycin	23000	451000	52500	26200	383000	37800	1800	91800	11000	400	14600	5500	500	9400	1100

*IC50 of Rivaroxaban for anti-Xa 21nM or 0.0092ng/mg

*IC50 of Argatroban for anti-IIa 21nM or 0.01ng/mg

*IC50 of Sirolimus for cell proliferation 0.1nM or 0.0001ng/mg

Table 4K

Tissue concentration of Rivaroxaban, Argatroban and Sirolimus number of orders of magnitude higher than IC50 for Anti-factor Xa/IIa or anti- cell proliferation for the respective drugs in the tissue of treated segment and adjacent tissue segment of 5mm proximal and 5mm distal to the implanted device for Slider II Arm2.

Ratio of Tissue content to IC50	1 Hour			3 Hours			1 Day			7 Days			28 days		
	Proximal	Treated	Distal	Proximal	Treated	Distal	Proximal	Treated	Distal	Proximal	Treated	Distal	Proximal	Treated	Distal
Rivaroxaban	165	4164	284	326	2851	296	10	142	8	1	116	1	2	57	N/A
Argatroban	61	1106	85	64	756	82	4	112	3	N/A	127	N/A	N/A	63	N/A
Rapamycin	13900	213400	18100	24900	273200	39500	2600	108500	8600	100	38000	3400	300	17300	1300

IC50 of Rivaroxaban for anti-Xa 21nM or 0.0092ng/mg

IC50 of Argatroban for anti-IIa 21nM or 0.01ng/mg

IC50 of Sirolimus for cell proliferation 0.1nM or 0.0001ng/mg

[0337] Tables 4D-4K show that all three drugs Apixaban, Argatroban, and rapamycin maintain therapeutic tissue concentrations in the tissue segment up to 28 days, up to 90 days or longer, and furthermore achieve therapeutic tissue concentration in the adjacent tissue segment (± 5 mm from the tissue segment such as Proximal and distal) at 1 hour, 3 hours and at/or up to 1 day. This can be important to inhibit thrombus formation in the stented segment, the device surface, and in the tissue adjacent to the stented segment as in many cases such tissue is injured by balloon deployment or stent edges.

[0338] Taking Apixaban IC50 for factor Xa inhibition to be about 0.08nM or 0.00004ng/mg; Rivaroxaban IC50 for factor Xa inhibition to be about 21nM or 0.0092ng/mg; Argatroban IC50 for factor IIa inhibition to be about 21nM or 0.01ng/mg; Sirolimus IC50 for cell proliferation to be about 0.1nM or 0.0001ng/mg. Table 4I-Table 4K are the tissue concentration of Apixaban or Rivaroxaban, Argatroban and Sirolimus are several order of magnitude higher (or times higher) than IC50 for Anti-factor Xa, anti-IIa, or anti- cell proliferation for the respective drugs in the tissue of treated segment and adjacent tissue segment of 5mm proximal and 5mm distal to the implanted device. It shows that Apixaban, Rivaroxaban, Argatroban and/or Sirolimus in tissue concentrations have one or more order of magnitudes higher concentration at the times specified, has from 1 to 6 orders of magnitude of tissue concentration for each of the drugs compared to their IC50, in the treated tissue segments up to 28 days, or up to 90 days.

Example 5: In vivo animal study of Anticoagulant1/Anticoagulant2/mTOR Eluting Stents (scaffolds)

[0339] The test drug eluting stent systems containing anticoagulants were prepared as described in Example 4 and were evaluated at 28 days and 90 days following implantation in a porcine coronary artery. The control device was the Novolimus (m-TOR) eluting DESyne X2 stent.

[0340] The porcine artery was chosen as this model has been used extensively for stent and angioplasty studies resulting in a large volume of data on the pulmonary response properties and its correlation to human pulmonary response (Schwartz et al, Circulation. 2002; 106:1867-1873). The animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals as established by the National Research Council.

[0341] All animals were pretreated with aspirin (325mg) and Clopidogrel (75mg) per oral dose beginning at least 3 days prior to the intervention and continuing for the duration of the study. After induction of anesthesia, the left or right femoral artery was accessed using standard techniques and an arterial sheath was introduced and advanced into the artery. Vessel angiography was performed under fluoroscopic guidance, a 7 Fr. guide catheter was inserted through the sheath and advanced to the appropriate location where intracoronary nitroglycerin was administered. An appropriate implantation segment of coronary artery was randomly selected and a 0.014" guidewire inserted. Quantitative Coronary Angiography (QCA) was performed to document the results. The appropriately sized stent (3.0 x 14 mm or 3.5 x 14 mm) was advanced to the deployment site. The balloon was inflated at a steady rate to a pressure sufficient to achieve a balloon to artery ratio of approximately 1.1 to 1.0 but less than 1.2:1. Pressure was maintained for approximately 10 seconds before the balloon was deflated. Each pig was implanted with 3 test devices and one control device in the coronary arteries. Each time point a whole blood was drawn from animals for blood drug concentration test.

[0342] Follow up angiography imaging was performed at the designated endpoint for each of the animals. Quantitative coronary angiographic analysis was performed and the average percent diameter stenosis values and late lumen loss for the test arms and control DESyne X2 for the 28 days and 3-month time points are shown in Table 5A.

[0343] Upon completion of follow-up angiography imaging, the animals were euthanized. The hearts were harvested from each animal. Any myocardial lesions or unusual observations were reported. The coronary arteries were perfused with 10% buffered formalin at 100 to 120 mm Hg with the animal's ear tag until processed for histology.

[0344] Stented portions of coronary arteries were embedded in methyl methacrylate (MMA), then divided into a target of three blocks of approximately similar length (about 4 mm),

identified as proximal, mid and distal segments. From three blocks, 3 to 5 cuts were made for histology evaluation.

[0345] Quantitative histopathological evaluation of stented artery sections was then performed and scored as indicated. The mean of each section was recorded and then averaged to provide a mean score per stent for the different parameters (Table 5A). The smaller the score, the better the efficacy.

[0346] Fibrin (strut-by-strut)

0 = absent, or rare minimal spotting around struts

1 = fibrin in small amounts, localized only around struts

2 = fibrin moderately abundant or denser, extending beyond struts

3 = abundant, dense fibrin, bridging between struts

Each strut in the section was scored; the mean fibrin score for each section was calculated and reported. The mean of the section means was calculated and reported, providing a mean fibrin score per stent.

[0347] Injury based on Schwartz et al. J Am Coll Cardiol 1992; 19:267-274. (strut-by-strut):

0 = IEL intact

1 = IEL lacerated

2 = media completely lacerated

3 = EEL lacerated

Each strut in the section as scored and the mean injury score for each section was calculated and reported. The mean of the section means was calculated and reported, providing a mean injury score per stent.

[0348] Inflammation (strut-by-strut)

0 = no or very few (≤ 3) inflammatory cells around strut

1 = few (~ 4 -10) inflammatory cells around strut

2 = many (> 10) inflammatory cells around strut, can extend into but does not efface surrounding tissue

3 = many (> 10) inflammatory cells, effacing surrounding tissue

Each strut in the section was scored and the mean inflammation score for each section was calculated and reported. The mean of the section means was calculated and reported, providing a mean inflammation score per device.

Table 5A

Histopathology Scores and Quantitative Coronary Angiography data Apixaban/Rivaroxaban, Argatroban and Sirolimus releasing 14mm stents at 7 days, 28 days and 3 month

Time Point	Device	Injury	Inflammation	Fibrin	Diameter Stenosis%	LLL, mm
7 Day	SS7(n=1)	0.15	1.52	0.62	N/A	N/A
	SS9(n=1)	0.61	1.66	0.44	N/A	N/A
28 Day	SS7(n=1)	0.21	0.49	0.94	16.7	0.71
	SS9(n=1)	0.36	0.32	0.64	21.3	0.76
	SS15 (n=3)	0.38 ± 0.34	0.62 ± 0.12	1.67 ± 0.38	19.8 ± 4.1	0.45 ± 0.15
	DESyne X2(n=1)	1.34	1.83	1.87	51.6	1.14
	SS16(Slider II Arm1, n=6)	0.91 ± 0.95	1.52 ± 1.03	1.90 ± 0.26	19.4 ± 13.8	0.72 ± 0.32
	DESyne X2(n=2)	1.17 ± 1.60	1.97 ± 1.46	1.32 ± 1.11	36.6 ± 27.3	0.99 ± 0.48
	SS17(Slider II Arm2, n=6)	1.01 ± 0.47	1.46 ± 0.43	1.88 ± 0.48	33.1 ± 12.8	0.86 ± 0.34
3 Month	DESyne X2 (n=2)	0.87 ± 0.74	1.59 ± 0.95	1.93 ± 0.31	21.9 ± 11.6	0.71 ± 0.62
	SS15 (n=3)	0.15 ± 0.18	0.52 ± 0.19	0.08 ± 0.06	18.2 ± 9.3	0.25 ± 0.29
	DESyne X2(n=1)	0.43	0.59	0.76	22.3	0.52

[0349] LLL is an indicator of the amount cell proliferation or inhibition potency. It is used to measure efficacy between drugs for proliferation inhibition in mammalian arteries. The smaller the LLL, the better the efficacy of the drug.

[0350] As shown in Table 5A, SS15 composition providing the combination of Sirolimus, Apixaban and Argatroban released from stents had a smaller LLL compared to control which only had m-TOR inhibitor (Novolimus) and thus was unexpectedly more effective at inhibiting smooth muscle cell proliferation compared to Novolimus releasing stents at 28 days, and at 90 days. This was an unexpected finding for the test SS15 stents in comparison to the control DESyne X2 stents at the 28-day time point and/or at 90 days.

[0351] In an unexpected finding, SS15 stents composition eluting Apixaban, Argatroban, and the M-Tor inhibitor rapamycin exhibited more efficacy at inhibiting one or more of the following at 28 days and/or 90 day time points: cell proliferation, inflammation, injury, fibrin formation inhibition, and fibrin dissolution acceleration.

[0352] The LLL is an indicator of the amount cell proliferation or inhibition potency. It is used to measure efficacy between drugs for proliferation inhibition in mammalian arteries. The smaller the LLL, the better the efficacy of the drug.

[0353] As shown in Table 5A, SS16 shows the combination of Sirolimus, Rivaroxaban and Argatroban released from stents had a smaller LLL compared to control which only had m-TOR inhibitor (Novolimus) and thus was unexpectedly more effective at inhibiting smooth muscle cell proliferation compared to Novolimus releasing stents at 28 days.

[0354] As shown in Table 5A, SS17 composition configured to delay the release and tissue concentration of rapamycin within the first 1 hour and/or within the first 3 hours by incorporating rapamycin in the base coating shows the combination of Sirolimus, and/or lower tissue concentration of Rivaroxaban and Argatroban within at least the first hour showed less inhibition of SMC proliferation at 28 days.

Table 5B

Whole Blood PK Results of Apixaban/Argatroban/Sirolimus Eluting Stents from SS15(from study with SS15; target dose Apixaban/Argatroban/Sirolimus=117/117/94; n=5)

Time Points	Apixaban (ng/mL)					Argatroban (ng/mL)					Sirolimus (ng/mL)				
	1 H	3 H	1 D	7 D	28D	1 H	3 H	1 D	7 D	28D	1 H	3 H	1 D	7 D	28D
Pre-implant	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL
Post 1 st implant	0.44 1	0.71	4.02	0.16	0.30	1.36	2.02	4.44	0.58	0.66	0.70	0.59	2.52	0.69	0.32
Post 2 nd implant	0.95	1.17	3.51	0.77	0.44	3.16	4.35	5.74	4.41	1.83	1.90	1.94	4.16	2.13	1.06
Post 3 rd implant	1.8	2.18	4.29	1.31	0.91	6.71	7.59	9.26	7.85	3.72	3.30	3.74	7.08	3.28	2.49
Post 4 th implant	2.56	2.91	5.01	1.72	1.65	10.2	11.0	11.4	10.9	7.33	4.80	5.22	8.06	4.17	4.73
Post 5 th implant	3.27	3.55	5.84	2.12	2.45	13.4	13.3	14.5	12.8	10.3	6.71	7.13	9.98	4.75	5.91
15 min.	3.91	4.60	-----	-----	-----	17.8	21.7	-----	-----	----	8.77	11.3	-----	-----	-----
30 min.	4.78	5.20	8.69	-----	-----	22.7	24.6	22.6	-----	----	11.2	12.3	17.2	-----	-----
45 min.	6.29	-----	-----	-----	-----	28.9	-----	-----	-----	----	13.4	-----	-----	-----	-----
60 min. (1hr)	6.32	6.28	9.91	-----	-----	28.2	29.0	24.4	-----	----	12.8	12.7	21.3	-----	-----
90 min.	-----	7.26	-----	-----	-----	-----	31.1	-----	-----	----	-----	13.7	-----	-----	-----
120 min.(2hr)	-----	7.76	12.7	-----	-----	-----	28.5	20.7	-----	----	-----	12.8	21.3	-----	-----
150 min.	-----	7.38	-----	-----	-----	-----	28.9	-----	-----	----	-----	12.4	-----	-----	-----
180 min.(3hr)	-----	6.89	12.8	-----	-----	-----	20.4	16.9	-----	----	-----	9.32	14.0	-----	-----
4hr	-----	-----	13.3	-----	-----	-----	-----	12.9	-----	----	-----	-----	12.4	-----	-----
5hr	-----	-----	14.7	-----	-----	-----	-----	9.25	-----	----	-----	-----	11.0	-----	-----
6hr	-----	-----	13.3	-----	-----	-----	-----	7.02	-----	----	-----	-----	10.5	-----	-----
24hr (Day 1)	-----	-----	1.51	-----	-----	-----	-----	0.38 3	-----	----	-----	-----	2.65	-----	-----

Time Points	Apixaban (ng/mL)					Argatroban (ng/mL)					Sirolimus (ng/mL)				
	1 H	3 H	1 D	7 D	28D	1 H	3 H	1 D	7 D	28D	1 H	3 H	1 D	7 D	28D
Day 7	-----	-----	-----	BQL	-----	-----	-----	-----	BQL	-----	-----	-----	-----	0.38 4	-----
Day 28	-----	-----	-----	-----	BQL	-----	-----	-----	-----	BQL	-----	-----	-----	-----	BQL

Note. Blood volume in porcine model is about 40%-50% of adult human. Thus drug concentrations in human would typically be lower than the figures shown in table 5B.

Table 5C

Whole Blood PK Results of Rivaroxaban /Argatroban/Sirolimus Eluting Stents from SS16
(Rivaroxaban Arm1; target dose Rivaroxaban/Argatroban/Sirolimus=117/117/94; n=5 except
D28 n= 6)

Time Points	Rivaroxaban (ng/mL)				Argatroban (ng/mL)				Sirolimus (ng/mL)			
	1 H	1 D	7 D	28 D	1 H	1 D	7 D	28 D	1 H	1 D	7 D	28 D
Pre-implant	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL
Post 1 st implant	0.72	1.14	1.07	0.69	1.96	2.94	2.22	1.89	0.52	0.69	0.66	0.48
Post 2 nd implant	1.34	2.16	2.14	1.44	5.05	6.92	5.91	4.76	1.56	1.47	1.75	1.29
Post 3 rd implant	2.10	2.57	2.94	2.17	10.3	10.1	10.2	8.22	3.53	2.22	3.35	2.32
Post 4 th implant	2.67	3.36	3.52	2.80	15.0	17.2	13.6	11.69	4.69	3.41	4.69	3.59
Post 5 th implant	2.92	4.32	3.95	3.33	18.9	19.9	19.1	14.42	6.15	5.37	7.24	5.08
Post 6 th implant	-----	-----	-----	4.32	-----	-----	-----	19.05	-----	-----	-----	6.58
15 min.	3.32	4.05	-----	-----	23.5	22.8	-----	-----	8.38	6.92	-----	-----
30 min.	3.34	3.76	-----	-----	27.5	27.0	-----	-----	10.1	8.56	-----	-----
45 min.	3.68	3.47	-----	-----	31.9	31.5	-----	-----	10.9	9.93	-----	-----
60 min. (1hr)	4.08	2.61	-----	-----	31.1	31.5	-----	-----	11.2	10.7	-----	-----
90 min.	-----	2.43	-----	-----	-----	30.8	-----	-----	-----	12.3	-----	-----
120 min.(2hr)	-----	2.30	-----	-----	-----	26.6	-----	-----	-----	12.1	-----	-----
150 min.	-----	2.08	-----	-----	-----	26.6	-----	-----	-----	11.7	-----	-----
180 min.(3hr)	-----	1.95	-----	-----	-----	25.1	-----	-----	-----	10.3	-----	-----
4hr	-----	1.23	-----	-----	-----	18.4	-----	-----	-----	7.62	-----	-----
5hr	-----	1.11	-----	-----	-----	13.5	-----	-----	-----	6.89	-----	-----
6hr	-----	1.03	-----	-----	-----	11.0	-----	-----	-----	6.09	-----	-----
24hr (Day 1)	-----	BQL	-----	-----	-----	1.92	-----	-----	-----	2.14	-----	-----
Day 7	-----	-----	BQL	-----	-----	-----	BQL	-----	-----	-----	0.44	-----
Day 28	-----	-----	-----	BQL	-----	-----	-----	BQL	-----	-----	-----	BQL

Note. Blood volume in porcine model is about 40%-50% of adult human. Thus drug concentrations in human would typically be lower than the figures shown in table 5C.

Table 5D

Whole Blood PK Results of Rivaroxaban /Argatroban/Sirolimus Eluting Stents from SS17

Rivaroxaban/Argatroban/Sirolimus=117/117/94; n=5 except D28 n= 6)

Time Points	Rivaroxaban (ng/mL)				Argatroban (ng/mL)				Sirolimus (ng/mL)			
	1 H	1 D	7 D	28 D	1 H	1 D	7 D	28 D	1 H	1 D	7 D	28 D
Pre-implant	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL	BQL
Post 1 st implant	3.77	3.62	3.18	3.20	11.7	11.1	11.0	14.05	BQL	0.11	BQL	BQL
Post 2 nd implant	5.27	6.16	5.90	5.79	20.5	24.7	25.9	32.44	0.17	0.36	BQL	0.06
Post 3 rd implant	9.27	11.4	8.41	8.66	41.3	45.2	44.3	52.20	0.28	0.44	0.22	0.21
Post 4 th implant	10.9	16.7	10.8	9.02	54.4	59.6	63.2	65.75	0.47	0.91	0.77	0.46
Post 5 th implant	12.5	22.3	12.0	10.70	65.7	70.3	73.6	79.38	0.98	1.38	1.08	0.69
Post 6 th implant	-----	-----	-----	13.42	-----	-----	-----	96.18	-----	-----	-----	1.20
15 min.	10.8	24.0	-----	-----	73.4	67.2	-----	-----	2.11	2.88	-----	-----
30 min.	8.44	20.1	-----	-----	51.9	51.7	-----	-----	2.79	4.65	-----	-----
45 min.	7.82	19.5	-----	-----	42.4	46.5	-----	-----	3.96	5.35	-----	-----
60 min. (1hr)	7.54	18.5	-----	-----	38.3	44.2	-----	-----	4.32	6.97	-----	-----
90 min.	-----	15.0	-----	-----	-----	32.9	-----	-----	-----	8.08	-----	-----
120 min.(2hr)	-----	11.6	-----	-----	-----	25.8	-----	-----	-----	8.12	-----	-----
150 min.	-----	11.3	-----	-----	-----	20.5	-----	-----	-----	7.97	-----	-----
180 min.(3hr)	-----	8.39	-----	-----	-----	16.3	-----	-----	-----	7.10	-----	-----
4hr	-----	3.74	-----	-----	-----	9.30	-----	-----	-----	5.63	-----	-----
5hr	-----	3.90	-----	-----	-----	6.78	-----	-----	-----	6.80	-----	-----
6hr	-----	3.10	-----	-----	-----	5.40	-----	-----	-----	6.30	-----	-----
24hr (Day 1)	-----	0.16	-----	-----	-----	0.48	-----	-----	-----	2.92	-----	-----
Day 7	-----	-----	BQL	-----	-----	-----	0.36	-----	-----	-----	0.49	-----
Day 28	-----	-----	-----	BQL	-----	-----	-----	BQL	-----	-----	-----	0.66

Note. Blood volume in porcine model is about 40%-50% of adult human. Thus drug concentrations in human would typically be lower than the figures shown in table 5D.

[0355] Tables 5B, 5C, and 5D show although local (tissue adjacent to the device) concentrations of Apixaban, Rivaroxaban, and Argatroban reached therapeutic levels, the systemic blood concentrations for each of the drugs were below one or more of the following to achieve systemic therapeutic concentrations: systemic C_{max}, Systemic C_{mean}, Systemic C_{trough}. These tables also show that each of these agents reached BQL levels within one of the following: 1 day, 7 days, 28 days, or 90 days

Example 6: Anti-proliferative activity of Apixaban, Argatroban and Rapamycin combination

[0356] Anti-proliferative activity of Apixaban, Argatroban, and Rapamycin was tested in Human Aortic SMC (HAoSMC, ATCC, PCS-100-012). Cell proliferation assay was done in 96-well format. Low passage cells were trypsinized and seeded in 96 well plates at a density of ~4000 cells/well. The cells are allowed to attach overnight in a CO₂ incubator. Next day, the medium was removed and replaced with fresh complete medium containing various concentrations of the test compounds. The final concentration of vehicle (DMSO) in the test medium was 0.1%. After adding test compounds, the cells were incubated for 72 hours. Following this period, the medium was removed and then added fresh medium (100µl) containing CellTiter Aqueous (1x concentration final) to the wells and incubated for 2 hours in the CO₂ incubator. At the end of incubation measured fluorescence with a plate-reader. Controlled incubations with untreated cells and blank incubations containing only medium were included and tested similarly. Based on the cell viability assay the percentage inhibition of the cell proliferation was determined at the different concentrations of the drug tested.

[0357] The cell proliferation assay was performed with different concentrations of Apixaban and Argatroban when combined with Rapamycin. Following the cell proliferation assay as indicated earlier, the percent cell proliferation inhibition was determined, and the assay results plotted to determine the IC₅₀.

[0358] FIGS. 1A-1C show HAoSMC proliferation inhibition in the presence of different drug combinations. The data shows the combination of Apixaban, Argatroban surprisingly and unexpectedly enhanced the anti-proliferative effects of rapamycin on smooth muscle cell proliferation as measured by cell proliferation test when Apixaban and Argatroban were combined with rapamycin, i.e the combination of Apixaban, Argatroban, and rapamycin were more potent than rapamycin alone at inhibiting SMS proliferation.

[0359] FIGS. 1D and 1E show HAoSMC proliferation in presence of different concentrations of Apixaban or Argatroban. In order to determine if Apixaban or Argatroban independently had inhibitory effect on the proliferation of HAoSMC, a proliferation assay in the presence of either of these two drugs at different concentrations were tested as described earlier. Various concentration of Apixaban alone or Argatroban alone had small to no inhibition of HAoSMC proliferation was observed as shown in FIGS. 1D-1E.

Example 7: Activated Clotting Time (ACT) evaluation of Apixaban, Argatroban or a combination of Apixaban and Argatroban

[0360] The activated clotting time (ACT) evaluation of anticoagulants was performed in Calcium-reconstituted sheep blood and recorded employing the Hemochron® Response device.

[0361] The ACT measurements were made in citrated sheep blood. 1.9ml of citrated sheep blood was added to a test tube containing an activator (Hemochron@Celite@ ACT tubes, Lot F8FTE026 from Accriva Diagnostics, Inc.). A target amount of drug solution was then added into the test tube. The test tube was gently swirled so that the blood and drug was well-mixed. 0.1ml of 0.3M calcium chloride was then added. The tube was gently shaken before being inserted into the Hemochron Response detector. The ACT read out was recorded and reported. The ACT of the control blood in the absence of any drug as first determined to establish a baseline. Then ACT was determined in the presence of different concentrations of the drug as a single component. Selected drug combinations, were then tested to evaluate for potential synergy in action between the two drugs.

[0362] As shown in FIGS. 2A-2D, the clotting time was observed to be significantly extended or increased at a higher drug combination concentration. The Apixaban/Argatroban combination achieved ACT levels that were higher than the sum of the individual ACT values, indicating a synergistic effect between these drug combinations. This may be particularly important when delivering these drugs locally (adjacent to injured tissue) to inhibit clot formation. The figures are presented in ng/mg wherein the density of blood and tissue are approximately the same.

[0363] It was found, unexpectedly, that the combination drug concentrations of 0.025ng/mg for each Apixaban and Argatroban drug extended the ACT by a larger time (as shown in FIG. 2B).

[0364] It was found, unexpectedly, that the combination drug concentrations of 0.3ng/mg for each drug (0.6ng/mg total) extended the ACT by a larger time (i.e., was more effective) than the ACT for each individual drug at 0.6ng/mg concentration (ACT of 976 for the combination versus 522 for Apixaban versus 301 for Argatroban as shown in FIG. 2C).

[0365] FIG. 2C further shows, unexpectedly, that the combination drug concentrations of 0.3ng/mg for each drug (0.6ng/mg total) extended the ACT by a larger time (i.e., was more effective) than the ACT for the sum of each individual drug ACT at 0.3ng/mg or at 0.6ng/mg concentration. (ACT of 976 for the combination versus 676 (for the sum of individual drugs having 0.3ng/mg concentrations).

[0366] It is important to note that drug tissue concentrations for factor Xa inhibitors like Rivaroxiban or Apixaban alone or in combination with factor IIa Argatroban to have sufficient tissue concentrations in the stented tissue segment and in the adjacent tissue segment to have therapeutic levels for each drug to be larger than 0.02ng/mg, larger than 0.1ng/mg, preferably larger than 0.2ng/mg of tissue, preferably 0.3ng/mg of tissue, more preferably larger than

1ng/mg of tissue at or within 3 hours after implantation, or at or within 1 day after implantation, to inhibit clot formation.

[0367] It was also shown that the combination of Apixaban and Argatroban with m-TOR inhibitor inhibited thrombus formation in a shunt model (e.g., as in Example 8).

Example 8: Ex vivo testing of Drug Eluting Stent compared with 2 anticoagulants and mTOR eluting stents

[0368] The thrombogenicity of a drug eluting stent system with two anticoagulant Apixaban and Argatroban in combination with rapamycin at two different loading drug doses was evaluated at 1 hour in an arteriovenous ex vivo shunt in a porcine model wherein the devices were deployed in a blood compatible polymeric tubing.

[0369] The control stents were 16-o-demethyl rapamycin m-TOR inhibitor (Novolimus) drug eluting coronary stent (DESyne, Elixir) and m-TOR inhibitor Zotarolimus eluting coronary stent (Resolute, Medtronic, USA).

[0370] The test arm for this experiment were SS9, SS9*, and SS10* and were manufactured as follows: Each polymer solution and each drug solutions were combined together ((Sirolimus and anticoagulant Apixaban and Argatroban was 1:1:1) to poly(L- lactide acid-co- glycolic acid) by weight ratio was 5:2 matrix) according to the target drug dose of 235 µg for each anticoagulant and 94 µg for Sirolimus for SS9, SS9* test arm was about 1/3 of each of the drugs dose as follows: Sirolimus and anticoagulant Apixaban and Argatroban was 1:1:1) to poly(L- lactide acid-co- glycolic acid) by weight ratio was 5:2 on matrix) according to the target drug dose of 78.3 µg for each anticoagulant and 31.3 µg for Sirolimus, and SS10* arm was Sirolimus and anticoagulant Apixaban and Argatroban was 1:1:3) to poly(L- lactide acid-co- glycolic acid) by weight ratio was 5:2 matrix) according to the target drug dose of 39.2 µg for Apixaban and 117.5 µg for Argatroban and 31.3 µg for Sirolimus.

[0371] A microprocessor controlled ultrasonic sprayer was used to coat each of the stents 14mm length uniformly with each of the drug/polymer matrix solution. After coating, the stents were placed in a 70°C oven for about 2 hours to remove the solvent. The stents were then mounted on balloon catheters and crimped. The catheters were then inserted in coils and packaged. The pouches were sterilized.

[0372] The ex-vivo shunt model to evaluate thrombogenicity has been extensively employed to evaluate the biocompatibility of different drug eluting stents (Waksman et al. Circ Cardiovasc Interv. 2017; 10:e004762, Otsuka et al. J Am Coll Cardiol Interv 2015; 8:1248–60, Lipinski et al. EuroInterv 2018; Jaa-369 2018) The animals were housed and cared for in

accordance with the Guide for the Care and Use of Laboratory Animals as established by the National Research Council.

[0373] The two pigs that were employed in this study did not receive any aspirin or clopidogrel pretreatment. Further all procedures were performed in the absence of any anticoagulant including heparin. After induction of anesthesia, an arterial bypass shunt from the femoral artery to the femoral vein was created.. Blood flow was established through the shunt. Flow rates through the shunt was continuously monitored during the procedure with a flow probe that was placed on the shunt tubing proximal to the arterial flow.

[0374] In the first pig three control devices were deployed in the first shunt and the blood flow through the shunt was performed for a period of 1 hour. Following perfusion, the shunt tubing containing the stents was rinsed with saline and then fixed in situ with 10% buffered formalin in order to capture the thrombus, if any, that are deposited on the stent surface.

[0375] Similar procedure with 3 shunts with only one stent SS9* or SS9 in each shunt was tested with a perfusion time of 1 hour for each of the shunts.

[0376] In the second pig three control devices were deployed in the first shunt and the blood flow through the shunt was performed for a period of 1 hour. Following perfusion, the shunt tubing containing the stents was rinsed with saline and then fixed in situ with 10% buffered formalin in order to capture the thrombus, if any, that are deposited on the stent surface.

[0377] Similar procedure with 2 shunts with only one stent SS10* in each shunt was tested with a perfusion time of 1 hour for each of the shunts.

[0378] Promptly following perfusion in each of the shunts, the tubing containing the stents was gently rinsed with saline under gravity flow and then fixed in situ with 10% buffered formalin in order to anchor the thrombus, if any, that are deposited on the stent surface.

[0379] The stents were then removed from the tubing and bisected longitudinally. Low magnification photographs of the luminal side of two halves of the control and test stents were recorded.

[0380] The two halves of the stents were then processed for scanning electron microscopy (SEM) so as to examine the thrombus on the luminal side of the stent. Low (15x) and high (200x) magnification images of the stent surface were captured to evaluate the extent of thrombus deposition on the luminal surface of the stent.

[0381] Significant number of thrombus was observed on the luminal surface (inner surface) of the control DES stents as seen on the low and high magnification SEM images whereas there was little or no thrombus deposits on the test stents with combinations of Apixaban and Argatroban and m-TOR. The number of thrombus deposits on the control and

test stents as evaluated from SEM images are shown in Table 6. The data shows that the combinations of Apixaban and Argatroban and m-TOR inhibited thrombus formation in the shunt model better than control.

[0382] Table 6 shows several therapeutic compositions of factor Xa inhibitor, factor IIa, and M-tor inhibitor releasing stents had less thrombus (clot formation) compared to M-Tor inhibitor alone releasing stents.

[0383] The composition comprising a combination of factor Xa inhibitor, a factor II inhibitor and an anti-proliferative were surprisingly more effective than the anti-proliferative alone.

Table 6

Thrombus deposits on the control and test stents as evaluated from SEM images

Animal #	Control/Test DES	Device	Number of Thrombus deposits
1	Control	DESyne-1	18
		DESyne-2	40
		Resolute	14
	Test – Apixaban:Argatroban(1:1)	SS9*	4
		SS9*	0
		SS9	3
2	Control	DESyne-1	17
		DESyne-2	17
		Resolute	15
	Test – Apixaban: Argatroban(1:3)	SS10*	2
		SS10*	2

Example 9 Preparation of Anticoagulant or mTOR inhibitors coated balloon

[0384] Rivaroxaban, Apixaban, Novolimus, or Rapamycin were dissolved into dichloromethane at room temperature and vortex until the drug was uniformly dissolved/dispersed; Ethylene Vinyl Acetate / Polyvinylpyrrolidone (MW=1.3M) was dissolved into 6.25% methanol in dichloromethane at room temperature and vortex until the polymer had uniformly dissolved/dispersed (100% Dichloromethane was used for Polyethylene oxide (MW=8M)).

[0385] Each polymer solution and each drug solutions were combined together (Rivaroxaban to Ethylene vinyl acetate / Polyvinylpyrrolidone (MW=1.3M) by weight ratio was 2:1:1 for SV300), (Apixaban to Polyethylene oxide (MW=8M)/ Polyvinylpyrrolidone (MW=1.3M) / Butylated hydroxytoluene by weight ratio was 2/1/1/0.01 for VGR), (Novolimus to Ethylene vinyl acetate / Polyvinylpyrrolidone (MW=1.3M) by weight ratio was 2:1:1

NEV250 and NEV200), and (Rapamycin to Ethylene vinyl acetate / Polyvinylpyrrolidone (MW=1.3M) by weight ratio was 2:1:1 for REV200) according to the target drug dose of 300 µg for Rivaroxaban in SV300, 800 µg for Apixaban in VGR, 250 µg for Novoliums in NEV250, 200 µg for Novoliums in NEV200 and 200 µg for Rapamycin in REV200.

[0386] A microprocessor controlled ultrasonic sprayer was used to coat each of the balloon 14mm or 18mm length uniformly (as shown in Table 7) with each of the drug/polymer matrix solutions. Balloons were inflated prior to coating and held by a rotating fixture. A rotational motor rotated the catheter and balloon 360 degrees while a mandrel and a clamp securely held the catheter tail in place and rotate. The coating parameter was adjusted to ideal coating texture and the morphology and the profile of the interface between drug and balloon surface. After coating, the balloons were placed in a vacuum chamber to remove the solvent. The balloons were then tri-folded before putting on the protective sheath. The balloon catheters were then inserted in coils and packaged. The pouches were sterilized.

Example 10: In vivo Pharmacokinetics of Drug Eluting Balloon with Anticoagulant

[0387] The pharmacokinetics of the drug eluting balloon systems with anticoagulant of Example 9 were evaluated in porcine coronary/internal thoracic arteries in the non-diseased porcine coronary artery model. The balloon (e.g., the balloon of a balloon-catheter of a stent-delivery system) was advanced to a site to be treated (e.g., an occluded or weakened section of a blood vessel to be opened up or supported by a stent) in the body of a subject and inflated to a desired inflation diameter. Before, during, and/or after inflation of the balloon, the balloon released the therapeutic composition to, into, or at the treatment site, or to, into, or at an area adjacent thereto, by any suitable mechanism (e.g., concentration gradient, diffusion, pressure, or mechanical force, or a combination thereof). Safety of the device was evaluated at the 7- and 28-day time points following treatment in the coronary arteries and the tissue pharmacokinetics were evaluated following treatment in the coronary/thoracic arteries at the 7 and 28 day time points. Following treatment with the 14 mm length test drug coated balloon or the control plain balloon, an 18 mm length drug eluting balloon was deployed over the balloon treated segment of the coronary/thoracic artery. The tissue concentration is shown in ng/mg tissue.

[0388] The coated balloons were evaluated for drug delivery efficiency in an animal study. Drug transfer of the coated balloons into arterial segments were evaluated using harvested pig arteries. The arterial wall was separated after animal study. The arterial walls were then stored in an individual labeled vial. All samples were kept on dry ice until stored in the -80°C freezer. All samples were then frozen to 70 °C prior to being analyzed. The tissue was extracted with Acetonitrile/methanol for Rivaroxaban, Apixaban, Novolimus and Rapamycin. The tissue

content of Rivaroxaban, Apixaban, Novolimus and Rapamycin from the different drug coated balloon were analyzed using liquid chromatography mass spectroscopy (LCMS) with corresponding reference standards.

Table 7

Coated Balloon tissue concentration in vivo by different time period.

Balloon type	Balloon Coating information	Artery type	Drug	Time period	Tissue concentration (ng/mg)
SV300 18mm Balloon	300µg Rivaroxaban in 150µg Ethylene vinyl acetate and 150µg Polyvinylpyrrolidone (MW=1.3M) matrix	Coronary Artery	Rivaroxaban	15min	1.23±0.37 (n=4)
		Superficial Femoral Artery (SFA)	Rivaroxaban	15min	40.58±64.71 (n=4)
VGR 14mm Balloon	800µg Apixaban in 400µg Polyethylene oxide (MW=8M), 400µg Polyvinylpyrrolidone (MW=1.3M) and 4µg Butylated hydroxytoluene matrix	Coronary Artery	Apixaban	Acute	60.09±80.49 (n=2)
				1D	0.08±0.08 (n=3)
				7D	0.0001±0.0001 (n=3)
				28D	0.003±0.005 (n=3)
NEV250 18mm Balloon	250µg Novolimus in 125µg Ethylene vinyl acetate and 125µg Polyvinylpyrrolidone (MW=1.3M) matrix	Coronary Artery	Novolimus	6H	6.52±3.68 (n=3)
				3D	3.77±1.62 (n=3)
				7D	1.47±0.37 (n=3)
		Superficial Femoral Artery (SFA)	Novolimus	6H	5.94±3.66 (n=4)
				3D	4.4±1.95 (n=4)
				7D	1.16±0.23
NEV200 14mm Balloon	200µg Novolimus in 100 µg Ethylene vinyl acetate and 100 µg Polyvinylpyrrolidone (MW=1.3M) matrix	Coronary Artery	Novolimus	7D	0.60±0.64 (n=7)
REV200 14mm Balloon	200µg Rapamycin in 100 µg Ethylene vinyl acetate and 100 µg Polyvinylpyrrolidone (MW=1.3M) matrix	Coronary Artery	Rapamycin	7D	1.45±1.23 (n=8)

[0389] As shown in Table 7, Rivaroxaban tissue concentration of tissue adjacent to the balloon treated segment ranges from at least 1.23 ng/mg within or at 15min in coronary artery

tissue to at least 40.58 ng/mg in Superficial Femoral Artery (SFA) tissue, within or at 15 minutes; Apixaban tissue concentration of tissue adjacent to the balloon treated segment in coronary artery tissue ranges from at least 60.09 ng/mg at acute to at least 0.08 ng/mg within or at 1 day to at least 0.0001 ng/mg within or at 7 days to at least 0.003 ng/mg within or at 28 days; Novolimus tissue concentration of tissue adjacent to the balloon treated segment in coronary artery tissue ranges from at least 6.52 ng/mg within or at 6 hours to at least 3.77 ng/mg within or at 3 day to at least 1.47 ng/mg within or at 7 days; Novolimus tissue concentration of tissue adjacent to the balloon treated segment in Superficial Femoral Artery (SFA) tissue ranges from at least 5.94 ng/mg within or at 6 hours to at least 4.4 ng/mg within or at 3 day to at least 1.16 ng/mg within or at 7 days; Rapamycin tissue concentration of tissue adjacent to the balloon treated segment in coronary artery tissue ranges from at least 1.45 ng/mg within or at 7 days. Rivaroxaban, Apixaban, Novolimus or Rapamycin released locally sufficiently from a coated balloon catheter to inhibit smooth muscle proliferation after vessel injury at sufficient tissue concentration up to 7 days.

Example 11: Preparation of Anticoagulant Eluting valve implant or part of the implant not covered by a sleeve with carrier

[0390] A valve or valve repair implant or part of the implant comprising the valve is coated with a coating containing anticoagulant Apixaban or Rivaroxaban and Argatroban..

[0391] Poly (L- lactide acid-co- glycolic acid) polymer is dissolved into dichloromethane at room temperature and vortex until the polymer had uniformly dissolved/dispersed. Anticoagulant (Apixaban or Rivaroxaban & Argatroban) are placed in a vial and dissolved in dichloromethane/Methanol at room temperature and vortex until all the drug was uniformly dissolved/dispersed.

[0392] Each polymer solution and each drug solutions are combined together (anticoagulant (Apixaban or Rivaroxaban & Argatroban with weight ratio 1 to 1) to poly (L- lactide acid-co- glycolic acid) by weight ratio was 3:1) according to the target drug dose.

[0393] The valve, and/or valve repair implant, and/or at least part of the implant comprising the valve optionally undergo surface treatment if the surface is not porous (i.e. plasma treatment or other surface friction treatment).

[0394] A microprocessor controlled ultrasonic sprayer was used to coat the valve of the drug containing carrier solution to the entire surface of the implant or part of the surface. After coating, the implant is placed in a 70°C oven for about 2 hours to remove the residue solvent. The transcatheter valve or valve repair implant is then mounted on the delivery catheter. The catheters is then inserted in coils and packaged. The pouches were sterilized.

Example 12: Preparation of Anticoagulant Eluting valve implant or part of the implant covered by a sleeve with carrier

[0395] A valve or valve repair implant or part of the implant covered by a sleeve can have a polymer coating containing anticoagulant Apixaban or Rivaroxaban, Argatroban or a combination of both on top, part of, or adjacent to the sleeve made from ePTFE, Dacron, knitted or weaved fabric, or those known in the art.

[0396] The sleeve is infused with a polymer coating in a solvent solution with anticoagulant Apixaban or Rivaroxaban, Argatroban or a combination of both drugs and drug solution into the said sleeve and said solvent is allowed to evaporate leaving either polymer coating or drug in the pores of the sleeve. The sleeve is placed prior to being attached to the valve or valve repair implant or after it has been attached to the implant.

[0397] Poly (L- lactide acid-co- glycolic acid) polymer is dissolved into dichloromethane at room temperature and vortex until the polymer is uniformly dissolved/dispersed. Anticoagulant (Apixaban or Rivaroxaban & Argatroban) is placed in a vial and dissolved in dichloromethane/Methanol at room temperature and vortex until all the drug is uniformly dissolved/dispersed.

[0398] Each polymer solution and each drug solutions is combined together (anticoagulant (Apixaban or Rivaroxaban and Argatroban with weight ratio 1 to 1) to poly (L- lactide acid-co- glycolic acid) by weight ratio was 3:1) according to the target drug dose.

[0399] The sleeve optionally undergoes surface treatment if the surface is not porous (i.e. plasma treatment or other friction surface treatment). After surface treatment, the coating is spray coated or dip coated. The coating can be inside the sleeve or dipped onto the sleeve or coated on the sleeve.

[0400] When spray coated, a microprocessor controlled ultrasonic sprayer is used to coat the sleeve containing drug/excipient solution to the entire surface of the implant. After coating, the sleeve is placed in a 70°C oven for about 2 hours to remove the residue solvent. The sleeve is then attached to the valve or valve repair implant if not prior to being attached to the implant. The valve or valve repair implant with sleeve attached is then mounted on the delivery catheter. The transcatheter valve or valve repair device is then inserted in a coil and packaged. The pouches were sterilized.

Example 13: Preparation of drug eluting stent having anticoagulant Argatroban crosslinked with poly N-(2-Hydroxypropyl) methacrylamide by ester linker

[0401] Example 13 includes methods for applying chemically crosslinked polymers and anticoagulant onto stent. The reactive polymer and anti-coagulant can be reacted and purified

before making the coating solution or can be mixed together with initiator, then coated one layer for slow release of anticoagulant.

[0402] Anticoagulant (Apixaban or Rivaroxaban, Argatroban, Rivaroxaban etc.) is conjugated with biocompatible polymers via a reversible covalent bond, which can slowly release anticoagulant in a controlled manner. For example, Argatroban is linked to poly N-(2-Hydroxypropyl) methacrylamide by a reversible ester bond as show in FIG. 3. When this ester bond is hydrolyzed, the drug Argatroban is released.

[0403] Argatroban reacted with poly N-(2-Hydroxypropyl) methacrylamide is dissolved in THF. This polymer solution is air sprayed onto a stainless-steel coronary stent by a microprocessor controlled ultrasonic sprayer until a target weight achieved. After coating, the stents is placed in a 70°C oven for about 2 hours to remove the solvent. The stents are then mounted on balloon catheters and crimped. The catheters are then inserted in coils and packaged. The pouches are sterilized.

[0404] In Vivo, when Argatroban - poly N-(2-Hydroxypropyl) methacrylamide ester bond is hydrolyzed, the drug Argatroban will be released.

Example 14: Preparation of drug eluting stent having anticoagulant Argatroban crosslinked with PAMAM-OH dendrimer by ester linker

[0405] PAMAM-OH dendrimer (generation 2) and Argatroban (1 to 1.2 mole ratio) are dissolved in THF, 4-Dimethylaminopyridine (DMAP) 1% was added as catalyst. The reaction mixture stirred overnight followed by purification. The purified Argatroban - PAMAM-OH dendrimer is dissolved in Tetrahydrofuran (THF). This polymer solution is air sprayed onto a stainless-steel coronary stent by a microprocessor controlled ultrasonic sprayer until a target weight achieved. After coating, the stents are placed in a 70°C oven for about 2 hours to remove the solvent. The stents are then mounted on balloon catheters and crimped. The catheters are then inserted in coils and packaged. The pouches were sterilized.

[0406] In Vivo, when Argatroban crosslinked with PAMAM-OH dendrimer ester bond is hydrolyzed, the drug Argatroban will be released.

Example 15: Preparation of drug eluting stent having anticoagulant and polymer microsphere

[0407] Anticoagulant (Apixaban, Argatroban, Rivaroxaban etc.) are embodied within biocompatible materials (such as polymers, metals, ceramics, natural plant and/or animal materials). The polymers can be selected from polyesters (poly lactic acid, poly glycolic acid, Polyurethanes), Poly methyl methacrylate (PMMA), poly N-(2-Hydroxypropyl) methacrylamide, Polyethylenimine (PEI), dextran, dextrin, chitosans, poly(L-lysine), and

poly(aspartamides), polyethylene, polypropylene, polyamides, Polyethylene glycol (PEG), Polytetrafluoroethylene (PTFE), Silicones, poly(anhydride), poly ortho esters etc. Anticoagulant (Apixaban, Argatroban, Rivaroxaban etc.) with polymers to form drug-polymer nano particles, microsphere, polymeric micelles as the polymer drug delivery systems, which can have high drug loading capacity in the hydrophobic core especially for hydrophobic drugs, and rapid cellular uptake facilitated by their nano-size characteristics.

[0408] 0.5 mL of poly(D,L-lactide) dichloromethane solution (0.5% w/v) and anticoagulant(Rivaroxaban, Apixaban or Argatroban) dichloromethane solution (0.5% w / v) are slowly added dropwise to polyvinyl alcohol water solution(5% w/w) with magnetic stirring at 1000-1500 rpm. These dispersions are continue stirred for 2 hours at 40 ° C. and 200 rpm until the microspheres are very small (ie less than 1 µm in diameter) to form colloids and therefore the suspension does not settle under gravity. This polymer-anticoagulant suspension is dip coated multiple times to the stent surface until target drug weight achieved. The stent is air dried first then the stents are placed in a 70°C oven for about 4 hours to remove the solvent. The stents are then mounted on balloon catheters and crimped. The catheters are then inserted in coils and packaged. The pouches were sterilized.

Example 16: Preparation and use of anticoagulant-impregnated balloon

[0409] A balloon made of a biodegradable or non-degradable polymeric material (e.g., a nylon) and having openings (e.g., pores, holes, etc.) in the body and/or at the surface of the balloon is immersed in a mixture containing a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent (e.g., an anticoagulant, such as Rivaroxaban or a derivative thereof, or a fibrinolytic or thrombolytic agent, such as a plasminogen activator, e.g. tPA) and a solvent, and optionally another kind of bioactive agent (e.g., an anti-proliferative agent, such as rapamycin or a derivative thereof, or an anti-inflammatory agent). The balloon can optionally have one or more coatings comprising a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and/or another kind of bioactive agent and can optionally have a coating comprising no bioactive agent.

[0410] The balloon (e.g., the balloon of a balloon-catheter of a stent-delivery system) is advanced to a site to be treated (e.g., an occluded or weakened section of a blood vessel to be opened up or supported by a stent) in the body of a subject and is inflated to a desired inflation diameter. Before, during and/or after inflation of the balloon, the balloon releases the fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and the optional other kind of bioactive agent (e.g., an anti-proliferative agent or an anti-inflammatory agent) to, into or at the treatment site, or to, into or at an area adjacent thereto, by any suitable

mechanism (e.g., concentration gradient, diffusion, pressure or mechanical force, or a combination thereof).

Example 17: Preparation and use of anticoagulant-coated catheter

[0411] A first mixture containing a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent (e.g., an anticoagulant, such as Rivaroxaban or a derivative thereof, or a fibrinolytic or thrombolytic agent, such as a plasminogen activator, e.g. tPA) and a solvent, and optionally a biodegradable or non-degradable polymeric material and/or another kind of bioactive agent (e.g., an anti-proliferative agent, such as rapamycin or a derivative thereof, or an anti-inflammatory agent), is applied (e.g., by spraying or dipping) to a catheter made of a biodegradable or non-degradable polymeric material (e.g., a nylon or a polyether block amide, such as PEBAX®) to form a first coating on the catheter. The first coating can cover any surfaces (e.g., the exterior surface, any other surfaces or all surfaces) of the catheter.

[0412] Optionally, a second mixture containing another kind of bioactive agent (e.g., an anti-proliferative agent, such as rapamycin or a derivative thereof, or an anti-inflammatory agent) and a solvent, and optionally a biodegradable or non-degradable polymeric material and/or a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent (e.g., an anticoagulant, such as Rivaroxaban or a derivative thereof, or a fibrinolytic or thrombolytic agent, such as a plasminogen activator, e.g. tPA), is applied to the catheter (e.g., by spraying or dipping) to form a second coating on the catheter. The optional second coating can cover any surfaces (e.g., the exterior surface, any other surfaces or all surfaces) of the catheter. The first mixture and the optional second mixture can be applied to the catheter in any order.

[0413] Optionally, a third mixture containing a biodegradable or non-degradable polymeric material and a solvent is applied to the catheter (e.g., by spraying or dipping) to form a third coating over the first coating and the optional second coating. The optional third coating can be, e.g., a top layer or coat or a diffusion barrier that controls release of the fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and the optional other kind of bioactive agent from the first coating and the optional second coating. The optional third coating can cover the exterior surface or any other surfaces, or all surfaces, of the catheter.

[0414] The coated catheter is optionally heated to stabilize the coating(s) and is placed in a container (e.g., a pouch) and sterilized (e.g., by exposure to e-beam radiation).

[0415] The catheter (e.g., the catheter of a stent-delivery system) is advanced to a site to be treated (e.g., an occluded or weakened section of a blood vessel to be opened up or supported by a stent) in the body of a subject. Before, while and/or after the catheter is positioned at the treatment site or at an area adjacent thereto, the catheter releases the fibrin/thrombus formation-

inhibiting or fibrin/thrombus dissolution-promoting agent and the optional other kind of bioactive agent (e.g., an anti-proliferative agent or an anti-inflammatory agent) to, into or at the treatment site, or to, into or at an area adjacent thereto, by any suitable mechanism (e.g., concentration gradient, diffusion, pressure or mechanical force, or a combination thereof).

Example 18: Preparation and use of anticoagulant-impregnated catheter

[0416] A catheter (e.g., an infusion catheter) made of a biodegradable or non-degradable polymeric material (e.g., a nylon or a polyether block amide, such as PEBAX®) and having openings (e.g., pores, holes, etc.) in the body and/or at the surface of the catheter is immersed in a mixture containing a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent (e.g., an anticoagulant, such as Rivaroxaban or a derivative thereof, or a fibrinolytic or thrombolytic agent, such as a plasminogen activator) and a solvent, and optionally another kind of bioactive agent (e.g., an anti-proliferative agent, such as rapamycin or a derivative thereof, or an anti-inflammatory agent). The catheter can optionally have one or more coatings comprising a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and/or another kind of bioactive agent and can optionally have a coating comprising no bioactive agent.

[0417] The catheter (e.g., the catheter of a stent-delivery system) is advanced to a site to be treated (e.g., an occluded or weakened section of a blood vessel to be opened up or supported by a stent) in the body of a subject. Before, while and/or after the catheter is positioned at the treatment site or at an area adjacent thereto, the catheter releases the fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and the optional other kind of bioactive agent (e.g., an anti-proliferative agent or an anti-inflammatory agent) to, into or at the treatment site, or to, into or at an area adjacent thereto, by any suitable mechanism (e.g., concentration gradient, diffusion, pressure or mechanical force, or a combination thereof).

Example 19: Preparation and use of anticoagulant-coated balloon-catheter

[0418] A first mixture containing a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent (e.g., an anticoagulant, such as Rivaroxaban or a derivative thereof, or a fibrinolytic or thrombolytic agent, such as a plasminogen activator) and a solvent, and optionally a biodegradable or non-degradable polymeric material and/or another kind of bioactive agent (e.g., an anti-proliferative agent, such as rapamycin or a derivative thereof, or an anti-inflammatory agent), is applied (e.g., by spraying or dipping) to the balloon portion and/or the catheter portion of a balloon-catheter made of a biodegradable or non-degradable polymeric material (e.g., a nylon) to form a first coating on the balloon portion

and/or the catheter portion. The first coating can cover any surfaces (e.g., the exterior surface, any other surfaces or all surfaces) of the balloon and/or the catheter.

[0419] Optionally, a second mixture containing another type of bioactive agent (e.g., an anti-proliferative agent, such as rapamycin or a derivative thereof, or an anti-inflammatory agent) and a solvent, and optionally a biodegradable or non-degradable polymeric material and/or a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent (e.g., an anticoagulant, such as Rivaroxaban or a derivative thereof, or a fibrinolytic or thrombolytic agent, such as a plasminogen activator), is applied to the balloon portion and/or the catheter portion (e.g., by spraying or dipping) to form a second coating on the balloon portion and/or the catheter portion. The optional second coating can cover any surfaces (e.g., the exterior surface, any other surfaces or all surfaces) of the balloon and/or the catheter. The first mixture and the optional second mixture can be applied to the balloon portion and/or the catheter portion in any order.

[0420] Optionally, a third mixture containing a biodegradable or non-degradable polymeric material and a solvent is applied to the balloon portion and/or the catheter portion (e.g., by spraying or dipping) to form a third coating over the first coating and the optional second coating. The optional third coating can be, e.g., a top layer or coat or a diffusion barrier that controls release of the fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and the optional other kind of bioactive agent from the first coating and the optional second coating. The optional third coating can cover the exterior surface or any other surfaces, or all surfaces, of the balloon and/or the catheter.

[0421] The coated balloon-catheter is optionally heated to stabilize the coating(s) and is placed in a container (e.g., a pouch) and sterilized (e.g., by exposure to e-beam radiation).

[0422] The balloon-catheter (e.g., the balloon-catheter of a stent-delivery system) is advanced to a site to be treated (e.g., an occluded or weakened section of a blood vessel to be opened up or supported by a stent) in the body of a subject, and the balloon is inflated to a desired inflation diameter. Before, during and/or after inflation of the balloon, the balloon portion and/or the catheter portion of the balloon-catheter releases the fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and the optional other kind of bioactive agent (e.g., an anti-proliferative agent or an anti-inflammatory agent) to, into or at the treatment site, or to, into or at an area adjacent thereto, by any suitable mechanism (e.g., concentration gradient, diffusion, pressure or mechanical force, or a combination thereof).

Example 20: Preparation and use of anticoagulant-impregnated balloon-catheter

[0423] The balloon portion and/or the catheter portion of a balloon-catheter (e.g., a weeping catheter) made of a biodegradable or non-degradable polymeric material (e.g., a nylon)

and having openings (e.g., pores, holes, etc.) in the body and/or at the surface of the balloon and/or the catheter is immersed in a mixture containing a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent (e.g., an anticoagulant, such as Rivaroxaban or a derivative thereof, or a fibrinolytic or thrombolytic agent, such as a plasminogen activator) and a solvent, and optionally another type of bioactive agent (e.g., an anti-proliferative agent, such as rapamycin or a derivative thereof, or an anti-inflammatory agent). The balloon portion and/or the catheter portion of the balloon-catheter can optionally have one or more coatings comprising a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and/or another kind of bioactive agent, and can optionally have a coating comprising no bioactive agent.

[0424] The balloon-catheter (e.g., the balloon-catheter of a stent-delivery system) is advanced to a site to be treated (e.g., an occluded or weakened section of a blood vessel to be opened up or supported by a stent) in the body of a subject, and the balloon is inflated to a desired inflation diameter. Before, during and/or after inflation of the balloon, the balloon portion and/or the catheter portion of the balloon-catheter releases the fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and the optional other kind of bioactive agent (e.g., an anti-proliferative agent or an anti-inflammatory agent) to, into or at the treatment site, or to, into or at an area adjacent thereto, by any suitable mechanism (e.g., concentration gradient, diffusion, pressure or mechanical force, or a combination thereof).

Example 21: Use of anticoagulant-delivering infusion catheter

[0425] An infusion catheter contains one or more lumens for delivering one or more drugs. The infusion catheter (e.g., the catheter of a stent-delivery system) is advanced to a site to be treated (e.g., an occluded or weakened section of a blood vessel to be opened up or supported by a stent) in the body of a subject. Before, while and/or after the catheter is positioned at the treatment site or at an area adjacent thereto, a first mixture containing a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent (e.g., an anticoagulant, such as Rivaroxaban or a derivative thereof, or a fibrinolytic or thrombolytic agent, such as a plasminogen activator) and a solvent (e.g., saline), and optionally another kind of bioactive agent (e.g., an anti-proliferative agent, such as rapamycin or a derivative thereof, or an anti-inflammatory agent), is injected through one or more drug-delivering lumens of the catheter. Before, while and/or after the catheter is positioned at the treatment site or at an area adjacent thereto, a second mixture containing another kind of bioactive agent (e.g., an anti-proliferative agent or an anti-inflammatory agent) and a solvent (e.g., saline), and optionally a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent, optionally is injected through one or more drug-delivering lumens of the catheter. The catheter delivers the

fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and the optional other kind of bioactive agent (e.g., an anti-proliferative agent or an anti-inflammatory agent) to, into or at the treatment site, or to, into or at an area adjacent thereto.

Example 22: Preparation and use of anticoagulant-coated surgical instrument

[0426] A surgical instrument (e.g., a cutting instrument, such as a knife) made of a biodegradable or non-degradable metal or metal alloy (e.g., stainless steel) optionally undergoes surface treatment (e.g., microblasting) to improve adhesion of a coating (e.g., a polymeric coating) to a metal surface.

[0427] A first mixture containing a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent (e.g., an anticoagulant, such as Rivaroxaban or a derivative thereof, or a fibrinolytic or thrombolytic agent, such as a plasminogen activator) and a solvent, and optionally a biodegradable or non-degradable polymeric material and/or another kind of bioactive agent (e.g., an anti-proliferative agent, such as rapamycin or a derivative thereof, or an anti-inflammatory agent), is applied to the surgical instrument (e.g., by dipping or spraying) to form a first coating on the surgical instrument. The first coating can cover the exterior surface, any other surfaces or all surfaces of the surgical instrument.

[0428] Optionally, a second mixture containing another kind of bioactive agent (e.g., an anti-proliferative agent, such as rapamycin or a derivative thereof, or an anti-inflammatory agent) and a solvent, and optionally a biodegradable or non-degradable polymeric material and/or a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent (e.g., an anticoagulant, such as Rivaroxaban or a derivative thereof, or a fibrinolytic or thrombolytic agent, such as a plasminogen activator), is applied to the surgical instrument (e.g., by dipping or spraying) to form a second coating on the surgical instrument. The optional second coating can cover the exterior surface, any other surfaces or all surfaces of the surgical instrument. The first mixture and the optional second mixture can be applied to the surgical instrument in any order.

[0429] Optionally, a third mixture containing a biodegradable or non-degradable polymeric material and a solvent is applied to the surgical instrument (e.g., by dipping or spraying) to form a third coating over the first coating and the optional second coating. The optional third coating can be, e.g., a top layer or coat or a diffusion barrier that controls release of the fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and the optional other kind of bioactive agent from the first coating and the optional second coating. The optional third coating can cover the exterior surface, any other surfaces or all surfaces of the surgical instrument.

[0430] The coated surgical instrument is optionally heated to stabilize the coating(s) and is placed in a container (e.g., a pouch) and sterilized (e.g., by exposure to e-beam radiation).

[0431] The surgical instrument (e.g., a cutting instrument, such as a knife) is advanced to a site in the body of a subject undergoing a surgery or intervention (e.g., a tissue to be cut or treated). Before, while and/or after the surgical instrument is positioned at the treatment site or at an area adjacent thereto, or before, while and/or after the surgical instrument contacts the tissue to be treated, the surgical instrument releases the fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and the optional other kind of bioactive agent (e.g., an anti-proliferative agent or an anti-inflammatory agent) to, into or at the treatment site, or to, into or at an area adjacent thereto, by any suitable mechanism (e.g., concentration gradient, diffusion, pressure or mechanical force, or a combination thereof).

Example 23: Preparation and use of anticoagulant-impregnated surgical instrument

[0432] A surgical instrument made of a biodegradable or non-degradable metal or metal alloy (e.g., stainless steel) and having openings (e.g., pores, holes, etc.) in the body and/or at the surface of the surgical instrument is immersed in a mixture containing a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent (e.g., an anticoagulant, such as Rivaroxaban or a derivative thereof, or a fibrinolytic or thrombolytic agent, such as a plasminogen activator) and a solvent, and optionally another kind of bioactive agent (e.g., an anti-proliferative agent, such as rapamycin or a derivative thereof, or an anti-inflammatory agent). The surgical instrument can optionally undergo surface treatment, can optionally have one or more coatings comprising a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and/or another kind of bioactive agent, and can optionally have a coating comprising no bioactive agent.

[0433] The surgical instrument (e.g., a cutting instrument, such as a knife) is advanced to a site in the body of a subject undergoing a surgery or intervention (e.g., a tissue to be cut or treated). Before, while and/or after the surgical instrument is positioned at the treatment site or at an area adjacent thereto, or before, while and/or after the surgical instrument contacts the tissue to be treated, the surgical instrument releases the fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and the optional other kind of bioactive agent (e.g., an anti-proliferative agent or an anti-inflammatory agent) to, into or at the treatment site, or to, into or at an area adjacent thereto, by any suitable mechanism (e.g., concentration gradient, diffusion, pressure or mechanical force, or a combination thereof).

Example 24: Use of anticoagulant-delivering infusion surgical instrument

[0434] An infusion surgical instrument [e.g., a cutting instrument (e.g., a knife) or an injection device (e.g., a needle)] contains one or more lumens for delivering one or more drugs.

The surgical instrument is advanced to a site in or on the body of a subject undergoing a surgery or intervention (e.g., a tissue to be cut or treated). Before, while and/or after the surgical instrument is positioned at the treatment site or at an area adjacent thereto, or before, while and/or after the surgical instrument contacts the tissue to be treated, a first mixture containing a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent (e.g., an anticoagulant, such as Rivaroxaban or a derivative thereof, or a fibrinolytic or thrombolytic agent, such as a plasminogen activator) and a solvent (e.g., saline), and optionally another kind of bioactive agent (e.g., an anti-proliferative agent, such as rapamycin or a derivative thereof, or an anti-inflammatory agent), is injected through one or more drug-delivering lumens of the surgical instrument. Before, while and/or after the surgical instrument is positioned at the treatment site or at an area adjacent thereto, or before, while and/or after the surgical instrument contacts the tissue to be treated, a second mixture containing another kind of bioactive agent (e.g., an anti-proliferative agent or an anti-inflammatory agent) and a solvent (e.g., saline), and optionally a fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent, optionally is injected through one or more drug-delivering lumens of the surgical instrument. The surgical instrument delivers the fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and the optional other kind of bioactive agent (e.g., an anti-proliferative agent or an anti-inflammatory agent) to, into or at the treatment site, or to, into or at an area adjacent thereto.

Example 25 Preparation of mTOR inhibitors and/or Anticoagulant coated balloon with excipient

[0435] The balloon can optionally adopt carrier excipient to coat to facilitate drug transfer to the vessel wall and control release rate. A variety of carrier excipients and techniques can be used. The selected excipient could be contrast agent (i.e. iopromide), urea, dextrane, shellac, shelloic acid, keratosis (a naturally derived protein), Plasticizer (i.e. butyryl-tri-hexyl citrate, acetyl tributyl citrate, citrate ester, glycerol, other organic ester), hydrophilic space, Polyvinylpyrrolidone (PVP) and its hydrogels, Surfactants, Non-ionic surfactant Polysorbate/sorbitol (i.e. Tween20, Tween60 or Tween80), nordihydroguaiaretic acid (NDGA), hydrophobic excipient such as phospholipid, amphiphilic polymer such as Poly(ethylene glycol) (i.e. PEG 8000), poly(ethylene oxide) (PEO) (molecular weight range from 100,000 to 10,000,000), Polyethylenimine (PEI) or polyaziridine linear or branched, amphiphilic block copolymers composed of poly(ethylene oxide) (PEO) as the hydrophilic block and poly(ether)s, poly(amino acid)s, hydrophobic polymer space, biodegradable polymers such as Poly DL lactide-co-glycolide, Poly L Lactide-co-caprolactone, durable polymers, individually or combinations thereof.

[0436] A balloon made of a biodegradable or non-degradable polymeric material (e.g., a nylon) and having openings (e.g., pores, holes, etc.) in the body and/or at the surface of the balloon was used. The drug was selected from a mixture containing a mTOR inhibitors /Anticoagulant1/ Anticoagulant2. The mTOR inhibitors was selected from Sirolimus, Novolimus, temsirolimus, zotarolimus and everolimus etc. The anticoagulants were selected from Apixaban, Argatroban, Rivaroxaban or a derivative thereof, and/or a fibrinolytic or thrombolytic agent, such as a plasminogen activator) individually or combinations thereof. For example, Siroliums and Anticoagulant1/ Anticoagulant2 (Apixaban and Argatroban) were placed in a vial and dissolved in dichloromethane or dichloromethane/Methanol combination at 2 to 10mg/ml. The carrier excipient was dissolved in a proper solvent. The solution and drug solutions were combined at a target ratio of 3 to 1, 1 to 1, 2 to1, or 1 to 3 ratios according to the target drug loading. Further dilution with dichloromethane was conducted if needed. Optionally anti-solvent was used to control the coating morphology of particles and drug release rate.

[0437] Optionally the balloon can undergo physical surface treatment before coated such that the surface has microspores, micro-holes, or chemical surface treatment before coated such that the balloon materials have photo-link or other chemical function group that can be reacted under UV or other techniques to easy coating. After surface treatment, the coating can be spray coat or dip coat or use other coating techniques (i.e. 3D printer).

[0438] The coating can optionally cover any surfaces (e.g., the exterior surface, any other surfaces or all surfaces) of the balloon. The coating solution can be homogeneous or non-homogeneous such as suspension or emulsions.

[0439] The coated balloon can optionally combine multi-strategies (e.g., electrospinning, plasma treatment, Layer-by-Layer Self-Assembly or a combination thereof) to form finely controlling structural, mechanical, and surface properties. With tuned coating techniques and solvent removal technique, the produces powder particles can be optionally homogenous, porous, and uniform in size and shape. The morphology of particles could be micro-crystalline, nanoparticles, Nano-encapsulated to provide release rate control.

[0440] When spray coat, a microprocessor controlled ultrasonic sprayer was used to apply the drug containing drug solution to cover any surface of a balloon. A mandrel was placed through catheter tips and underneath an ultrasonic spray nozzle (Micromist System with Ultrasonic Atomizing Nozzle Sprayer, Sono-Tek, N.Y.), which was rotating at 80 rpm and move longitudinally at a rate of 0.050 inches/minutes. The coating parameter can optionally adjusted to ideal coating texture and the morphology and the profile of the interface between drug and balloon surface.

[0441] After coating, the balloon was placed in a vacuum chamber to remove the residue solvent. Optionally the coated balloon can be tri-folded to protect coated drug with a folded and/or wrapped balloon thereon to a pre-annealing step to induce a fold/wrap memory in the resulting pre-annealed balloon and/or coated balloon has a protector which need to peel off before use.

[0442] The balloon catheter was then inserted in a coil and packaged. The pouch was sterilized by Ethylene oxide or E-beam. The pouch was further packaged in a foil pouch with oxygen scavengers and nitrogen purge and vacuum sealed.

[0443] The balloon (e.g., the balloon of a balloon-catheter of a balloon-delivery system) was advanced to a site to be treated (e.g., an occluded or weakened section of a blood vessel to be opened up or supported by a balloon) in the body of a subject and was inflated to a desired inflation diameter. Before, during and/or after inflation of the balloon, the balloon releases the fibrin/thrombus formation-inhibiting or fibrin/thrombus dissolution-promoting agent and the optional other kind of bioactive agent (e.g., an anti-proliferative agent or an anti-inflammatory agent) to, into or at the treatment site, or to, into or at an area adjacent thereto, by any suitable mechanism (e.g., concentration gradient, diffusion, pressure or mechanical force, or a combination thereof).

Example 26: Preparation of drug coated balloon having anticoagulant and polymer microsphere

[0444] Anticoagulant (Apixaban, Argatroban, Rivaroxaban etc.) are embodied within biocompatible materials (such as polymers, metals, ceramics, albumin, liposome, natural plant and/or animal materials). The polymers can be selected from polyesters (poly lactic acid, poly glycolic acid, poly lactic acid-co- glycolic acid, poly lactic acid-co-caprolactone, poly ethylene glycol-block- poly caprolactone, Polyurethanes etc.), Poly methyl methacrylate (PMMA), poly N-(2-Hydroxypropyl) methacrylamide, Polyethylenimine (PEI), dextran, dextrin, chitosans, poly(L-lysine), and poly(aspartamides), polyethylene, polypropylene, polyamides, Polyethylene glycol (PEG) , Silicones, poly(anhydride), poly ortho esters etc. Anticoagulant (Apixaban, Argatroban, Rivaroxaban etc.) with polymers to form drug-polymer nano particles, microsphere, polymeric micelles as the polymer drug delivery systems, which can have high drug loading capacity in the hydrophobic core especially for hydrophobic drugs, and rapid cellular uptake facilitated by their micro/nano-size characteristics.

[0445] 0.5 mL of poly(D,L-lactide) dichloromethane solution (0.5% w/v) and anticoagulant(Rivaroxaban, Apixaban or Argatroban) dichloromethane solution (0.5% w / v) are slowly added dropwise to polyvinyl alcohol water solution(5% w/w) with magnetic stirring at 1000-1500 rpm. These dispersions are continue stirred for 2 hours at 40 ° C. and 200 rpm until the microspheres

are very small (ie less than 1 μm in diameter) to form colloids and therefore the suspension does not settle under gravity. This polymer-anticoagulant suspension is dip coated multiple times to the balloon surface until target drug weight achieved. After coating, the balloons were air dried first then were placed in a vacuum chamber to remove the solvent. The balloons were then tri-folded before putting on the protective sheath. The balloon catheters were then inserted in coils and packaged. The pouches were sterilized.

Example 27: Preparation of drug coated balloon having anticoagulant and polymer self-assembly hollow nanoparticles

[0446] Anticoagulant (Apixaban, Argatroban, Rivaroxaban etc.) are monodispersed within hollow polymer nanoparticle. The polymers with molecular range from 100K to 10K (optimally from 40k to 20K) can be selected from block degradable polymers of poly lactic acid, poly glycolic acid, poly lactic acid-co- glycolic acid, poly lactic acid-co-caprolactone, poly ethylene glycol-block- poly caprolactone etc., or block non-degradable polymers selected from Polyvinylpyridine block with Poly methyl methacrylate (PMMA), poly N-(2-Hydroxypropyl) methacrylamide, Polyethylenimine (PEI), dextran, dextrin, chitosans, poly(L-lysine), and poly(aspartamides), polyamides, Polyethylene glycol (PEG), Silicones, poly(anhydride), poly ortho esters, polystyrene-b-polyvinylpyridine, poly(isoprene)-b-poly(vinyl pyridine), poly(vinyl pyridine)-b-poly(styrene)-b-poly(vinyl pyridine), poly(styrene)-b-polyvinyl pyridine-b-poly(styrene), poly(styrene-b-poly(acrylic acid), poly(styrene)-b-poly(butadiene)-b-poly(vinyl pyridine), poly(styrene-b-poly(methacrylic acid), poly(styrene)-b-poly(ethylene oxide), poly(butadiene)-b-poly(acrylic acid), poly(butadiene)-b-poly(ethylene oxide), poly(vinyl pyridine)-b-poly(butadiene)-b-poly(vinyl pyridine), poly(ethylene)-b-poly(ethylene oxide), and poly(styrene)-b-poly(vinyl pyridine)-b-poly(ethylene oxide) etc. Anticoagulant (Apixaban, Argatroban, Rivaroxaban etc.) with polymers to form drug-polymer nano particles as the polymer drug delivery systems, which can have high drug loading capacity in the hydrophobic core especially for hydrophobic drugs, and rapid cellular uptake facilitated by their nano-size characteristics.

[0447] Poly(ethylene glycol)-block-poly(4-vinyl pyridine) or poly(styrene)-block-poly(4-vinyl pyridine) or other poly(4-vinyl pyridine) block polymers is dissolved in DMF or 1,4-dioxane to prepare a solution of 5mg/ml; this solution is added to solutions containing varied amount of Azo compounds in the same solvent(the monomer molar ratio of 4-vinyl pyridine: Azo compounds from 1:0.2 to 1:2). Azo compounds are selected from Metanil Yellow, Orange II sodium salt, 2,2'-Dihydroxyazobenzene, 2-(4-Hydroxyphenylazo) benzoic acid, 5-[(2-Carboxyphenyl) azo]-2-hydroxybenzoic acid, Olsalazine, 5-[(4-aminophenyl)azo]-2-hydroxy-Benzoic acid as hydrogen bonding agent for self-assembly. After stirring and reflux overnight,

the self-assembly nanoparticles were collected by centrifuging. Using ethanol wash to remove hydrogen bonding agent results in monodisperse hollow nanoparticles with tunable hollow cavity size and internal surface reactivity. The resulting nanoparticles are redispersed in chloroform and mixed with anticoagulant (Rivaroxaban, Apixaban or Argatroban) solution in the same solvent; the balloon can be coated with this solution by dip- or spin-coating method to the balloon surface until target drug weight achieved with anticoagulant (Rivaroxaban, Apixaban or Argatroban) is hydrogen bonding with this hollow nanoparticle polymers.

[0448] After coating, the balloons were air dried first then were placed in a vacuum chamber to remove the solvent. The balloons were then tri-folded before putting on the protective sheath. The balloon catheters were then inserted in coils and packaged. The pouches were sterilized.

Example 28: Preparation of drug coated balloon having colocalized synergized delivery of m-TOR and paclitaxel self-assembly hollow nanoparticles

[0449] m-TOR (Sirolimus, biolimus, everolimus, myolimus, novolimus, ridaforolimus, temsirolimus, zotarolimus, or salts, isomers, solvates, analogs, derivatives, metabolites etc.) and paclitaxel are embodied within hollow polymer nanoparticles. The polymers with molecular range from 100K to 10K (optimally from 40k to 20K) can be selected from block degradable polymers of poly lactic acid, poly glycolic acid, poly lactic acid-co- glycolic acid, poly lactic acid-co-caprolactone, poly ethylene glycol-block- poly caprolactone etc., or block non-degradable polymers selected from Polyvinylpyridine block with Poly methyl methacrylate (PMMA), poly N-(2-Hydroxypropyl) methacrylamide, Polyethylenimine (PEI), dextran, dextrin, chitosans, poly(L-lysine), and poly(aspartamides), polyamides, Polyethylene glycol (PEG), Silicones, poly(anhydride), poly ortho esters, polystyrene-b-polyvinylpyridine, poly(isoprene)-b-poly(vinyl pyridine), poly(vinyl pyridine)-b-poly(styrene)-b-poly(vinyl pyridine), poly(styrene)-b-polyvinyl pyridine-b-poly(styrene), poly(styrene-b-poly(acrylic acid), poly(styrene)-b-poly(butadiene)-b-poly(vinyl pyridine), poly(styrene-b-poly(methacrylic acid), poly(styrene)-b-poly(ethylene oxide), poly(butadiene)-b-poly(acrylic acid), poly(butadiene)-b-poly(ethylene oxide), poly(vinyl pyridine)-b-poly(butadiene)-b-poly(vinyl pyridine), poly(ethylene)-b-poly(ethylene oxide), and poly(styrene)-b-poly(vinyl pyridine)-b-poly(ethylene oxide). M-TOR with paclitaxel and polymers to form drug-polymer nano particles as the polymer drug delivery systems, which can have high drug loading capacity in the hydrophobic core especially for hydrophobic drugs, and rapid cellular uptake facilitated by their nano-size characteristics.

[0450] Poly(ethylene glycol)-block-poly(4-vinyl pyridine) or poly(styrene)-block-poly(4-vinyl pyridine) or other poly(4-vinyl pyridine) block polymers is dissolved in DMF or 1,4-dioxane to prepare a solution of 5mg/ml; this solution is added to solutions containing varied

amount of Azo compounds in the same solvent(the monomer molar ratio of 4-vinyl pyridine: Azo compounds from 1:0.2 to 1:2). Azo compounds are selected from Metanil Yellow, Orange II sodium salt, 2,2'-Dihydroxyazobenzene,2-(4-Hydroxyphenylazo) benzoic acid,5-[(2-Carboxyphenyl) azo]-2-hydroxybenzoic acid, Olsalazine, 5-[(4-aminophenyl) azo]-2-hydroxy-Benzoic acid as hydrogen bonding agent. After stirring and reflux overnight, the nanoparticles were collected by centrifuging. Using ethanol wash to remove hydrogen bonding agent results in self-assembly monodisperse hollow nanoparticles with tunable hollow cavity size and internal surface reactivity. The resulting nanoparticles are redispersed in chloroform and mixed with m-TOR and paclitaxel (ranging from 3:1 to 1:3 by weight) solution in the same solvent; the balloon can be coated with this solution by dip- or spin-coating method to the balloon surface until target drug weight achieved with m-TOR and paclitaxel are hydrogen bonding with this self-assembly hollow nanoparticle.

[0451] After coating, the balloons were air dried first then were placed in a vacuum chamber to remove the solvent. The balloons were then tri-folded before putting on the protective sheath. The balloon catheters were then inserted in coils and packaged. The pouches were sterilized.

Example 29: Preparation of Anticoagulant and/or mTOR coated sleeve in a covered stent

[0452] A stent covered by a sleeve can have a polymer coating containing anticoagulant and/or a combination with mTOR on top, part of, and /or adjacent to the sleeve made from polymer selected from non-degradable polymers such as polytetrafluoroethylene, fluorinated ethylene propylene, Dacron, polyethylene terephthalate, polyurethanes, polycarbonate, polypropylene, Pebax, polyethylene and biological polymers such as modified cellulose, collagen, fibrin, and elastin, and biodegradable polymer such as poly(alpha-hydroxy acid), poly-L-lactide (PLLA), poly-D-lactide (PDLA), polyglycolide (PGA), polydioxanone, polycaprolactone (PCL), polygluconate, polylactic acid-polyethylene oxide copolymers, poly(hydroxybutyrate), polyanhydride, polyphosphoester, or poly(aminoacides), knitted or weaved fabric material or film material which have been previously cast by brushing, dipping, electrospun or electrospray technique or other means onto the metallic bare or polymer stent, or those known in the art, which coated with anticoagulant and/or mTOR. The sleeve surface can be porous or non-porous.

[0453] The sleeve can be infused with a polymer coating in a solvent solution with anticoagulant such as Apixaban, Rivaroxaban, Argatroban or a combination with mTOR such as rapamycin, everolimus, biolimus, temsirolimus, ridaforolimus, zotarolimus, myolimus and novolimus drug solution into the said sleeve and said solvent can evaporate leaving either polymer coating or drug in the pores of the sleeve. This process also applicable to stent graft.

[0454] Poly (L- lactide acid-co- glycolic acid) polymer is dissolved into dichloromethane at room temperature and vortex until the polymer is uniformly dissolved/dispersed.

Anticoagulant (Apixaban or Rivaroxaban & Argatroban) is placed in a vial and dissolved in dichloromethane/Methanol at room temperature and vortex until all the drug is uniformly dissolved/dispersed.

[0455] Each polymer solution and each drug solutions is combined anticoagulant (Apixaban or Rivaroxaban) and Argatroban and/or mTOR with weight ratio 1 to 1 or other ratio) to poly (L- lactide acid-co- glycolic acid) by weight ratio was 3:1 or other ratio according to the target drug dose.

[0456] The sleeve can optionally undergo surface treatment if the surface is not porous (i.e. plasma treatment or other friction surface treatment). After surface treatment, the coating could be spray coated or dip coated. The coating can be inside the sleeve or dipped onto the sleeve or coated on the sleeve.

[0457] When spray coated, a microprocessor controlled ultrasonic sprayer is used to coat the sleeve containing drug/excipient solution to the entire surface of the implant. After coating, the sleeve is placed in a 70°C oven for about 2 hours to remove the residue solvent. The covered stent is then mounted on the delivery catheter and then is inserted in a coil and packaged. The pouches were sterilized.

Example 30: Preparation of m-TOR organic formulation with self-emulsifying agent for use in eye injection

[0458] Novolimus at concentration of about 200 mg/mL was dissolved in 95% PEG 300 NF with 5% Ethanol 190 Proof USP. Myolimus at concentration of about 175 mg/mL was dissolved in 95% PEG 300 NF with 5% Ethanol 190 Proof USP. These drug solutions were injected (10 to 23 µL) into rabbit and swine eye with drug concentrations in target eye tissues and whole blood as shown in Tables 7A, 7C, 8A, and 8C. Drug concentrations in the eye tissues were measured using High Performance Liquid Chromatography (HPLC). Drug concentrations in the blood were measured using HPLC-Mass Spectrometry (HPLC-MS). In addition, ocular pressure was measured during study as shown in Tables 7B and 8B. Ocular pressure was measured using a Model 30 Classic™ pneumatonometer.

[0459] Table 7A: Tissue Concentrations from Novolimus Formulation I Injection into Rabbit Eye and Swine Eye

Animal Group	Formulation (Dose in mg)	Time Point (Days)	Average Amount of Drug per Tissue Mass (ng/g)			
			Vitreous	Retina	Choroid	Sclera
G	Formulation I	1	25328	10948	3264	760

rabbit	(20µl; <u>4mg</u>)					
C and D rabbits	Formulation I (20µl; <u>4mg</u>)	72	332	76	92	52
A and B rabbits	Formulation I (10µl; <u>2mg</u>)	72	2	4	8	4
E swine	Formulation I (20µl; <u>4mg</u>)	1	101621	135598	44736	4331

[0460] Table 7B: Intraocular Pressure of Rabbit Eye from Novolimus Formulation I Injection

Animal Group	Formulation (Dose in mg)	Intraocular Pressure (mm Hg)			
		Day 1		Later Time Point	
		pre-treatment*	post-treatment*	(Day)	
G rabbit	Formulation I (20µl; <u>4mg</u>)	12.0	13.0	<u>Day 2</u> 24.3	
C and D rabbits	Formulation I (20µl; <u>4mg</u>)	17.0 (Control)	26.5 (Control)	<u>Day 8/10</u> 29.3 (Control)	<u>Day 71</u> 25.5 (Control)
		16.8 (Test Article)	29.5 (Test Article)	10.8 (Test Article)	26.5 (Test Article)
A and B rabbits	Formulation I (10µl; <u>2mg</u>)	21.5 (Control)	25.3 (Control)	<u>Day 8/10</u> 27.3 (Control)	<u>Day 71</u> 21.0 (Control)
		23.0 (Test Article)	24.0 (Test Article)	23.5 (Test Article)	24.3 (Test Article)

* Under anesthetic and dilation

[0461] Table 7C: Whole Blood Concentration from Novolimus Formulation I Injection into Rabbit Eye

Animal Group	Formulation (Dose in mg)	Time Point (Days)	Whole Blood Concentration (ng/mL)
G rabbit	Formulation I (20µl; <u>4mg</u>)	1	5.23
C and D rabbits	Formulation I (20µl; <u>4mg</u>)	72	0.639 (average of two)
A and B rabbits	Formulation I (10µl; <u>2mg</u>)	72	0.120 (average of two)

[0462] As shown in Tables 7A and 7C, Novolimus with formulation I for eye injection with 2mg to 4 mg dose efficiently delivered to eyes and generated Novolimus tissue concentration range from 135598 ng/g to 760 ng/g at day 1 and generated Novolimus tissue concentration range from 332 ng/g to 2 ng/g at day 72. The blood concentration was 5.23 ng/ml at day 1 and 0.120 ng/ml to 0.639 ng/ml at day 72. Both the tissue concentration and blood concentration were well within the therapeutically effective dose range.

[0463] Table 8A: Tissue Concentrations from Myolimus Formulation I Injection into Rabbit Eye

Animal Group	Formulation (Dose in mg)	Time Point (Days)	Average Amount of Drug per Tissue Mass (ng/g)			
			Vitreous	Retina	Choroid	Sclera
J	Formulation I (20µl; <u>3.5mg</u>)	1	877017	2070	12099	24928
F	Formulation I (23µl; <u>4.0mg</u>)	72	480072	9424	3114	209
E	Formulation I (10µl; <u>1.75mg</u>)	72	275793	17254	22206	3293

[0464] Table 8B: Intraocular Pressure of Rabbit Eye from Myolimus Formulation I Injection

Animal Group	Formulation (Dose in mg)	Intraocular Pressure (mm Hg)			
		Day 1		Later Time Point (Day)	
		pre-treatment*	post-treatment*		
J	Formulation I (20µl; <u>3.5mg</u>)	14.5	29.0	<u>Day 2</u> 25.0	
F	Formulation I (23µl; <u>4.0mg</u>)	19.8	26.5	<u>Day 8/10</u> 25.5	<u>Day 71</u> 25.5
E	Formulation I (10µl; <u>1.75mg</u>)	22.8	28.0	<u>Day 8/10</u> 22.8	<u>Day 71</u> 26.0

* Under anesthetic and dilation

[0465] Table 8C: Whole Blood Concentration from Myolimus Formulation I Injection into Rabbit Eye

Animal Group	Formulation (Dose in mg)	Time Point (Days)	Whole Blood Concentration (ng/mL)
J	Formulation I (20µl; <u>3.5mg</u>)	1	1.25

Animal Group	Formulation (Dose in mg)	Time Point (Days)	Whole Blood Concentration (ng/mL)
F	Formulation I (23µl; <u>4.0mg</u>)	72	0.177
E	Formulation I (10µl; <u>1.75mg</u>)	72	0.155

As shown in Tables 8A and 8C, Myolimus with formulation I for eye injection with 1.75 mg to 4 mg dose efficiently delivered to eyes and generated Myolimus tissue concentration range from 877017 ng/g to 2070 ng/g at day 1 and generated Myolimus tissue concentration range from 480072 ng/g to 209 ng/g at day 72. The blood concentration was 1.25 ng/ml at day 1 and 0.155 ng/ml at day 72. Both the tissue concentration and blood concentration were well within the therapeutically effective dose range.

Example 31: Preparation of m-TOR organic formulation with self-emulsifying agent in saline for use in eye injection.

[0466] Novolimus was dissolved in 95% PEG 300 NF with 5% Ethanol 190 Proof USP initially. Prior to injection into animal, this organic formulation with self-emulsifying agent was diluted with saline such that the final composition of this formulation was about 40 mg/mL of Novolimus in 63.3% PEG 300 NF, 3.3% Ethanol 190 Proof USP, and 33.3% saline. Myolimus was dissolved in 95% PEG 300 NF with 5% Ethanol 190 Proof USP initially. Prior to injection into animal, this organic formulation with self-emulsifying agent was diluted with saline such that the final composition of this formulation was about 4 mg/mL of Myolimus in 63.3% PEG 300 NF, 3.3% Ethanol 190 Proof USP, and 33.3% saline. These drug solutions were injected (20 to 50 µL) into rabbit's eye and swine's eye with the drug concentrations in target eye tissues and whole blood as shown in Tables 9A, 9C, 10A, and 10C. Drug concentrations in the eye tissues were measured using HPLC. Drug concentrations in the blood were measured using HPLC-MS. In addition, ocular pressure was measured during study as shown in Tables 9B and 10B. Ocular pressure was measured using a Model 30 Classic™ pneumatonometer.

[0467] Table 9A: Tissue Concentrations from Novolimus Formulation II Injection into Rabbit Eye and Swine Eye

Animal Group	Formulation (Dose in mg)	Time Point (Days)	Average Amount of Drug per Tissue Mass (ng/g)			
			Vitreous	Retina	Choroid	Sclera
H rabbit	Formulation II (50µl; <u>2mg</u>)	1	60554	32338	3534	7207

N rabbit	Formulation II (50µl; <u>2mg</u>)	37	1038	1176	591	236
A rabbit	Formulation II (20µl; <u>0.8mg</u>)	1	80991	77291	22411	3782
C rabbit	Formulation II (20µl; <u>0.8mg</u>)	36	80	228	265	64
B rabbit	Formulation II (50µl; <u>2mg</u>)	1	167460	182312	85417	14610
F swine	Formulation II (50µl; <u>2mg</u>)	1	70509	86662	45047	1150
H swine	Formulation II (50µl; <u>2mg</u>)	36	314	469	455	70
J swine	Formulation II (50µl; <u>2mg</u>)	72	5	3	12	10

Table 9B: Intraocular Pressure of Rabbit Eye from Novolimus Formulation II Injection

Animal Group	Formulation (Dose in mg)	Intraocular Pressure (mm Hg)			
		Day 1		Later Time Point (Day)	
		pre-treatment*	post-treatment*		
H rabbit	Formulation II (50µl; <u>2mg</u>)	14.0	36.5	<u>Day 2</u> 15.3	
N rabbit	Formulation II (50µl; <u>2mg</u>)	18.0	40.3	<u>Day 8/10</u> 15.5	<u>Day 36</u> 31.0

* Under anesthetic and dilation

[0468] Table 3C: Whole Blood Concentration from Novolimus Formulation II Injection into Rabbit Eye and Swine Eye

Animal Group	Formulation (Dose in mg)	Time Point (Days)	Whole Blood Concentration (ng/mL)
H rabbit	Formulation II (50µl; <u>2mg</u>)	1	31.9 (average of two)
N rabbit	Formulation II (50µl; <u>2mg</u>)	37	4.88
C rabbit	Formulation II (20µl; <u>0.8mg</u>)	36	0.895 (average of two)
H swine	Formulation II (50µl; <u>2mg</u>)	36	1.05

[0469] As shown in Tables 9A and 9C, Novolimus with formulation II for eye injection with 0.8 mg to 2 mg dose efficiently delivered to eyes and generated Novolimus tissue concentration range from 182312 ng/g to 1150 ng/g at day 1 and efficiently generated Novolimus tissue concentration range from 1176 ng/g to 64 ng/g at day 36 or day 37 and efficiently generated Novolimus tissue concentration range from 12 ng/g to 3 ng/g at day 72. The blood concentration was 31.9 ng/ml at day1 and 0.895 ng/ml to 4.88 ng/ml at day 36 or day 37. Both the tissue concentration and blood concentration were well within the therapeutically effective dose range.

[0470] Table 10A: Tissue Concentrations from Myolimus Formulation II Injection into Rabbit Eye

Animal Group	Formulation (Dose in mg)	Time Point (Days)	Average Amount of Drug per Tissue Mass (ng/g)			
			Vitreous	Retina	Choroid	Sclera
K	Formulation II (20µl; <u>0.2mg</u>)	1	33524	7583	6202	3525

[0471] Table 10B: Intraocular Pressure of Rabbit Eye from Myolimus Formulation II Injection into Rabbit Eye

Animal Group	Formulation (Dose in mg)	Intraocular Pressure (mm Hg)		
		Day 1		Day 2
		pre-treatment*	post-treatment*	
K	Formulation II (20µl; <u>0.2mg</u>)	15.8	47.3	12.3

* Under anesthetic and dilation

[0472] Table 10C: Whole Blood Concentration from Myolimus Formulation III Injection into Rabbit Eye and Swine Eye (Studies NP100203* and NP100904)

Animal Group	Formulation (Dose in mg)	Time Point (Days)	Whole Blood Concentration (ng/mL)
K	Formulation II (20µl; <u>0.2mg</u>)	1	0.652

As shown in Tables 10A and 10C, Myolimus with formulation II for eye injection with 0.2 mg dose efficiently delivered to eyes and generated Myolimus tissue concentration range from 33524 ng/g to 3525 ng/g at day 1 and with blood concentration of 0.652 ng/ml at day 1. Both the

tissue concentration and blood concentration were well within the therapeutically effective dose range.

Example 32: Preparation of m-TOR formulation with combination of self-emulsifying and complexing agents in saline for use in eye injection

[0473] Novolimus was dissolved in 36% PEG 400 NF with 9% absolute ethanol and 55% of an aqueous sodium phosphate buffer (pH 5) with Capitsol(35% w/v). The final Novolimus drug concentration was 2.7 mg/mL. Myolimus was dissolved in 45% PEG 400 NF with 10% absolute ethanol and 45% of an aqueous sodium phosphate buffer (pH 5) with Capitsol (35% w/v). The final Myolimus drug concentration was 0.9 mg/mL. These drug solutions were injected (50 µL) into rabbit's eye and swine's eye with the drug concentrations in target eye tissues and whole blood as shown in Tables 11A, 11C, 12A, and 12C. Drug concentrations in the eye tissues were measured using HPLC. Drug concentrations in the blood were measured using HPLC-MS. In addition, ocular pressure was measured during study as shown in Tables 11B and 12B. Ocular pressure was measured using a Model 30 Classic™ pneumatonometer.

[0474] Table 11A: Tissue Concentrations from Novolimus Formulation III Injection into Rabbit Eye and Swine Eye

Animal Group	Formulation (Dose in mg)	Time Point (Days)	Average Amount of Drug per Tissue Mass (ng/g)			
			Vitreous	Retina	Choroid	Sclera
I rabbit	Formulation III (50µl <u>0.14 mg</u>)	1	757	1591	2519	4022
O rabbit	Formulation III (50µl <u>0.14 mg</u>)	37	1.4	3.2	32	19
G swine	Formulation III (50µl <u>0.14 mg</u>)	1	10137	131076	32010	334
I swine	Formulation III (50µl <u>0.14 mg</u>)	36	57	39	60	14

[0475] Table 11B: Intraocular Pressure of Rabbit Eye from Novolimus Formulation III Injection

Animal Group	Formulation (Dose in mg)	Intraocular Pressure (mm Hg)		
		Day 1		Later Time Point (Day)
		pre-treatment*	post-treatment*	
I rabbit	Formulation III (50µl <u>0.14 mg</u>)	16.0	36.3	<u>Day 2</u> 16.3

O rabbit	Formulation III (50µl <u>0.14 mg</u>)	23.5	48.3	<u>Day 8/10</u> 20.0	<u>Day 36</u> 22.3
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* Under anesthetic and dilation

[0476] Table 11C: Whole Blood Concentration from Novolimus Formulation III Injection into Rabbit Eye and Swine Eye

Animal Group	Formulation (Dose in mg)	Time Point (Days)	Whole Blood Concentration (ng/mL)
I rabbit	Formulation III (50µl <u>0.14 mg</u>)	1	5.75 (average of two)
O rabbit	Formulation III (50µl <u>0.14 mg</u>)	37	BQL [†]
G swine	Formulation III (50µl <u>0.14 mg</u>)	1	5.23
I swine	Formulation III (50µl <u>0.14 mg</u>)	36	BQL [†]

[†] < 5 ng/mL (Novolimus)

[0477] As shown in Tables 11A and 11C, Novolimus with formulation III for eye injection with 0.14 mg dose efficiently delivered to eyes and generated Novolimus tissue concentration range from 131076 ng/g to 334 ng/g at day 1 and efficiently generated Novolimus tissue concentration range from 60 ng/g to 14 ng/g at day 36 or day 37. The blood concentration was 5.23 ng/ml to 5.75 ng/ml at day 1. Both the tissue concentration and blood concentration were well within the therapeutically effective dose range.

[0478] Table 12A: Tissue Concentrations from Myolimus Formulation III Injection into Rabbit Eye

Animal Group	Formulation (Dose in mg)	Time Point (Days)	Average Amount of Drug per Tissue Mass (ng/g)			
			Vitreous	Retina	Choroid	Sclera
L	Formulation III (50µl <u>0.05 mg</u>)	1	843	5314	5033	18923
D	Formulation III (50µl <u>0.05 mg</u>)	36	6	57	81	38

[0479] Table 12B: Intraocular Pressure of Rabbit Eye from Myolimus Formulation III Injection

Animal Group	Formulation (Dose in mg)	Time Point	Intraocular Pressure (mm Hg)	
			Day 1	Day 2

		(Days)	pre-treatment*	post-treatment*	
L	Formulation III (50µl 0.05 mg)	1	16.3	38.3	13.3

* Under anesthetic and dilation

[0480] Table 12C: Whole Blood Concentration from Myolimus Formulation III Injection into Rabbit Eye

Animal Group	Formulation (Dose in mg)	Time Point (Days)	Whole Blood Concentration (ng/mL)
L	Formulation III (50µl 0.05 mg)	1	0.708
D	Formulation III (50µl 0.05 mg)	36	BQL [†]

[†] < 0.25 ng/mL (Myolimus)

As shown in Tables 12A and 12C, Myolimus with formulation III for eye injection with 0.05 mg dose efficiently delivered to eyes and generated Myolimus tissue concentration range from 18923 ng/g to 843 ng/g at day 1 and range from 81 ng/g to 6 ng/g at day 36 and with blood concentration of 0.708 ng/ml at day 1. Both the tissue concentration and blood concentration were well within the therapeutically effective dose range.

Example 33: Preparation of direct factor Xa inhibitor injection formulation for ophthalmic application.

[0481] Direct factor Xa inhibitor (such as Apixaban or Rivaroxaban) at target concentration in USP proof alcohol dissolves in PEG and/or copolymer of PEG with USP proof alcohol. The drug solution is injected (10 to 20 µL) into eye. This formulation, following local application into the eye, is sufficient to provide a therapeutically effective dose of the direct factor Xa inhibitor at day 1 through day 72 or more. Some formulations are sufficient to generate a therapeutically effective dose of the direct factor Xa lasting through 30 days locally, 60 days, 90 days, and even 6 months while below in the systemic circulation therapeutic levels at or after day 1, at day 2, at day 3, at day 7, and even longer from introduction in the eye.

Example 34: Preparation of direct factor Xa inhibitor suspension formulation for ophthalmic application with excipients.

[0482] Direct factor Xa inhibitor (such as Apixaban or Rivaroxaban) at target concentration formulated as ophthalmic suspension or solution that contains excipients (all, less, or more) such as suspending agents such as carboxymethylcellulose, hypromellose, povidone, buffers, preservatives, surfactants, caffeine, nicotinamide derivatives and complex/encapsulating agents such as derivatives of cyclodextrin (hydroxylpropyl cyclodextrin, captisol). This formulation can use as short release or longer release depending on the delivery method. Some of the

delivery methods are sufficient to generate a therapeutically effective dose of the direct factor Xa lasting through 30 days locally, 60 days, 90 days, and even 6 months while negligible in the systemic circulation at day 1, at day 2, at day 3, at day 7, and even longer.

Example 35: Preparation of direct factor Xa inhibitor lipid emulsions formulation for ophthalmic application with excipients.

[0483] Direct Factor Xa inhibitor (such as Apixaban or Rivaroxaban) at target concentration formulated as lipid emulsions (all, less, or more) such as medium chain triglycerides with polymeric surface modifiers such as chitosan (CS) and poloxamer 407 (P407) with other components such as surfactants, caffeine, and/or nicotinamide derivatives. This formulation can use as short release or longer release depending on the delivery method. This formulation, following local application into the eye, is sufficient to provide a therapeutically effective dose of the direct factor Xa inhibitor at day 1 through day 72. Some of the delivery methods are sufficient to generate a therapeutically effective dose of the direct factor Xa lasting through 30 days locally, 60 days, 90 days, and even 6 months while negligible in the systemic circulation at day 1, at day 2, at day 3, at day 7, and even longer.

Example 36: Preparation of direct factor Xa inhibitor combined with m-TOR suspension formulation for ophthalmic application with excipients.

[0484] Direct Factor Xa inhibitor (such as Apixaban or Rivaroxaban) at target concentration formulated with m-TOR (such as Novolimus or Myolimus) as ophthalmic suspension or solution that contains excipients (all, less, or more), suspending agents such as carboxymethylcellulose, hypromellose, povidone, buffers, preservatives, surfactants, caffeine, nicotinamide derivatives and complex/encapsulating agents such as derivatives of cyclodextrin (hydroxypropyl cyclodextrin, captisol). This formulation can use as short release or longer release depending on the delivery method. Some of the delivery method applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the direct factor Xa inhibitor and m-TOR at day 1 through day 72. Some of formulation generates sufficient therapeutically effective dose of the direct factor Xa inhibitor and m-TOR lasting through 30 days locally, 60 days, 90 days, and even 6 months while below the systemic circulation therapeutic level at day 1, at day 2, at day 3, or at day 7.

Example 37: Preparation of direct factor Xa inhibitor combined with m-TOR lipid emulsions formulation for ophthalmic application with excipients.

[0485] Direct Factor Xa inhibitor (such as Apixaban or Rivaroxaban) at target concentration formulated with m-TOR (such as Novolimus or Myolimus) in lipid emulsions (all, less, or more) such as medium-chain medium chain triglycerides with polymeric surface modifiers such as chitosan (CS) and poloxamer 407 (P407) with other components such as surfactants, caffeine,

nicotinamide derivatives. This formulation can use as short release or longer release depending on the delivery method. Some of the delivery method applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the direct factor Xa inhibitor and m-TOR at day 1 through day 72. Some of formulation generates sufficient therapeutically effective dose of the direct factor Xa inhibitor and m-TOR lasting through 30 days locally, 60 days, 90 days, and even 6 months below the systemic circulation therapeutic level at day 1, at day 2, at day 3, at day 7.

Example 38: Preparation of direct factor Xa inhibitor combined with aflibercept formulation for ophthalmic application with excipients.

[0486] Direct Factor Xa inhibitor (such as Apixaban or Rivaroxaban) at target concentration formulated (such as Apixaban or Rivaroxaban) will be formulated with Eylea (aflibercept) with excipients (all, less, or more) such as buffers, surfactant (such as tween), NaCl, sugar (such as trehalose), amino acid (such as arginine) and complex/encapsulating agents such as derivatives of cyclodextrin (hydroxylpropyl cyclodextrin, captisol). This formulation can use as short release or longer release depending on the delivery method. Some of the delivery method applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the direct factor Xa inhibitor and aflibercept at day 1 through day 72. Some of formulation generates sufficient therapeutically effective dose of the direct factor Xa inhibitor and aflibercept lasting through 30 days locally, 60 days, 90 days, and even 6 months while below the systemic circulation therapeutic level at day 1, at day 2, at day 3, or at day 7.

Example 39: Preparation of direct factor Xa inhibitor combined with Ranibizumab formulation for ophthalmic application with excipients.

[0487] Direct Factor Xa inhibitor (such as Apixaban or Rivaroxaban) at target concentration formulated with Ranibizumab with excipients (all, less, or more) such as buffers, surfactant (such as tween), NaCl, sugar (such as trehalose), amino acid (such as arginine) and complex/encapsulating agents such as derivatives of cyclodextrin (hydroxylpropyl cyclodextrin, captisol). This formulation can use as short release or longer release depending on the delivery method. Some of the delivery method applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the direct factor Xa inhibitor and Ranibizumab at day 1 through day 72. Some of the delivery method generates sufficient therapeutically effective dose of the direct factor Xa inhibitor and Ranibizumab lasting through 30 days locally, 60 days, 90 days, and even 6 months while below the systemic circulation therapeutic level at day 1, at day 2, at day 3, or at day 7.

Example 40: Preparation of direct factor Xa inhibitor eye drop formulation for ophthalmic application with excipients.

[0488] Direct Factor Xa inhibitor (such as Apixaban or Rivaroxaban) at target concentration formulated in sterile water and/or artificial tears and/or buffer that contains (all, less, or more) suspending agents such as carboxymethylcellulose, hypromellose, povidone, complex/ encapsulating agents such as derivatives of cyclodextrin (hydroxylpropyl cyclodextrin, captisol) and other components such as surfactants, caffeine, nicotinamide derivatives as needed. This formulation applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the direct factor Xa inhibitor while negligible in the systemic circulation.

Example 41: Preparation of direct factor Xa inhibitor eye drop polymeric micelles formulation for ophthalmic application with excipients.

[0489] Direct Factor Xa inhibitor (such as Apixaban or Rivaroxaban) at target concentration formulated in sterile water and/or artificial tears and/or buffer that contains (all, less, or more) polymeric micelles such as poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) and other components such as surfactants, caffeine, nicotinamide derivatives. This formulation applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the direct factor Xa inhibitor while negligible in the systemic circulation.

Example 42: Preparation of Anticoagulant (rivaroxaban, argatroban, or Apixaban) drug coated contact lens

[0490] Poly (methyl methacrylate) or Poly (2-hydroxyethyl methacrylate) or similar polymers is dissolved into proper solvent at room temperature and vortex or stir until the polymer have been uniformly dissolved/dispersed. Anticoagulant (rivaroxaban, argatroban, or Apixaban or combinations) is dissolved into solvent at room temperature and vortex/ or stir until the drug is uniformly dissolved/dispersed. Each polymer solution and each drug solution are combined at a target weight ratio according to the target drug dose for each drug. This mixed solution can be coated on the commercially available contact lens or commercially available therapeutically soft lens or commercially available therapeutically scleral lens by dipping method or spray method. If by a spray method, a microprocessor-controlled ultrasonic sprayer will be used to coat each of the commercially available contact lens or commercially available therapeutically soft lens or commercial available therapeutically scleral lens until a uniformly dispense with each of the drug/polymer matrix . After coating, the contact lens will be placed in a vacuum chamber to remove the solvent. The contact lens is then packaged into a pouch. The pouches are sterilized. This drug eluting lens can be used either at night or daytime depending on the target using drug release profile. This drug delivery system comprising anticoagulant

(rivaroxaban, argatroban, or Apixaban) coated contact lens could have a dispersed particle size less than about 200 nm, an anticoagulant (rivaroxaban, argatroban, or Apixaban) is nanoencapsulated in a material and is capable of diffusion into and migration through contact lens and into the post-lens tear film when contact lens is placed on the eye. This contact lens applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the direct factor Xa inhibitor and/or the direct factor IIa inhibitor while below the systemic circulation therapeutic level.

Example 43: Preparation of Anticoagulant (rivaroxaban, argatroban, or Apixaban) combined with metformin drug coated contact lens

[0491] Poly (methyl methacrylate) or Poly (2-hydroxyethyl methacrylate) or similar polymer is dissolved into proper solvent at room temperature and vortex or stir until the polymer have been uniformly dissolved/dispersed. Anticoagulant (rivaroxaban, argatroban, or Apixaban or combinations) and metformin or metformin.HCl is dissolved into proper solvent at room temperature and vortex/ or stir until the drug is uniformly dissolved/dispersed. Each polymer solution and each drug solution are combined at a target weight ratio according to the target drug dose for each drug. This mixed solution can be coated on the commercially available contact lens or commercially available therapeutically soft lens or commercially available therapeutically scleral lens by dipping method or spray method. If by a spray method, a microprocessor-controlled ultrasonic sprayer will be used to coat each of the commercially available contact lens or commercially available therapeutically soft lens or commercially available therapeutically scleral lens until a uniformly dispense with each of the drug/polymer matrix. After coating, the contact lens will be placed in a vacuum chamber to remove the solvent. The contact lenses are then packaged into a pouch. The pouches are sterilized. This drug eluting lens can be used either at night or daytime depending on the target using drug release profile. This drug delivery system comprising anticoagulant (rivaroxaban, argatroban, or Apixaban) combined with metformin coated contact lens could have a dispersed particle size less than about 200 nm, anticoagulant (rivaroxaban, argatroban, or Apixaban) with metformin are nanoencapsulated in a material and are capable of diffusion into and migration through contact lens and into the post-lens tear film when contact lens is placed on the eye. This contact lens applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the direct factor Xa inhibitor/ the direct factor IIa inhibitor/metformin while negligible in the systemic circulation.

Example 44: Preparation of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban combined with 25% Capitsol in citrate for application in ophthalmic injection or eye drops

[0492] 6600 mg Capitsol was dissolved in 15mM sodium citrate in a 25.0 mL volumetric flask and the solution was sonicated and mixed until clear. This solution labeled as 25% (250 mg/mL) Captisol (Solution A). 100 mg of apixaban was weighed into a separate vial and 20.0 mL of the 25% Captisol solution(Solution A) prepared above was added. This 20 mL of apixaban solution was sonicated with heat at 40°C for approximately 30 minutes to maximally dissolve the apixaban. This “saturated apixaban solution” was cooled and filtered to create a clear solution of apixaban in 25% Captisol (Solution B). 51 mg of argatroban was added to a separate vial, and 10 mL of the saturated apixaban solution(Solution B) prepared above was added to the same vial. The final combination solution was sonicated with heat at 40°C for approximately 30 minutes until clear. The cooled solution was filtered, and pH as measured was pH 7.5. The concentration of the anticoagulants were measured as 1.5 mg/mL of apixaban and 5.0 mg/mL of argatroban. These drug solutions are injected (10 to 50 µL) into rabbit’s eye or swine’s eye with the drug concentrations in target eye tissues and whole blood. Drug concentrations in the eye tissues and in the blood is measured. In addition, ocular pressure is measured during study.

[0493] If formulated as eye drops, other than preservatives, the other inactive ingredient may be added to adjust drug concentration and tonicity such as buffers for adjusting pH, drug stabilizers such as anti-oxidants, solubility enhancers, viscosity adjusters, and the like. These inactive ingredients include but not limit to benzalkonium chloride, chlorobutanol, sodium dehydroacetate, phenethyl alcohol, benzethonium chloride, and the like; Boric Acid, Edetate Disodium, Potassium Chloride, Sodium Borate, Sodium Chloride and the like; methyl cellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methyl cellulose, polyvinyl alcohol and the like; polyoxyethylene hydrogenated castor oil, polyethylene glycol, polysorbate 80, polyoxyethylene monostearate and the like; sodium edetate and citric acid, sodium edetate and sodium bisulfite and the like.

[0494] This formulation applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the drug while below the systemic circulation therapeutic level.

Example 45: Preparation of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban combined with 30% Capitsol in citrate for application in ophthalmic injection or eye drops

[0495] 7900 mg Capitsol was dissolved in 15mM sodium citrate in a 25.0 mL volumetric flask, and the solution was sonicated and mixed until clear. This solution labeled as 30%

(300mg/mL) Captisol(Solution C). 100 mg of apixaban was weighed into a separate vial and 20.0 mL of the 30% Captisol solution (Solution C) prepared above was added. This 20 mL of apixaban solution was sonicated with heat at 40°C for approximately 30 minutes to maximally dissolve the apixaban. This “saturated apixaban solution” was cooled and filtered to create a clear solution of apixaban in 30% Captisol(Solution D). 51 mg of argatroban was added to a separate vial, and 10 mL of the saturated apixaban solution prepared above (Solution D) was added to the same vial. The final combination solution was sonicated with heat at 40°C for approximately 30 minutes until clear. The cooled solution was filtered, and pH as measured was pH 7.3. The concentration of the anticoagulants were measured as 2.0 mg/mL of apixaban and 5.0 mg/mL of argatroban. These drug solutions are injected (10 to 50 µL) into rabbit’s eye or swine’s eye with the drug concentrations in target eye tissues and whole blood. Drug concentrations in the eye tissues and in the blood is measured. In addition, ocular pressure is measured during study.

[0496] If formulated as eye drops, other than preservatives, the other inactive ingredient may be added to adjust drug concentration and tonicity such as buffers for adjusting pH, drug stabilizers such as anti-oxidants, solubility enhancers, viscosity adjusters, and the like. These inactive ingredients include but not limit to benzalkonium chloride, chlorobutanol, sodium dehydroacetate, phenethyl alcohol, benzethonium chloride, and the like; Boric Acid, Edetate Disodium, Potassium Chloride, Sodium Borate, Sodium Chloride and the like; methyl cellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methyl cellulose, polyvinyl alcohol and the like; polyoxyethylene hydrogenated castor oil, polyethylene glycol, polysorbate 80, polyoxyethylene monostearate and the like; sodium edetate and citric acid, sodium edetate and sodium bisulfite and the like.

[0497] This formulation applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the drug while negligible in the systemic circulation.

Example 46: Preparation of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban combined with 40% Captisol in citrate for application in ophthalmic injection or eye drops

[0498] 10,560 mg Captisol was dissolved in 15mM sodium citrate in a 25.0 mL volumetric flask, and the solution was sonicated and mixed until clear. This solution labeled as 40% (400 mg/mL) Captisol(Solution E). 100.2 mg of apixaban was weighed into a separate vial and 20.0 mL of the 40% Captisol solution(Solution E) prepared above was added. This 20 mL of apixaban solution was sonicated with heat at 40°C for approximately 30 minutes to maximally dissolve the apixaban. This “saturated apixaban solution” was cooled and filtered to create a clear solution of apixaban in 40% Captisol (Solution F). 51 mg of argatroban was added to a

separate vial, and 10 mL of the saturated apixaban solution(Solution F) prepared above was added to the same vial. The final combination solution was sonicated with heat at 40°C for approximately 30 minutes until clear. The cooled solution was filtered, and pH as measured was pH 6.9. The concentration of the anticoagulants was measured as 3.2 mg/mL of apixaban and 5.0 mg/mL of argatroban. These drug solutions are injected (10 to 50 μ L) into rabbit's eye or swine's eye with the drug concentrations in target eye tissues and whole blood. Drug concentrations in the eye tissues and in the blood is measured. In addition, ocular pressure is measured during study.

[0499] If formulated as eye drops, other than preservatives, the other inactive ingredient may be added to adjust drug concentration and tonicity such as buffers for adjusting pH, drug stabilizers such as anti-oxidants, solubility enhancers, viscosity adjusters, and the like. These inactive ingredients include but not limit to benzalkonium chloride, chlorobutanol, sodium dehydroacetate, phenethyl alcohol, benzethonium chloride, and the like; Boric Acid, Edetate Disodium, Potassium Chloride, Sodium Borate, Sodium Chloride and the like; methyl cellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methyl cellulose, polyvinyl alcohol and the like; polyoxyethylene hydrogenated castor oil, polyethylene glycol, polysorbate 80, polyoxyethylene monostearate and the like; sodium edetate and citric acid, sodium edetate and sodium bisulfite and the like.

[0500] This formulation applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the drug while negligible in the systemic circulation.

Example 47: Preparation of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban combined with 50% Captisol in citrate for application in ophthalmic injection or eye drops

[0501] 13,200 mg Captisol was dissolved in 15mM sodium citrate in a 25.0 mL volumetric flask, and the solution was sonicated and mixed until clear. This solution labeled as 50% (500 mg/mL) Captisol(Solution G). 120.2 mg of apixaban was weighed into a separate vial and 20.0 mL of the 50% Captisol solution(Solution G) prepared above was added. This 20 mL of apixaban solution was sonicated with heat at 40°C for approximately 30 minutes to maximally dissolve the apixaban. This "saturated apixaban solution" was cooled and filtered to create a clear solution of apixaban in 50% Captisol(Solution H). 62 mg of argatroban was added to a separate vial, and 10 mL of the saturated apixaban solution (Solution H) prepared above was added to the same vial. The final combination solution was sonicated with heat at 40°C for approximately 30 minutes until clear. The cooled solution was filtered, and pH as measured was pH 7.6. The concentration of the anticoagulants were measured as 3.7 mg/mL of apixaban and 6.0 mg/mL of argatroban. These drug solutions are injected (10 to 50 μ L) into rabbit's eye

or swine's eye with the drug concentrations in target eye tissues and whole blood. Drug concentrations in the eye tissues and in the blood is measured. In addition, ocular pressure is measured during study.

[0502] If formulated as eye drops, other than preservatives, the other inactive ingredient may be added to adjust drug concentration and tonicity such as buffers for adjusting pH, drug stabilizers such as antioxidants, solubility enhancers, viscosity adjusters, and the like. These inactive ingredients include but not limit to benzalkonium chloride, chlorobutanol, sodium dehydroacetate, phenethyl alcohol, benzethonium chloride, and the like; Boric Acid, Edetate Disodium, Potassium Chloride, Sodium Borate, Sodium Chloride and the like; methyl cellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methyl cellulose, polyvinyl alcohol and the like; polyoxyethylene hydrogenated castor oil, polyethylene glycol, polysorbate 80, polyoxyethylene monostearate and the like; sodium edetate and citric acid, sodium edetate and sodium bisulfite and the like.

[0503] This formulation applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the drug while negligible in the systemic circulation.

Example 48: Preparation of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban combined with Azelastine (antihistamine) in Capitsol in citrate for application in ophthalmic injection or eye drops

[0504] In a formulation using example 18, one additional drug of azelastine hydrochloride can be added to the final vial. All the excipients and drugs can be varied within a range.

[0505] 10,560 mg Capitsol was dissolved in 15mM sodium citrate in a 25.0 mL volumetric flask, and the solution was sonicated and mixed until clear. This solution labeled as 40% (400 mg/mL) Captisol(Solution E). 100.2 mg of apixaban was weighed into a separate vial and 20.0 mL of the 40% Captisol solution(Solution E) prepared above was added. This 20 mL of apixaban solution was sonicated with heat at 40°C for approximately 30 minutes to maximally dissolve the apixaban. This "saturated apixaban solution" was cooled and filtered to create a clear solution of apixaban in 40% Captisol (Solution F). Two drugs (51 mg of argatroban and 1.0 mg of Azelastine hydrochloride) are weighed and added into the same vial, and finally 10 mL of the saturated apixaban solution (Solution F) prepared above is added to the same vial. The final 3 drug combination solution is sonicated with heat if needed until clear. The cooled solution is filtered, and pH is measured and adjusted to the suitable physiological pH's. The concentration of the anticoagulants are projected to be at 3.2 mg/mL of apixaban and 5.0 mg/mL of argatroban, and azelastine is confirmed around 0.1 mg/mL. These drug solutions are injected (10 to 50 µL) into rabbit's eye or swine's eye with the drug concentrations in target eye tissues

and whole blood. Drug concentrations in the eye tissues and in the blood is measured. In addition, ocular pressure is measured during study.

[0506] If formulated as eye drops, other than preservatives, the other inactive ingredient may be added to adjust drug concentration and tonicity such as buffers for adjusting pH, drug stabilizers such as antioxidants, solubility enhancers, viscosity adjusters, and the like. These inactive ingredients include but not limit to benzalkonium chloride, chlorobutanol, sodium dehydroacetate, phenethyl alcohol, benzethonium chloride, and the like; Boric Acid, Edetate Disodium, Potassium Chloride, Sodium Borate, Sodium Chloride and the like; methyl cellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methyl cellulose, polyvinyl alcohol and the like; polyoxyethylene hydrogenated castor oil, polyethylene glycol, polysorbate 80, polyoxyethylene monostearate and the like; sodium edetate and citric acid, sodium edetate and sodium bisulfite and the like.

[0507] This formulation applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the drug while negligible in the systemic circulation.

Example 49: Preparation of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban combined with Hydroxychloroquine in Capitsol in citrate for application in ophthalmic injection or eye drops

[0508] In a formulation using example 18, one additional drug of hydroxychloroquine sulfate can be added to the final vial. All the excipients and drugs can be varied within a range.

[0509] 10,560 mg Capitsol was dissolved in 15mM sodium citrate in a 25.0 mL volumetric flask, and the solution was sonicated and mixed until clear. This solution labeled as 40% (400 mg/mL) Captisol (Solution E). 100.2 mg of apixaban was weighed into a separate vial and 20.0 mL of the 40% Captisol solution(Solution E) prepared above was added. This 20 mL of apixaban solution was sonicated with heat at 40°C for approximately 30 minutes to maximally dissolve the apixaban. This “saturated apixaban solution” was cooled and filtered to create a clear solution of apixaban in 40% Captisol (Solution F). In a separate vial, two drugs(51 mg of argatroban and 15 mg of hydroxychloroquine sulphate) can be weighed and added to this single vial and finally 10 mL of the saturated apixaban solution (Solution F) prepared above is added to this same vial. The final 3 drug combination solution is sonicated with heat if needed until clear. The cooled solution is filtered, and pH is measured and adjusted to the suitable physiological pH's. The concentration of the anticoagulants are projected to be at 3.2 mg/mL of apixaban and 5.0 mg/mL of argatroban, and 1.5 mg/mL of hydroxychloroquine sulphate. These drug solutions are injected (10 to 50 µL) into rabbit's eye or swine's eye with the drug concentrations in target eye tissues and whole blood. Drug concentrations in the eye tissues and in the blood is measured. In addition, ocular pressure is measured during study.

[0510] If formulated as eye drops, other than preservatives, the other inactive ingredient may be added to adjust drug concentration and tonicity such as buffers for adjusting pH, drug stabilizers such as anti-oxidants, solubility enhancers, viscosity adjusters, and the like. These inactive ingredients include but not limit to benzalkonium chloride, chlorobutanol, sodium dehydroacetate, phenethyl alcohol, benzethonium chloride, and the like; Boric Acid, Edetate Disodium, Potassium Chloride, Sodium Borate, Sodium Chloride and the like; methyl cellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methyl cellulose, polyvinyl alcohol and the like; polyoxyethylene hydrogenated castor oil, polyethylene glycol, polysorbate 80, polyoxyethylene monostearate and the like; sodium edetate and citric acid, sodium edetate and sodium bisulfite and the like.

[0511] This formulation applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the drug while negligible in the systemic circulation.

Example 50: Preparation of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban combined with anti-VEGF (brolucizumab, aflibercept or ranibizumab or pegaptanib sodium or bevacizumab) in Capitsol in citrate for application in ophthalmic injection or eye drops

[0512] In a formulation as in example 18, one additional drug of commercial medications such as brolucizumab or aflibercept or ranibizumab or pegaptanib sodium or bevacizumab can be added to the final vial. All the excipients and drugs can be varied within a range.

[0513] 10,560 mg Capitsol is dissolved in 15mM sodium citrate in a 25.0 mL volumetric flask, and the solution was sonicated and mixed until clear. This solution labeled as 40% (400 mg/mL) Captisol(Solution E). 100.2 mg of apixaban was weighed into a separate vial and 20.0 mL of the 40% Captisol solution(Solution E) prepared above was added. This 20 mL of apixaban solution was sonicated with heat at 40°C for approximately 30 minutes to maximally dissolve the apixaban. This “saturated apixaban solution” was cooled and filtered to create a clear solution of apixaban in 40% Captisol (Solution F). In a separate single vial, 51 mg of argatroban and 1201mg of brolucizumab or 401mg of aflibercept or 101mg of ranibizumab or 34mg of pegaptanib sodium or 251mg bevacizumab is weighed into the same vial, and finally 10 mL of the saturated apixaban solution prepared above is added to the vial. The final 3 drug combination solution is sonicated with heat if needed until clear. The cooled solution is filtered, and pH is measured and adjusted to the suitable physiological pH's. The concentration of the anticoagulants are projected to be at 3.2 mg/mL of apixaban and 5.0 mg/mL of argatroban, and other drugs is confirmed at around 120 mg/mL of brolucizumab or 40 mg/mL of aflibercept or 10 mg/mL of ranibizumab or 3.3 mg/mL of pegaptanib sodium or 25 mg/mL bevacizumab. These drug solutions are injected (10 to 50 µL) into rabbit's eye or swine's eye with the drug

concentrations in target eye tissues and whole blood. Drug concentrations in the eye tissues and in the blood is measured. In addition, ocular pressure is measured during study.

[0514] If formulated as eye drops, other than preservatives, the other inactive ingredient may be added to adjust drug concentration and tonicity such as buffers for adjusting pH, drug stabilizers such as anti-oxidants, solubility enhancers, viscosity adjusters, and the like. These inactive ingredients include but not limit to benzalkonium chloride, chlorobutanol, sodium dehydroacetate, phenethyl alcohol, benzethonium chloride, and the like; Boric Acid, Edetate Disodium, Potassium Chloride, Sodium Borate, Sodium Chloride and the like; methyl cellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methyl cellulose, polyvinyl alcohol and the like; polyoxyethylene hydrogenated castor oil, polyethylene glycol, polysorbate 80, polyoxyethylene monostearate and the like; sodium edetate and citric acid, sodium edetate and sodium bisulfite and the like.

[0515] This formulation applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the drug while negligible in the systemic circulation.

Example 51: Preparation of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban and m-TOR self-assembly nanomicellar formulation for ophthalmic drug delivery

[0516] Anticoagulant (Apixaban, Argatroban, Rivaroxaban etc.) and m-TOR are dispersed within nano scale range from 10nm to 200nm. The most used carriers in nanomedicine include but not limit to lipids (liposomes), proteins (albumin nanoparticles), cyclic oligosaccharides (cyclodextrins), synthetic polymers (polymeric micelles, dendrimers, hydrogel), castor oil, octoxynol, and inorganic compounds (cerium oxide nanoparticles). This nanocarriers increase the solubility of hydrophobic drugs, their capability to provide sustained drug release with reduced toxicity and improved efficacy, their ability to prolong drug retention time and enhancement of drug penetration through ocular barriers, and their proficiency to direct drugs to specific tissues and cells.

[0517] Self-assembly of anticoagulant(Apixaban, Argatroban, Rivaroxaban etc.) and m-TOR can be formulated nanomicelles at target concentration using amphiphilic molecules such as surfactants, polymer amphiphiles, amphiphilic PEG-Hydrodenated castor oil, Octyxonal-40 amphiphilic polymers and some lipid molecules, which containing both hydrophilic and hydrophobic components. This allows entrapment of hydrophobic drugs.

Anticoagulant(Apixaban, Argatroban, Rivaroxaban etc.) and m-TOR can be formulated in the core of the nanomicelles using solvent evaporation-film rehydration method. PEG-Hydrogenated castor oil and Octoxynol-40 are weighed and dissolved in ethanol or methanol at 1%(w/w) to 5%(w/w) in a round bottle flask and is mixed above critical micellar concentration (CMC), then add 0.1%(w/w) to 0.5%(w/w) of ethanolic or methanolic solution of anticoagulant

(Apixaban, Argatroban, Rivaroxaban etc.) and m-TOR in the same round bottle flask. The organic solvent was evaporated at a high speed under vacuum to obtain a thin solid film of polymer and drugs. The resulting film was rehydrated with HPLC grade water and sonicated. This solution is followed by filtration through a 0.22 μm or less nylon syringe filter or the similar filter system. This results anticoagulant (Apixaban, Argatroban, Rivaroxaban etc.) and m-TOR in nanomicelles in water solution. Some of the delivery method applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the direct factor Xa inhibitor and/or the director factor IIa inhibitor and m-TOR at day 1 through day 72. Some of the delivery method generates sufficient therapeutically effective dose of the direct factor Xa inhibitor and/or director factor IIa inhibitor and m-TOR lasting through 30 days locally, 60 days, 90 days, and even 6 months while negligible in the systemic circulation at day 1, at day 2, at day 3, at day 7, and even longer.

Example 52: Preparation of Anticoagulant Apixaban crystal fine particles composition in PEG 300 for ophthalmic injection

[0518] 10 mg of apixaban powder was weighed into a vial and 50 μL of 190 proof USP grade ethanol was added first in the same vial, then 950 μL of PEG 300 (polyethylene glycol) was added to solubilize the drug. The vial was sonicated with 40°C heat for 30 minutes and then was placed in the 40°C oven overnight for 20 hours. The cloudy solution was transferred to a polyethylene centrifuge tube and centrifuged for 15 minutes to pelletize the undissolved apixaban. The clear supernatant was analyzed by HPLC and the concentration was found to be 6200 $\mu\text{g/mL}$ apixaban in 95% PEG 300 solution. This clear solution is ready for eye injection. After injection into eyes, these drugs will form a crystal fine particle in PEG 300 and the drug has extended release profiles. The crystal fine particles were verified by following.

[0519] 100 μL of the clear PEG 300 solution was added to a vial with 4.3 mL of Dulbecco's phosphate buffered saline to mimic the ophthalmic vitreous humor. The solution immediately cleared upon mixing. The solution stands overnight for 24 hours at room temperature. Upon close inspection, the apixaban had precipitated producing a small carpet of crystals on the bottom of the glass vial. The solution was then centrifuged, and the supernatant was analyzed by HPLC. The concentration was found to be 94 $\mu\text{g/mL}$ of apixaban compared to the theoretical concentration in the PBS at 141 $\mu\text{g/mL}$. Therefore 67% of the apixaban was in solution and 33 % had precipitated out to crystalline form.

[0520] This clear periocular injection which late forms fine particles containing Apixaban and enables Apixaban to deliver to the posterior segments. Apixaban can be efficiently delivered to the posterior segments (such as a retina, a choroid and an optic nerve) while scarcely injuring ophthalmic tissues by administering the fine particles containing Apixaban periocularly. This

solution after injection into a biological system could be used to extend the pharmacokinetics of the drug profile, thus creating a sustained release profile in patients.

[0521] Some of the delivery method applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the direct factor Xa inhibitor at day 1 through day 72. Some of the delivery method generates sufficient therapeutically effective dose of the direct factor Xa inhibitor lasting through 30 days locally, 60 days, 90 days, and even 6 months while negligible in the systemic circulation at day 1, at day 2, at day 3, at day 7, and even longer.

Example 53: Preparation of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban crystal fine particles composition in PEG 300 for ophthalmic injection

[0522] In a similar formulation as in Example 52, combined Anticoagulant solution of apixaban and argatroban was created by taking 400uL of the 6200 ug/mL clear supernatant of apixaban from example 52, and adding it to a vial containing 2.44 mg of argatroban. The solution was sonicated 10 minutes until clear. The clear solution was analyzed by HPLC and the concentration was found to be 6200 ug/mL apixaban and 5690ug/mL argatroban. This clear solution is ready for eye injection.. After injection into eyes, these drugs will form a crystal fine particle in PEG 300 and the drug has sustained release profiles. The crystal fine particles were verified by following.

[0523] Similar procedure can be used to verify a crystalline form of anticoagulant apixaban and argatroban as in Example 23.

[0524] This clear periocular injection comprises fine particles containing Apixaban and Argatroban and enables both anticoagulant deliver to the posterior segments. The drug can be efficiently delivered to the posterior segments (such as a retina, a choroid and an optic nerve) while scarcely injuring ophthalmic tissues by administering the fine particles containing the drug periocularly. This solution after injection into a biological system could be used to extend the pharmacokinetics of the drug profile, thus creating a sustained release profile in patients.

[0525] Some of the delivery method applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the direct factor Xa inhibitor and/or the director factor IIa inhibitor at day 1 through day 72. Some of the delivery method generates sufficient therapeutically effective dose of the direct factor Xa inhibitor and/or director factor IIa inhibitor lasting through 30 days locally, 60 days, 90 days, and even 6 months while below the systemic circulation therapeutic level at day 1, at day 2, at day 3, or at day 7.

Example 54: Preparation of Ranibizumab and Apixaban in port delivery system for sustained release of drug for ophthalmic applications

[0526] The therapeutic agent delivery device (port delivery system) combined with a formulation containing Ranibizumab and Apixaban offers a unique advantage of Ranibizumab and Apixaban to release therapeutic ingredients for extended periods of time and controlled release. The formulations described in Example 24 to Example 27 and Example 52 to Example 53 can be combined with Ranibizumab and Apixaban in the sustained release device (port delivery system). The drugs can be injected separately to the eye while the device is implanted or combined into solution vials with Ranibizumab. Further, long-acting drug delivery system with the potential to reduce treatment burden while maintaining optimal vision outcomes by enabling the continuous delivery of a customized formulation of ranibizumab and apixaban into the target tissue. The range of concentration in formulations is adjustable.

[0527] Equal parts of a solution of containing 40% Captisol and 3200 ug/mL Apixaban can be combined with 100 mg/mL Ranibizumab solution to create 1600 ug/mL Apixaban and 50 mg/mL Ranibizumab. The solution is filtered, and pH is measured and adjusted to the suitable physiological pH's. then fill into the port delivery system sustained release device.

[0528] Therapeutic agent release testing is performed by measuring the amount of therapeutic agent released by the port delivery system into a fluid representative of vitreous, maintained at 37° C in an incubator. The port delivery system is suspended in a container containing phosphate buffered saline. Periodically, the port delivery system is transferred into a new container and the concentration of therapeutic agent is measured in the fluid of the previous container. Rates are calculated from the amount of therapeutic agent released divided by the sample collection duration. The percent cumulative release is calculated from the cumulative amount of therapeutic agent divided by the amount of therapeutic agent initially filled into the therapeutic device (port delivery system).

[0529] The therapeutic agent delivery device (port delivery system) combined with a formulation containing Ranibizumab and Apixaban into the eye that follows the local dose generates sufficient therapeutically effective dose lasting through 1 day, 7 days, 14 days, 30 days locally, 60 days, 90 days, 6 months or even longer while negligible in the systemic circulation. Extended or sustained release is achieved with the therapeutic agent released from the therapeutic device after implantation.

Example 55: Preparation of Ranibizumab and Argatroban in port delivery system for sustained release of drug for ophthalmic applications

[0530] The therapeutic agent delivery device (port delivery system) combined with a formulation containing Ranibizumab and Argatroban offers a unique advantage of Ranibizumab

and Apixaban to release therapeutic ingredients for extended periods of time and controlled release. The formulations described in Example 53 can also be combined with Ranibizumab and Argatroban in the sustained release device(port delivery system). The drugs can be injected separately to the eye while the device is implanted or combined into solution vials with Ranibizumab and Argatroban. Further, long-acting drug delivery system with the potential to reduce treatment burden while maintaining optimal vision outcomes by enabling the continuous delivery of a customized formulation of ranibizumab and Argatroban into the target tissue. The range of concentration in formulations is adjustable.

[0531] Equal parts of a solution of containing 40% Captisol and 5000 ug/mL Argatroban can be combined with Ranibizumab 100 mg/mL solution to create 1600 ug/mL apixaban and 50 mg/mL Ranibizumab before instilling into the port delivery system reservoir chamber sustained delivery system.

[0532] Therapeutic agent release testing is performed by measuring the amount of therapeutic agent released by the port delivery system into a fluid representative of vitreous, maintained at 37° C in an incubator. The port delivery system is suspended in a container containing phosphate buffered saline. Periodically, the port delivery system is transferred into a new container and the concentration of therapeutic agent is measured in the fluid of the previous container. Rates are calculated from the amount of therapeutic agent released divided by the sample collection duration. The percent cumulative release is calculated from the cumulative amount of therapeutic agent divided by the amount of therapeutic agent initially filled into the therapeutic device (port delivery system).

[0533] The therapeutic agent delivery device (port delivery system) combined with a formulation containing Ranibizumab and Argatroban into the eye that follows the local dose generates sufficient therapeutically effective dose lasting through 1 day, 7 days, 14 days, 30 days locally, 60 days, 90 days, 6 months or even longer while negligible in the systemic circulation. Extended or sustained release is achieved with the therapeutic agent released from the therapeutic device after implantation.

Example 56: Preparation of Ranibizumab and Apixaban and Argatroban in port delivery system for sustained release of drug for ophthalmic applications

[0534] The therapeutic agent delivery device (port delivery system) combined with a formulation containing Ranibizumab and Apixaban and Argatroban also offers a unique advantage over all previous applications of Ranibizumab and Apixaban and Argatroban to release therapeutic ingredients for extended periods of time and controlled release. The formulations described in Example 53 to Example 54 can also be combined with Ranibizumab and Apixaban and Argatroban in the sustained release device(port delivery system). The drugs

can be injected separately to the eye while the device is implanted or combined into solution vials with Ranibizumab. Further, long-acting drug delivery system with the potential to reduce treatment burden while maintaining optimal vision outcomes by enabling the continuous delivery of a customized formulation of ranibizumab and apixaban and Argatroban into the target tissue. The range of concentration in formulations is adjustable.

[0535] Equal parts of a solution of containing 40% Captisol and 3200 ug/mL Apixaban and 5000ug/mL Argatroban can be combined with Ranibizumab 100 mg/mL solution to create 1600 ug/mL Apixaban 2500ug/mL Argatroban and 50 mg/mL Ranibizumab before instilling into the port delivery system reservoir chamber sustained release device.

[0536] Therapeutic agent release testing is performed by measuring the amount of therapeutic agent released by the port delivery system into a fluid representative of vitreous, maintained at 37° C in an incubator. The port delivery system is suspended in a container containing phosphate buffered saline. Periodically, the port delivery system is transferred into a new container and the concentration of therapeutic agent is measured in the fluid of the previous container. Rates are calculated from the amount of therapeutic agent released divided by the sample collection duration. The percent cumulative release is calculated from the cumulative amount of therapeutic agent divided by the amount of therapeutic agent initially filled into the therapeutic device (port delivery system).

[0537] The therapeutic agent delivery device (port delivery system) combined with a formulation containing Ranibizumab and Apixaban and Argatroban into the eye that follows the local dose generates sufficient therapeutically effective dose lasting through 1 day, 7 days, 14 days, 30 days locally, 60 days, 90 days, 6 months or even longer while below the systemic circulation therapeutic level. Extended or sustained release is achieved with the therapeutic agent released from the therapeutic device after implantation.

Example 57: Preparation of direct factor IIa inhibitor injection formulation for ophthalmic application.

[0538] Direct factor IIa inhibitor (such as Argatroban etc.) at target concentration in USP proof alcohol dissolves in PEG and/or copolymer of PEG with USP proof alcohol. The drug solution is injected (10 to 20 µL) into eye. This formulation, following local application into the eye, is sufficient to provide a therapeutically effective dose of the direct factor IIa inhibitor at day 1 through day 72 or more. Some formulations are sufficient to generate a therapeutically effective dose of the direct factor IIa inhibitor lasting through 30 days locally, 60 days, 90 days, and even 6 months while below in the systemic circulation therapeutic levels at or after day 1, at day 2, at day 3, at day 7, from introduction in the eye.

Example 58: Preparation of direct factor IIa inhibitor suspension formulation for ophthalmic application with excipients.

[0539] Direct factor IIa inhibitor (such as Argatroban etc.) at target concentration formulated as ophthalmic suspension or solution that contains excipients (all, less, or more) such as suspending agents carboxymethylcellulose, hypromellose, povidone, buffers, preservatives, surfactants, caffeine, nicotinamide derivatives and complex/encapsulating agents or derivatives of cyclodextrin (hydroxypropyl cyclodextrin, captisol). This formulation can use as short release or longer release depending on the delivery method. Some of the delivery methods are sufficient to generate a therapeutically effective dose of the direct factor IIa inhibitor lasting through 1 hour, 1 day, 30 days locally, 60 days, 90 days, and even 6 months while being below the systemic circulation therapeutic levels at day 1, at day 2, at day 3, at day 7.

Example 59: Preparation of Argatroban combined with 40% Captisol in citrate for application in ophthalmic injection or eye drops

[0540] 10,560 mg Captisol was dissolved in 15mM sodium citrate in a 25.0 mL volumetric flask, and the solution was sonicated and mixed until clear. This solution labeled as 40% (400 mg/mL) Captisol(Solution E). 51 mg of argatroban was weighed into a separate vial and 10.0 mL of the 40% Captisol solution(Solution E) prepared above was added. This 10 mL of argatroban solution was sonicated with heat at 40°C for approximately 30 minutes to maximally dissolve the argatroban. This “saturated argatroban solution” was cooled and filtered to create a clear solution of argatroban in 40% Captisol (Solution H) and pH is measured and adjusted to the suitable physiological pH's. The concentration of the anticoagulants was 5.0 mg/mL of argatroban. These drug solutions are injected (10 to 50 µL) into rabbit's eye or swine's eye with the drug concentrations in target eye tissues and whole blood. Drug concentrations in the eye tissues and in the blood is measured. In addition, ocular pressure is measured during study.

[0541] If formulated as eye drops, other than preservatives, the other inactive ingredient may be added to adjust drug concentration and tonicity such as buffers for adjusting pH, drug stabilizers such as anti-oxidants, solubility enhancers, viscosity adjusters, and the like. These inactive ingredients include but not limit to benzalkonium chloride, chlorobutanol, sodium dehydroacetate, phenethyl alcohol, benzethonium chloride, and the like; Boric Acid, Edetate Disodium, Potassium Chloride, Sodium Borate, Sodium Chloride and the like; methyl cellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methyl cellulose, polyvinyl alcohol and the like; polyoxyethylene hydrogenated castor oil, polyethylene glycol, polysorbate 80, polyoxyethylene monostearate and the like; sodium edetate and citric acid, sodium edetate and sodium bisulfite and the like. This formulation applies into the eye that follows the local

dose generates sufficient therapeutically effective dose of the direct factor IIa inhibitor while below the systemic circulation therapeutic levels.

Example 60: Preparation of organic formulation containing direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban for application in ophthalmic injection

[0542] 250 mg of Apixaban is weighed into a 20 mL scintillation vial. To the same vial, 518.13 mg of Argatroban is added, and 10 mL of Dimethyl sulfoxide (DMSO) is added to the vial containing weighed drug. Mix thoroughly by applying stirring, shaking, sonication. Allow the solution to equilibrate to room temperature. Filter the formulation through a PTFE membrane filter. Transfer the filtered solution into sterilized storage vials. The concentration of the anticoagulants is measured as 25.0 mg/mL of Apixaban and 50.0 mg/mL of Argatroban. These drug solutions are injected (10 to 50 μ L) into rabbit's eye or swine's eye with the drug concentrations in target eye tissues and whole blood. Drug concentrations in the eye tissues and in the blood is measured. In addition, ocular pressure is measured during study. This formulation applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the drug while below the systemic circulation therapeutic level.

Example 61: Preparation of organic formulation containing direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban and m-TOR Sirolimus for application in ophthalmic injection

[0543] 125 mg of Apixaban is weighed into a 20 mL scintillation vial. To the same vial, 259.07 mg of Argatroban and 125 mg of Sirolimus are added, and 5 mL of Dimethyl sulfoxide (DMSO) is added to the vial containing weighed drug. Mix thoroughly by applying stirring, shaking, sonication. Allow the solution to equilibrate to room temperature. Filter the formulation through a PTFE membrane filter. Transfer the filtered solution into sterilized storage vials. The concentration of the drugs is measured as 25.0 mg/mL of Apixaban and 50.0 mg/mL of Argatroban and 25.0 mg/mL of Sirolimus. These drug solutions are injected (10 to 50 μ L) into rabbit's eye or swine's eye with the drug concentrations in target eye tissues and whole blood. Drug concentrations in the eye tissues and in the blood is measured. In addition, ocular pressure is measured during study. This formulation applies into the eye that follows the local dose generates sufficient therapeutically effective dose of the drug while below the systemic circulation therapeutic level.

Example 62: Preparation of tissue plasminogen activator (tPA) formulation for application in ophthalmic eye drops or injection

[0544] The tPA therapy could be effective for the rapid dissolution of fibrin clots in the anterior chamber of the human eye, as well as for lysis of fibrin clots after vitrectomy and failed blebs after glaucoma filtering surgery or wet age-related macular degeneration (AMD). The

tissue plasminogen activator (tPA) eye drops administered topically to patients with postoperative hyphema could give rapid clearance of the clot. Intracameral tPA injection is a safe and effective way to help lyse fibrin and dissolve blood clots to reopen occluded glaucoma drainage devices. tPA is a nonsurgical eye injection that's often used to treat patients suffering from wet age-related macular degeneration (AMD). It's also used to treat a common side effect of AMD, submacular hemorrhages. An amount of 25 mg or more of tPA has been widely used intracamerally or intravitreally. The tPA therapeutic agent can be, for example, tPA, a tPA derivative, a small molecule direct or indirect tPA agonist, or a gene therapy vector. The most common used ones are recombinant tissue plasminogen activators (r-tPA) which are manufactured in labs. Examples of these commercial drugs include alteplase, reteplase, and tenecteplase.

[0545] The tPA eye drops is prepared by reconstitution of 0.5 mg of lyophilised tissue plasminogen activator (The commercially available r-TPA such as alteplase or reteplase or Tenecteplase) in 0.5 ml of sterile water according to the manufacturer's recommendations. The reconstituted r-TPA is diluted 1:4 in sterile balanced salt solution. Using balanced salt solution, tPA is diluted to achieve a 250 µg per mL concentration. The eyedrops are then stored at -70°C in an ultralow freezer and before use. The eye drops are thawed to room temperature when use.

[0546] The tPA eye injection is prepared by reconstitution of 1 mg of lyophilised tissue plasminogen activator (The commercially available r-TPA such as alteplase or reteplase or Tenecteplase) in 1 ml of sterile water according to the manufacturer's recommendations. All preparation is under a sterile hood. The reconstituted r-TPA is diluted 1:4 in sterile balanced salt solution which was then divided into multiple aliquots of 1 ml (250 µg) of r-TPA. Using balanced salt solution, tPA is diluted to achieve a 250 µg per mL concentration. The syringes are then stored at -70°C in an ultralow freezer and before use the syringes are thawed to room temperature.

[0547] The injections are performed with the patient in the supine position with a lid speculum inserted to separate the lids. A sterile 30 gauge cannula is placed on the syringe and 0.1 ml (25 µg) r-TPA is injected into the anterior chamber. Slit lamp examination was performed postoperatively after 2 hours, 1 day, 2 days, 3 days, and then weekly to assess complications and dissolution of the fibrin. Visual acuity was measured by Snellen or Sheridan-Gardiner acuity tests depending on the age of the patient.

[0548] tPA use could have prevented the need for additional glaucoma surgery. One or more injections of tPA may have been used to clear or prevent tube blockage by blood and/or fibrin to result in a successful ocular outcome, or decrease eye pressure.

[0549] The tPA eye drops or injections can benefit eye disease such as vitreous opacity, wet age-related macular degeneration (AMD), glaucoma and anterior chamber fibrinoid syndrome after cataract extraction etc.

Example 63: Preparation of tissue plasminogen activator (tPA) and direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban in captisol eye drops for ophthalmic application

[0550] The tPA eye drops is prepared by reconstitution of 0.5 mg of lyophilised tissue plasminogen activator (The commercially available r-TPA such as alteplase or reteplase or Tenecteplase) in 0.5 ml of sterile water according to the manufacturer's recommendations. The reconstituted r-TPA is diluted 1:4 in sterile balanced salt solution. Using balanced salt solution, tPA is diluted to achieve a 250 µg per mL concentration. The eyedrops are then stored at -70°C in an ultralow freezer and before use. The eye drops are thawed to room temperature when use.

[0551] The direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban in captisol eye drops was prepared as below: 10,560 mg Captisol was dissolved in 15mM sodium citrate in a 25.0 mL volumetric flask to make 40% Captisol in citrate buffer. 100.2 mg of apixaban was weighed into a separate vial and 20.0 mL of the 40% Captisol citrate buffer solution prepared above was added. This 20 mL of apixaban solution was sonicated with heat at 40°C for approximately 30 minutes to maximally dissolve the apixaban. This "saturated apixaban solution" was cooled and filtered to create a clear solution of apixaban in 40% Captisol. 51 mg of argatroban is weighed and added into the same vial, and finally 10 mL of the saturated apixaban solution prepared above is added to the same vial. The final 2 drug combination solution is sonicated with heat if needed until clear. The cooled solution is filtered, and pH is measured and adjusted to the suitable physiological pH's. The concentration of the anticoagulants are projected to be at 3.2 mg/mL of apixaban and 5.0 mg/mL of argatroban. The eye drops, other than preservatives, the other inactive ingredient may be added to adjust drug concentration and tonicity such as buffers for adjusting pH, drug stabilizers such as anti-oxidants, solubility enhancers, viscosity adjusters, and the like.

[0552] The tPA eye drops and the direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban eye drops can apply in alternative time to clear fibrin in eyes over times.

Example 64: Preparation of tissue plasminogen activator (tPA) and direct factor Xa inhibitor Apixaban crystal fine particle in PEG 300 formulation for application in ophthalmic injection with burst release of tPA and sustained release of direct factor Xa inhibitor Apixaban

[0553] The tPA eye injection is prepared by reconstitution of 1mg of lyophilised tissue plasminogen activator (The commercially available r-TPA such as alteplase or reteplase or Tenecteplase) in 1 ml of sterile water according to the manufacturer's recommendations. All

preparation is under a sterile hood. The reconstituted r-TPA is diluted 1:4 in sterile balanced salt solution which was then divided into multiple aliquots of 1 ml (250 µg) of r-TPA. Using balanced salt solution, tPA is diluted to achieve a 250 µg per mL concentration. The syringes are then stored at -70°C in an ultralow freezer and before use the syringes are thawed to room temperature. This tPA injection can be efficiently delivered to the posterior segments and could be created a burst release profile in patients to dissolve/dissolute most of fibrin in eyes.

[0554] The direct factor Xa inhibitor Apixaban crystal fine particle in PEG 300 was prepared as below: 10 mg of apixaban powder was weighed into a vial and 50µL of 190 proof USP grade ethanol was added first in the same vial, then 950uL of PEG 300 (polyethylene glycol) was added to solubilize the drug. The vial was sonicated with 40°C heat for 30 minutes and then was placed in the 40°C oven overnight for 20 hours. The cloudy solution was transferred to a polyethylene centrifuge tube and centrifuged for 15 minutes to pelletize the undissolved apixaban. The clear supernatant was analyzed by HPLC and the concentration was found to be 6200 µg/mL apixaban in 95% PEG 300 solution. This clear solution is ready for eye injection. After injection into eyes, these drugs will form a crystal fine particle in PEG 300 and the drug has sustained release profiles. The crystal fine particles were verified by following.

[0555] 100 µL of the clear PEG 300 solution was added to a vial with 4.3 mL of Dulbecco's phosphate buffered saline to mimic the ophthalmic vitreous humor. The solution immediately cleared upon mixing. The solution stands overnight for 24 hours at room temperature. Upon close inspection, the apixaban had precipitated producing a small carpet of crystals on the bottom of the glass vial. The solution was then centrifuged, and the supernatant was analyzed by HPLC. The concentration was found to be 94 µg/mL of apixaban compared to the theoretical concentration in the PBS at 141 µg/mL. Therefore 67% of the apixaban was in solution and 33 % had precipitated out to crystalline form.

[0556] This clear periocular injection comprises fine particles containing Apixaban and enables Apixaban to deliver to the posterior segments. Apixaban can be efficiently delivered to the posterior segments and could be used to extend the pharmacokinetics of the drug profile, thus creating a sustained release profile in patients and continue to clear fibrin in eyes over times.

[0557] tPA injection apply first then follows apixaban eye injections in a proper time interval. These ophthalmic injections with burst release of tPA and sustained release of direct factor Xa inhibitor Apixaban to clear the fibrin in eyes over time.

Example 65: Preparation of tissue plasminogen activator (tPA) and direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban crystal fine particle in PEG 300 formulation for application in ophthalmic injection with burst release of tPA and sustained release of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban

[0558] The tPA eye injection is prepared by reconstitution of 1 mg of lyophilised tissue plasminogen activator (The commercially available r-TPA such as alteplase or reteplase or Tenecteplase) in 1 ml of sterile water according to the manufacturer's recommendations. All preparation is under a sterile hood. The reconstituted r-TPA is diluted 1:4 in sterile balanced salt solution which was then divided into multiple aliquots of 1 ml (250 µg) of r-TPA. Using balanced salt solution, tPA is diluted to achieve a 250 µg per mL concentration. The syringes are then stored at -70°C in an ultralow freezer and before use the syringes are thawed to room temperature. This tPA injection can be efficiently delivered to the posterior segments and could be created a burst release profile in patients to dissolve/dissolute most of fibrin in eyes.

[0559] The direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban crystal fine particle in PEG 300 were prepared as below: In a similar formulation as in Example 64, combined Anticoagulant solution of apixaban and argatroban was created by taking 400uL of the 6200 ug/mL clear supernatant of apixaban from example 64, and adding it to a vial containing 2.44 mg of argatroban. The solution was sonicated 10 minutes until clear. The clear solution was analyzed by HPLC and the concentration was found to be 6200 ug/mL apixaban and 5690ug/mL argatroban. This clear solution is ready for eye injection. After injection into eyes, these drugs will form a crystal fine particle in PEG 300 and the drug has extended-release profiles. The crystal fine particles were verified by following.

[0560] Similar procedure can be used to create a crystalline form of anticoagulant apixaban and argatroban as in Example 64. This clear periocular injection comprises fine particles containing Apixaban and Argatroban and enables both anticoagulant deliver to the posterior segments.

[0561] tPA, Apixaban and Argatroban can be efficiently delivered to the posterior segments and could be used to extend the pharmacokinetics of the drug profile, thus creating a sustained release profile in patients and continue to clear fibrin in eyes over times.

[0562] tPA injection apply first then follows anticoagulant eye injection in a proper time interval. These ophthalmic injections with burst release of tPA and sustained release of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor to clear the fibrin in eyes over time.

Example 66: Preparation of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban eye drops and crystal fine particle eye injection formulation with burst release and sustained release of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban

[0563] The direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban in captisol eye drops was prepared as in example 63.

[0564] The direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban crystal fine particle in PEG 300 were prepared as in Example 64. The eye injection solution was analyzed by HPLC and the concentration was found to be 6200 ug/mL apixaban and 5690ug/mL argatroban. This clear solution is ready for eye injection.. After injection into eyes, these drugs will form a crystal fine particle in PEG 300 and the drug has sustained release profiles. The crystal fine particles were verified by following.

[0565] Similar procedure can be used to create a crystalline form of anticoagulant apixaban and argatroban as in Example 64. This clear periocular injection comprises Apixaban and Argatroban and enables both anticoagulant deliver to the posterior segments to form crystal fine particles after injection with extended-release profiles. Apixaban and Argatroban eye drops can be efficiently delivered to drug and have a burst release profile in patients.

Apixaban and Argatroban eye injection can be efficiently delivered to the posterior segments and could be used to extend the pharmacokinetics of the drug profile, thus creating a sustained release profile in patients. The direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban eye drops apply first then followed by anticoagulant the direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban eye injection in a proper time interval. These eye drops/injections have a burst release of the direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban and a sustained release of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor to clear the fibrin in eyes over time.

Example 67: Preparation of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban drug coated microneedle implant for sustained release of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban

[0566] The direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban can be coated in a metal microneedle. The metal microneedle is fabricated by laser cut from 50-80 micron thick stainless steel sheet following an acid electropolishing. The final size of microneedle is 400 µm in length and 40x100 µm in cross section with 40-50° in tip angle to avoid penetration through eye corneal tissue.

[0567] The microneedle can optionally undergo surface treatment if the surface is not porous (i.e. plasma treatment or other friction surface treatment). After surface treatment, the coating could be spray coated or dip coated.

[0568] When spray coated, a microprocessor controlled ultrasonic sprayer is used to coat the microneedle containing drug/excipient solution to the entire surface of the implant. After coating, the microneedle is placed in a 70°C oven for about 2 hours to remove the residue solvent. The microneedle is then put in a coil and packaged. The pouches were sterilized.

[0569] The microneedle implant provides a sustained release of direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban. This microneedle implant may use for treatment of inflammation or fibrin related of uveitis, glaucoma, diabetic macular edema, age-related macular degradation, corneal infection, cytomegalovirus retinitis or anterior chamber fibrinoid syndrome after cataract extraction etc.

Example 68: Preparation of the complement factor C3 inhibitor eye injection for ocular inflammation.

[0570] Complement is the main driver of disease for AMD, Uveitis, Glaucoma. Local inhibition of complement activation has been considered a promising approach for treating these diseases. Complement pathway has three pathways which involves various factors, B, D, H & I, which interact with each other, and with C3b, to form a C3 convertase, C3bBb, that can activate more C3. C3 is the converging point for all 3 complement pathways and involves in the amplification loop.

[0571] Compstatin is a C3 inhibitor and could be a potent inhibitor, blocking all three complement systems. A fourth generation Compstatin complement C3 inhibitor (Cp40-KKK, AMY106) can block C3 for at least 3 months with a single intravitreal injection. Compstatin and its analogs (Cp40-KKK, AMY106) are synthesized in a peptide synthesizer using F-moc amide resin (4-(2',4'-dimethoxyphenyl)-F-moc-aminomethyl)-phenoxy resin). Cp40-KKK or AMY106 eye injection is prepared by reconstitution of 100mg of Cp40-KKK or AMY106 in 1 ml of Dulbecco's phosphate buffered saline. All preparation is under a sterile hood. Using balanced salt solution, the syringes are then stored at -70°C in an ultralow freezer and before use the syringes are thawed to room temperature. The injections are performed with the patient in the supine position with a lid speculum inserted to separate the lids. A sterile 30 gauge cannula is placed on the syringe and 0.1 ml (10mg of Cp40-KKK or AMY106) of solution will be injected. The above solution is injected into the anterior chamber. Compstatin or its analogs eye injections can benefit eye disease such as AMD, Uveitis, Glaucoma, etc.

Example 69: Preparation of the complement factor C3 inhibitor Compstatin or its analog and direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban in PEG 300 eye injection for ocular inflammation

[0572] The combination of complement factor C3 inhibitor Compstatin or its analog and direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban in PEG 300 for eye injection would be a powerful therapy for AMD, Uveitis, Glaucoma and other ocular inflammation.

[0573] Cp40-KKK or AMY106 eye injection is prepared by reconstitution of 200mg of Cp40-KKK or AMY106 in 1 ml of Dulbecco's phosphate buffered saline. All preparation is under a sterile hood. Using balanced salt solution, the syringes are then stored at -70°C in an ultralow freezer and before use the syringes are thawed to room temperature.

[0574] The direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban in PEG 300 was prepared as below: 15 mg of apixaban powder 10 mg of Argatroban powder were weighed into a vial and 50 μL of 190 proof USP grade ethanol was added first in the same vial, then 950 μL of PEG 300 (polyethylene glycol) was added to solubilize the drug. The vial was sonicated with 40°C heat for 30 minutes and then was placed in the 40°C oven overnight for 20 hours. The cloudy solution was transferred to a polyethylene centrifuge tube and centrifuged for 15 minutes to pelletize the undissolved apixaban. The clear supernatant was analyzed by HPLC and the concentration was found to be 5299 $\mu\text{g/mL}$ Apixaban and 10363 $\mu\text{g/mL}$ Argatroban in 95% PEG 300 solution.

[0575] 100 μL of the clear direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban PEG 300 solution and 100 μL of Cp40-KKK or AMY106(200mg/ml) of Dulbecco's phosphate buffered saline solution are diluted to 200 μL of Dulbecco's phosphate buffered saline to mimic the ophthalmic vitreous humor. The solution immediately will be cleared upon mixing and is ready for injection.

[0576] The injections are performed with the patient in the supine position with a lid speculum inserted to separate the lids. A sterile 30-gauge cannula is placed on the syringe and 0.1 ml (25 μg of Apixaban, 50 μg of Argatroban, 10 mg of Cp40-KKK or AMY106) of solution will be injected. The above solution is injected into the anterior chamber.

[0577] Optionally, direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban PEG 300 and C3 inhibitor can be administrated separately. For example, AMY106 can be injected monthly; direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban PEG 300 can be injected weekly.

[0578] After injection into eyes, these drugs will form a crystal fine particles in PEG 300 and the drug has extended release profiles. The complement factor C3 inhibitor Compstatin or

its analog and direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban in PEG 300 can benefit eye disease such as AMD, Uveitis, Glaucoma, etc. The rate of progression of atrophy in the eyes of patients with dry AMD might be slowed down or stopped with the combination of the complement factor C3 inhibitor Compstatin or its analog and direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban in PEG 300 eye injection.

Example 70: Preparation of the complement factor C5 inhibitor and direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban in PEG 300 eye injection for ocular inflammation

[0579] Complement is the main driver of disease for AMD, Uveitis, Glaucoma. Local inhibition of complement activation has been considered a promising approach for treating this disease. Immunosuppressive drug such as Eculizumab or Ravulizumab specifically binds to the terminal complement component 5, or C5, which acts at a late stage in the complement cascade. When activated, C5 is involved in activating host cells, thereby attracting pro-inflammatory immune cells, while also destroying cells by triggering pore formation. By inhibiting the complement cascade at this point, the normal, disease-preventing functions of proximal complement system are largely preserved, while the properties of C5 that promote inflammation and cell destruction are impeded.

[0580] The complement factor C5 inhibitor eye injection is prepared with the commercially available Eculizumab or Ravulizumab according to the manufacturer's recommendations and is diluted with commercially available Saline if needed. All preparation is under a sterile hood. The dose is range from 600 mg to 1200mg weekly.

[0581] The direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban in PEG 300 was prepared as in example 69. The dose is range from 25µg to 0.5mg of Apixaban and 50µg to 1mg of Argatroban. The injections are performed with the patient in the supine position with a lid speculum inserted to separate the lids. A sterile 30 gauge cannula is placed on the syringe. The above solution is injected into the anterior chamber.

[0582] Optionally, direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban PEG 300 and C3 inhibitor can be administrated separately. For example, Eculizumab or Ravulizumab can be injected biweekly; direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban PEG 300 can be injected weekly.

[0583] After injection into eyes, these drugs will form a crystal fine particle in PEG 300 and the drug has extended-release profiles. The above eye injections can benefit eye disease such as AMD, Uveitis, Glaucoma, etc. The rate of progression of atrophy in the eyes of patients with dry AMD might be slowed with combination of Eculizumab or Ravulizumab and direct factor Xa inhibitor Apixaban and direct factor IIa inhibitor Argatroban.

[0584] When a feature or element is herein referred to as being “on” another feature or element, it can be directly on the other feature or element or intervening features and/or elements may also be present. In contrast, when a feature or element is referred to as being “directly on” another feature or element, there are no intervening features or elements present. It will also be understood that, when a feature or element is referred to as being “connected”, “attached” or “coupled” to another feature or element, it can be directly connected, attached, or coupled to the other feature or element or intervening features or elements may be present. In contrast, when a feature or element is referred to as being “directly connected”, “directly attached” or “directly coupled” to another feature or element, there are no intervening features or elements present. Although described or shown with respect to one embodiment, the features and elements so described or shown can apply to other embodiments. It will also be appreciated by those of skill in the art that references to a structure or feature that is disposed “adjacent” another feature may have portions that overlap or underlie the adjacent feature.

[0585] Terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the present disclosure. For example, as used herein, the singular forms “a”, “an” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. It will be further understood that the terms “comprises” and/or “comprising,” when used in this specification, specify the presence of stated features, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, steps, operations, elements, components, and/or groups thereof. As used herein, the term “and/or” includes any and all combinations of one or more of the associated listed items and may be abbreviated as “/”.

[0586] Spatially relative terms, such as “under”, “below”, “lower”, “over”, “upper” and the like, may be used herein for ease of description to describe one element or feature’s relationship to another element(s) or feature(s) as illustrated in the figures. It will be understood that the spatially relative terms are intended to encompass different orientations of the device in use or operation in addition to the orientation depicted in the figures. For example, if a device in the figures is inverted, elements described as “under” or “beneath” other elements or features would then be oriented “over” the other elements or features. Thus, the exemplary term “under” can encompass both an orientation of over and under. The device may be otherwise oriented (rotated 90 degrees or at other orientations) and the spatially relative descriptors used herein interpreted accordingly. Similarly, the terms “upwardly”, “downwardly”, “vertical”, “horizontal” and the like are used herein for the purpose of explanation only unless specifically indicated otherwise.

[0587] Although the terms “first” and “second” may be used herein to describe various features/elements (including steps), these features/elements should not be limited by these

terms, unless the context indicates otherwise. These terms may be used to distinguish one feature/element from another feature/element. Thus, a first feature/element discussed below could be termed a second feature/element, and similarly, a second feature/element discussed below could be termed a first feature/element without departing from the teachings of the present disclosure.

[0588] Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising” means various components can be co-jointly employed in the methods and articles (e.g., compositions and apparatuses including device and methods). For example, the term “comprising” will be understood to imply the inclusion of any stated elements or steps but not the exclusion of any other elements or steps.

[0589] As used herein in the specification and claims, including as used in the examples and unless otherwise expressly specified, all numbers may be read as if prefaced by the word “about” or “approximately,” even if the term does not expressly appear. The phrase “about” or “approximately” may be used when describing magnitude and/or position to indicate that the value and/or position described is within a reasonable expected range of values and/or positions. For example, a numeric value may have a value that is +/- 0.1% of the stated value (or range of values), +/- 1% of the stated value (or range of values), +/- 2% of the stated value (or range of values), +/- 5% of the stated value (or range of values), +/- 10% of the stated value (or range of values), etc. Any numerical values given herein should also be understood to include about or approximately that value, unless the context indicates otherwise. For example, if the value “10” is disclosed, then “about 10” is also disclosed. Any numerical range recited herein is intended to include all sub-ranges subsumed therein. It is also understood that when a value is disclosed that “less than or equal to” the value, “greater than or equal to the value” and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value “X” is disclosed the “less than or equal to X” as well as “greater than or equal to X” (e.g., where X is a numerical value) is also disclosed. It is also understood that the throughout the application, data is provided in a number of different formats, and that this data, represents endpoints and starting points, and ranges for any combination of the data points. For example, if a particular data point “10” and a particular data point “15” are disclosed, it is understood that greater than, greater than or equal to, less than, less than or equal to, and equal to 10 and 15 are considered disclosed as well as between 10 and 15. It is also understood that each unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

[0590] While preferred embodiments of the present disclosure have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

WHAT IS CLAIMED IS:

1. A method of treating an ophthalmic condition or disease or an inflammatory ophthalmic condition or disease in a patient, the method comprising:
providing a therapeutic composition comprising at least one of a direct factor Xa inhibitor and a factor IIa inhibitor; and
delivering a therapeutically effective dose of the therapeutic composition to the patient's eye.
2. The method of claim 1, wherein delivering the therapeutically effective dose of the therapeutic composition comprises administering the therapeutically effective dose via injection, an implant, an eye drop, an emulsion, a reservoir, a suspension, an ointment, a nanomicelle, a nanoparticle, a nanosuspension, a liposome, an in-situ gel, a contact lens, or a microneedle.
3. The method of any one of the preceding claims, wherein the inflammatory ophthalmic condition or disease comprises one or more of angiogenesis, leaking blood vessel, protein deposition, fibrosis, extracellular matrix (ECM) deposition, thrombin generation, clot formation, or fibrin formation, wet age-related macular degeneration (AMD), dry AMD, diabetic retinopathy, glaucoma, uveitis, cataracts, conjunctivitis, and dry eye disease.
4. The method of any one of the preceding claims, wherein therapeutic composition is delivered to a site of the inflammatory ophthalmic condition or disease selected from the group consisting of an eyelid, a meibomian gland, a trabecular meshwork, Schlemm's canal, a cornea, a sclera, a vitreous, a choroid, a uvea, and a retina.
5. The method of any one of claims 1 to 4, wherein therapeutic composition is delivered non-site-specifically to the patient's eye.
6. The method of any one of the preceding claims, further comprising diagnosing the patient as having the inflammatory ophthalmic condition or disease prior to delivering the therapeutically effective dose of the therapeutic composition.
7. The method of any one of the preceding claims, wherein the therapeutic composition includes at least a direct factor Xa inhibitor is selected from the group consisting of apixaban, betrixaban, edoxaban, otamixaban, razaxaban, rivaroxaban, (r)-n-(2-(4-(1-methylpiperidin-4-yl)piperazin-1-yl)-2-oxo-1-phenylethyl)-1h-indole-6-carboxamide(LY-517717), daraxaban (YM-150), 2-[(7-carbamimidoylnaphthalen-2-yl)methyl]-[4-(1-ethanimidoylpiperidin-4-yl)oxyphenyl]sulfamoyl]acetic acid (YM-466 or YM-60828),

eribaxaban (PD 0348292), 2-(5-carbamimidoyl-2-hydroxy-phenyl) 4-[5-(2,6-dimethyl-piperidin-1-yl)-pentyl]-3-oxo-3, 4-dihydro-quinoxaline-6-carboxylic acid(PD0313052), (S)-3-(7-carbamimidoylnaphthalen-2-yl)-2-(4-(((S)-1-(1-iminoethyl)pyrrolidin-3-yl)oxy)phenyl)propanoic acid hydrochloride pentahydrate(DX9065a), letaxaban (TAK-442), GW813893, JTV-803, KFA-144, DPC-423, RPR-209685, MCM-09, or antistasin.

8. The method of claim 7, wherein the direct factor Xa inhibitor comprises rivaroxaban, or a salt, isomer, solvate, analog, derivative, metabolite, or prodrugs thereof.

9. The method of claim 7, wherein the direct factor Xa inhibitor comprises apixaban, or a salt, isomer, solvate, analog, derivative, metabolite, or prodrugs thereof.

10. The method of any one of the preceding claims, wherein an IC₅₀ of the direct factor Xa inhibitor is within a range of about 0.01 nM to about 1000 nM.

11. The method of claim 10, wherein the IC₅₀ of the direct factor Xa inhibitor is within a range of about 0.1 nM to about 1000 nM.

12. The method of claim 10, wherein the IC₅₀ of the direct factor Xa inhibitor is within a range of about 1 nM to about 1000 nM.

13. The method of claim 10, wherein the IC₅₀ of the direct factor Xa inhibitor is within a range of about 10 nM to about 1000 nM.

14. The method of any one of the preceding claims, wherein the therapeutically effective dose of the direct factor Xa inhibitor is within a range of about 50 micrograms to about 10 mg.

15. The method of claim 14, wherein the therapeutically effective dose of the direct factor Xa inhibitor is within a range of about 0.1 mg to about 10 mg.

16. The method of claim 14, wherein the therapeutically effective dose of the direct factor Xa inhibitor is within a range of about 1 mg to about 5 mg.

17. The method of any one of the preceding claims, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue.

18. The method of claim 17, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 0.2 ng/g tissue to about 100 mg/g tissue

19. The method of claim 17, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 0.5 ng/g tissue to about 100 mg/g tissue.

20. The method of claim 17, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 1 ng/g tissue to about 100 mg/g tissue.

21. The method of claim 17, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 10 ng/g tissue to about 100 mg/g tissue.

22. The method of claim 17, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 100 ng/g tissue to about 100 mg/g tissue.

23. The method of claim 17, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor Xa inhibitor which is less than about 200 ng/ml, 100 ng/ml, 50 ng/ml 25 ng/ml, or 10 ng/ml.

24. The method of claim 17, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor Xa inhibitor which is less than a systemic therapeutic concentration of the direct factor Xa inhibitor for any systemic indication.

25. The method of claim 17, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor Xa inhibitor which is smaller than a median maximum serum concentration (C_{max}) of the direct factor Xa inhibitor generated by systemic delivery of the direct factor Xa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease.

26. The method of claim 17, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor Xa inhibitor which does not exceed a median maximum serum concentration (C_{max}) of the direct factor Xa inhibitor generated by systemic delivery of the direct factor Xa inhibitor to achieve the same tissue

concentration at the site of the inflammatory ophthalmic condition or disease for more than about 6 hours to about 3 days.

27. The method of any one of the preceding claims, wherein the therapeutically effective dose is sufficient to maintain a tissue concentration of the direct factor Xa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

28. The method of any one of the preceding claims, wherein the therapeutically active substance comprises at least a direct factor IIa inhibitor selected from the group consisting of argatroban, dabigatran, ximelagatran, melagatran, efegatran, inogatran, atecagatran metoxil (AZD-0837), hirudin, hirudin analogs, bivalirudin, desirudin, or lepirudin.

29. The method of claim 28, wherein the direct factor IIa inhibitor comprises argatroban.

30. The method of claim 28, wherein the direct factor IIa inhibitor comprises a salt, isomer, solvate, analog, derivative, metabolite, or prodrugs of argatroban.

31. The method of claim 28 or 29, wherein the therapeutically effective dose of the direct factor IIa inhibitor is within a range of about 50 micrograms to about 10 mg.

32. The method of claim 28 or 29, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue.

33. The method of claim 32, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 0.2 ng/g tissue to about 100 mg/g tissue.

34. The method of claim 32, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 0.5 ng/g tissue to about 100 mg/g tissue.

35. The method of claim 32, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 1 ng/g tissue to about 100 mg/g tissue.

36. The method of claim 32, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 10 ng/g tissue to about 100 mg/g tissue.

37. The method of claim 32, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 100 ng/g tissue to about 100 mg/g tissue.

38. The method of claim 32, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which is less than about 200 ng/ml, 100 ng/ml, 50 ng/ml 25 ng/ml, or 10 ng/ml.

39. The method of claim 32, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which is smaller than a median maximum serum concentration (C_{max}) of the direct factor IIa inhibitor generated by systemic delivery of the direct factor IIa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease.

40. The method of claim 32, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which does not exceed a median maximum serum concentration (C_{max}) of the direct factor IIa inhibitor generated by systemic delivery of the direct factor IIa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease for more than about 6 hours to about 3 days.

41. The method of claim 28 or 29, wherein the therapeutically effective dose is sufficient to maintain a tissue concentration of the direct factor IIa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

42. The method of any one of the preceding claims, wherein the therapeutic composition comprises each of a direct factor Xa inhibitor and a factor IIa inhibitor.

43. The method of claim 42, wherein a weight ratio of the direct factor Xa inhibitor to the direct factor IIa inhibitor in the therapeutic composition is within a range of about 3:1 to about 1:3.

44. The method of claim 43, wherein a weight ratio of the direct factor Xa inhibitor to the direct factor IIa inhibitor in the therapeutic composition is about 1:1.

45. The method of any one of the preceding claims, wherein the therapeutic composition comprises or is administered in combination with one or more additional pharmaceutical agents.

46. The method of claim 45, wherein the one or more additional pharmaceutical agents comprises one or more anti-fibrotic agents, anti-proliferative agents, serine protease, complement activation inhibitor, anti-diabetic agents, anti-viral agents, anti-angiogenic agents, anti-VEGF, or combinations thereof.

47. The method of claim 46, wherein the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration sufficient to generate a blood concentration of pirfenidone of less than about 20 µg/ml, 12 µg/ml, 7 µg/ml, or 6.5 µg/ml.

48. The method of claim 46, wherein the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pirfenidone of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

49. The method of claim 46, wherein the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pirfenidone of within a range of about 1 ng/g tissue to about 100 mg/g tissue.

50. The method of claim 46, wherein the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pirfenidone of within a range of about 10 ng/g tissue to about 100 mg/g tissue.

51. The method of claim 46, wherein the therapeutically effective dose is sufficient to maintain a tissue concentration of pirfenidone of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

52. The method of claim 46, wherein the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a blood concentration of nintedanib of less than about 100 ng/ml, 50 ng/ml, 40 ng/ml, or 30 ng/ml.

53. The method of claim 46, wherein the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of nintedanib of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

54. The method of claim 46, wherein the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of nintedanib of within a range of about 1 ng/g tissue to about 100 mg/g tissue.

55. The method of claim 46, wherein the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of nintedanib of within a range of about 10 ng/mg tissue to about 100 mg/g tissue.

56. The method of claim 46, wherein the therapeutically effective dose is sufficient to maintain a tissue concentration of nintedanib of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

57. The method of claim 46, wherein the anti-fibrotic agent comprises nintedanib and the therapeutically effective dose comprises nintedanib within a range of about 0.1 mg to about 5 mg.

58. The method of claim 46, anti-viral agent or anti-diabetic agent comprises metformin or its salt and is present in the therapeutic composition at a concentration sufficient to generate a blood concentration of metformin or its salt of less than about 1800 µg/ml, 900 µg/ml, 500 µg/ml, 50 µg/ml, 10 µg/ml, 1.3 µg/ml, 1 µg/ml, or 0.8 µg/ml.

59. The method of claim 46, wherein the anti-viral agent or anti-diabetic agent comprises metformin or its salt and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of metformin or its salt of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

60. The method of claim 46, wherein the anti-viral agent or anti-diabetic agent comprises metformin or its salt and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of metformin or its salt of within a range of about 1 ng/g tissue to about 100 mg/g tissue.

61. The method of claim 46, wherein the therapeutically effective dose is sufficient to maintain a tissue concentration of metformin or its salt of about 0.1 ng/mg tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

62. The method of claim 46, wherein the anti-angiogenic agent comprises an anti-VEGF agent.

63. The method of claim 62, wherein the anti-VEGF agent comprises ranibizumab, aflibercept, pegaptanib sodium, bevacizumab, brolucizumab, lapatinib, sorafenib, axitinib, pazopanib, sunitinib, cabozantinib, lenvatinib, ponatinib, ramucirumab, regorafenib, vandetanib, cediranib, thiazolidinediones or RGX-314.

64. The method of claim 63, wherein the anti-VEGF agent comprises ranibizumab and is present in the therapeutic composition at a concentration within a range of about 6 mg/ml to about 40 mg/ml.

65. The method of claim 63, wherein the anti-VEGF agent comprises ranibizumab and the therapeutically effective dose comprises ranibizumab within a range of about 0.3 mg to about 2 mg.

66. The method of claim 63, wherein the anti-VEGF agent comprises ranibizumab and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of ranibizumab of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

67. The method of claim 63, wherein the anti-VEGF agent comprises ranibizumab and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of ranibizumab of within a range of about 1 ng/g tissue to about 100 mg/g tissue.

68. The method of claim 63, wherein the therapeutically effective dose is sufficient to maintain a tissue concentration of ranibizumab within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

69. The method of claim 63, wherein the anti-VEGF agent comprises aflibercept and is present in the therapeutic composition at a concentration within a range of about 10 mg/ml to about 100 mg/ml.

70. The method of claim 63, wherein the anti-VEGF agent comprises aflibercept and the therapeutically effective dose comprises aflibercept within a range of about 0.5 mg to about 5 mg.

71. The method of claim 63, wherein the anti-VEGF agent comprises aflibercept and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of aflibercept of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

72. The method of claim 63, wherein the anti-VEGF agent comprises aflibercept and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of aflibercept of within a range of about 1 ng/g tissue to about 100 mg/g tissue.

73. The method of claim 63, wherein the therapeutically effective dose is sufficient to maintain a tissue concentration of aflibercept within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

74. The method of claim 63, wherein the anti-VEGF agent comprises pegaptanib and is present in the therapeutic composition at a concentration within a range of about 1 mg/ml to about 10 mg/ml.

75. The method of claim 63, wherein the anti-VEGF agent comprises pegaptanib and the therapeutically effective dose comprises pegaptanib within a range of about 0.1 mg to about 1 mg.

76. The method of claim 63, wherein the anti-VEGF agent comprises pegaptanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pegaptanib of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

77. The method of claim 63, wherein the anti-VEGF agent comprises pegaptanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pegaptanib of within a range of about 1 ng/g tissue to about 100 mg/g tissue.

78. The method of claim 63, wherein the therapeutically effective dose is sufficient to maintain a tissue concentration of pegaptanib within a range of about 0.1 ng/g

tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

79. The method of claim 46, wherein the anti-proliferative agent comprises an mTOR inhibitor.

80. The method of claim 79, wherein the mTOR inhibitor comprises rapamycin, everolimus, biolimus, temsirolimus, ridaforolimus, zotarolimus, myolimus, or novolimus.

81. The method of claim 80, wherein the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration within a range of 1 mg/ml to about 250 mg/ml.

82. The method of claim 80, wherein the anti-proliferative agent comprises sirolimus and the therapeutically effective dose comprises sirolimus within a range of about 50 µg to about 5 mg, about 0.1 mg to about 5 mg, or about 0.5 mg to about 3 mg.

83. The method of claim 80, wherein the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of sirolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

84. The method of claim 80, wherein the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of sirolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 1 day.

85. The method of claim 80, wherein the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of sirolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 72 days.

86. The method of claim 80, wherein the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to maintain a tissue concentration of sirolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year

87. The method of claim 80, wherein the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration within a range of 1 mg/ml to about 250 mg/ml.

88. The method of claim 80, wherein the anti-proliferative agent comprises novolimus and the therapeutically effective dose comprises novolimus within a range of about 50 µg to about 5 mg, about 0.1 mg to about 5 mg, or about 0.5 mg to about 3 mg.

89. The method of claim 80, wherein the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of novolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

90. The method of claim 80, wherein the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of novolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 1 day.

91. The method of claim 80, wherein the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of novolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 72 days.

92. The method of claim 80, wherein the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient to maintain a tissue concentration of novolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

93. The method of claim 80, wherein the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration within a range of 1 mg/ml to about 250 mg/ml.

94. The method of claim 80, wherein the anti-proliferative agent comprises myolimus and the therapeutically effective dose comprises myolimus within a range of about 50 µg to about 5 mg, about 0.1 mg to about 5 mg, or about 0.5 mg to about 3 mg.

95. The method of claim 80, wherein the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to generate

a tissue concentration of myolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

96. The method of claim 80, wherein the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of myolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 1 day.

97. The method of claim 80, wherein the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of myolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 72 days.

98. The method of claim 80, wherein the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to maintain a tissue concentration of myolimus of within a range of about 0.1ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

99. The method of claim 80, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the anti-proliferative agent which is smaller than a median maximum serum concentration (C_{max}) of the anti-proliferative agent generated by systemic delivery of the anti-proliferative agent to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease.

100. The method of claim 80, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the anti-proliferative agent which does not exceed a median maximum serum concentration (C_{max}) of the anti-proliferative agent generated by systemic delivery of the anti-proliferative agent to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease for more than about 6 hours to about 7 days.

101. The method of claim 80, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the anti-proliferative agent which is less than about 10 ng/ml.

102. The method of any one of the preceding claims, wherein the therapeutic composition comprises one or more of a pharmaceutically acceptable carrier, a diluent, an

adjuvant, an excipient, a vehicle, a preserving agent, a filler, a polymer, a disintegrating agent, a glidant, a wetting agent, an emulsifying agent, a suspending agent, a sweetening agent, a flavoring agent, a perfuming agent, a lubricating agent, an acidifying agent, and a dispensing agent.

103. The composition of any one of the preceding claims wherein delivery of the therapeutically effective dose of the therapeutic composition is effective to suppress or prevent initiation, progression, or relapses of disease, including the progression of established disease.

104. The method of any one of the preceding claims, wherein the therapeutically effective dose of the therapeutic composition is effective to inhibit thrombosis, inhibit clot formation, or inhibit fibrin formation in the patient's eye.

105. A therapeutic composition for inhibiting an inflammatory ophthalmic condition or disease in a patient, the composition comprising:

at least one of a direct factor Xa inhibitor and a factor IIa inhibitor formulated for delivery to an eye of the patient.

106. The composition of claim 105, wherein the at least one of a direct factor Xa inhibitor and a factor IIa inhibitor is formulated as one of a solution, an implant, an eye drop, an emulsion, a suspension, an ointment, a nanomicelle, a nanoparticle, a nanosuspension, a liposome, or an in-situ gel.

107. The composition of any one of claims 105 and 106, wherein the at least one of a direct factor Xa inhibitor and a factor IIa inhibitor is formulated to treat an inflammatory ophthalmic condition or disease comprising one or more of angiogenesis, leaking blood vessel, protein deposition, fibrosis, extracellular matrix (ECM) deposition, thrombin generation, clot formation, or fibrin formation, wet age-related macular degeneration (AMD), dry AMD, diabetic retinopathy, glaucoma, uveitis, cataracts, conjunctivitis, and dry eye disease.

108. The composition of any one of claims 105 to 107, wherein the at least one of a direct factor Xa inhibitor and a factor IIa inhibitor comprises at least a direct factor Xa inhibitor is selected from the group consisting of apixaban, betrixaban, edoxaban, otamixaban, razaxaban, rivaroxaban, (r)-n-(2-(4-(1-methylpiperidin-4-yl)piperazin-1-yl)-2-oxo-1-phenylethyl)-1h-indole-6-carboxamide(LY-517717), daraxaban (YM-150), 2-[(7-

carbamimidoylnaphthalen-2-yl)methyl-[4-(1-ethanimidoylpiperidin-4-yl)oxyphenyl]sulfamoyl]acetic acid (YM-466 or YM-60828), or eribaxaban (PD 0348292), 2-(5-carbamimidoyl-2-hydroxy-phenyl) 4-[5-(2,6-dimethyl-piperidin-1-yl)-pentyl]-3-oxo-3, 4-dihydro-quinoxaline-6-carboxylic acid(PD0313052), (S)-3-(7-carbamimidoylnaphthalen-2-yl)-2-(4-(((S)-1-(1-iminoethyl)pyrrolidin-3-yl)oxy)phenyl)propanoic acid hydrochloride pentahydrate(DX9065a), letaxaban (TAK-442), GW813893, JTV-803, KFA-144, DPC-423, RPR-209685, MCM-09, or antistasin.

109. The composition of claim 108, wherein the direct factor Xa inhibitor comprises rivaroxaban, or a salt, isomer, solvate, analog, derivative, metabolite, or prodrugs thereof.

110. The composition of claim 108, wherein the direct factor Xa inhibitor comprises apixaban, or a salt, isomer, solvate, analog, derivative, metabolite, or prodrugs thereof.

111. The composition of any one of claims 105 to 110, wherein an IC₅₀ of the direct factor Xa inhibitor is within a range of about 0.01 nM to about 1000 nM.

112. The composition of claim 111, wherein the IC₅₀ of the direct factor Xa inhibitor is within a range of about 0.1 nM to about 1000 nM.

113. The composition of claim 111, wherein the IC₅₀ of the direct factor Xa inhibitor is within a range of about 1 nM to about 1000 nM.

114. The composition of claim 111, wherein the IC₅₀ of the direct factor Xa inhibitor is within a range of about 10 nM to about 1000 nM.

115. The composition of any one of claims 105 to 114, wherein a therapeutically effective dose of the direct factor Xa inhibitor is within a range of about 50 micrograms to about 5 mg.

116. The composition of claim 115, wherein the therapeutically effective dose of the direct factor Xa inhibitor is within a range of about 0.1 mg to about 5 mg.

117. The composition of claim 115, wherein the therapeutically effective dose of the direct factor Xa inhibitor is within a range of about 1 mg to about 5 mg.

118. The composition of any one of claims 105 to 117, wherein a therapeutically effective dose of the direct factor Xa inhibitor is sufficient to achieve a tissue concentration of the direct factor Xa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue.

119. The composition of claim 118, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 0.2 ng/g tissue to about 100 mg/g tissue

120. The composition of claim 118, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 0.5 ng/g tissue to about 100 mg/g tissue.

121. The composition of claim 118, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 1 ng/g tissue to about 100 mg/g tissue.

122. The composition of claim 118, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 10 ng/g tissue to about 100 mg/g tissue.

123. The composition of claim 118, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor Xa inhibitor of about 100 ng/g tissue to about 100 mg/g tissue.

124. The composition of claim 118, wherein the therapeutically effective dose of the direct factor Xa inhibitor is sufficient to achieve a blood concentration of the direct factor Xa inhibitor which is less than about 200 ng/ml, 100 ng/ml, 50 ng/ml 25 ng/ml, or 10 ng/ml.

125. The composition of claim 118, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor Xa inhibitor which is less than a systemic therapeutic concentration of the direct factor Xa inhibitor for any systemic indication.

126. The composition of claim 118, wherein the therapeutically effective dose of the direct factor Xa inhibitor is sufficient to achieve a blood concentration of the direct factor Xa inhibitor which is smaller than a median maximum serum concentration (C_{max}) of the direct factor Xa inhibitor generated by systemic delivery of the direct factor Xa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease.

127. The composition of claim 118, wherein the therapeutically effective dose of the direct factor Xa inhibitor is sufficient to achieve a blood concentration of the direct factor Xa inhibitor which does not exceed a median maximum serum concentration (C_{max}) of the direct factor Xa inhibitor generated by systemic delivery of the direct factor Xa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease for more than about 6 hours to about 3 days.

128. The composition of any one of claims 105 to 127, wherein a therapeutically effective dose of the direct factor Xa inhibitor is sufficient to maintain a tissue concentration of the direct factor Xa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

129. The composition of any one of claims 105 to 128, wherein the at least one of a direct factor Xa inhibitor and a factor IIa inhibitor comprises at least a direct factor IIa inhibitor selected from the group consisting of argatroban, dabigatran, ximelagatran, melagatran, efegatran, inogatran, atecegatran metoxil (AZD-0837), hirudin, hirudin analogs, bivalirudin, desirudin, or lepirudin.

130. The composition of claim 129, wherein the direct factor IIa inhibitor comprises argatroban, or a salt, isomer, solvate, analog, derivative, metabolite, or prodrugs thereof.

131. The composition of claim 129 or 130, wherein the direct factor IIa inhibitor is present at a therapeutically effective dose within a range of about 50 micrograms to about 5 mg.

132. The composition of claim 131, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue.

133. The composition of claim 131, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 0.2 ng/g tissue to about 100 mg/g tissue.

134. The composition of claim 131, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 0.5 ng/g tissue to about 100 mg/g tissue.

135. The composition of claim 131, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 1 ng/g tissue to about 100 mg/g tissue.

136. The composition of claim 131, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 10 ng/g tissue to about 100 mg/g tissue.

137. The composition of claim 131, wherein the therapeutically effective dose is sufficient to generate a tissue concentration of the direct factor IIa inhibitor of about 100 ng/g tissue to about 100 mg/g tissue.

138. The composition of claim 131, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which is less than about 200 ng/ml, 100 ng/ml, 50 ng/ml 25 ng/ml, or 10 ng/ml.

139. The composition of claim 131, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which is smaller than a median maximum serum concentration (C_{max}) of the direct factor IIa inhibitor generated by systemic delivery of the direct factor IIa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease.

140. The composition of claim 131, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the direct factor IIa inhibitor which does not exceed a median maximum serum concentration (C_{max}) of the direct factor IIa inhibitor generated by systemic delivery of the direct factor IIa inhibitor to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease for more than about 6 hours to about 3 days.

141. The composition of claim 131, wherein the therapeutically effective dose is sufficient to maintain a tissue concentration of the direct factor IIa inhibitor of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

142. The composition of any one of claims 105 to 141, wherein the therapeutic composition comprises both a direct factor Xa inhibitor and a factor IIa inhibitor .

143. The composition of claim 142, wherein the direct factor Xa inhibitor comprises apixaban or a salt, isomer, solvate, analog, derivative, metabolite, or a prodrug

thereof or rivaroxaban, or a salt, isomer, solvate, analog, derivative, metabolite, or prodrug thereof and the direct factor IIa inhibitor comprises argatroban or a salt, isomer, solvate, analog, derivative, metabolite, or prodrugs thereof.

144. The composition of claim 142 or 143, wherein the weight compositional ratio of the direct factor Xa inhibitor to the direct factor IIa inhibitor in the therapeutic composition is within a range of about 3:1 to about 1:3.

145. The composition of claim 144, wherein the weight compositional ratio of the direct factor Xa inhibitor to the direct factor IIa inhibitor in the therapeutic composition is about 1:1.

146. The composition of any one of claims 142 to 145, wherein the therapeutic composition is administered in combination with one or more additional pharmaceutical agents.

147. The composition of claim 146, wherein the one or more additional pharmaceutical agents comprises one or more anti-fibrotic agents, anti-proliferative agents, anti-diabetic agents, anti-viral agents, anti-VEGF, anti-angiogenic agents, complement inhibitors, or combinations thereof.

148. The composition of claim 147, wherein the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration within a range of sufficient to generate a blood concentration of pirfenidone of less than about 20 µg/ml, 12 µg/ml, 7 µg/ml, or 6.5 µg/ml.

149. The composition of claim 147, wherein the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pirfenidone of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

150. The composition of claim 147, wherein the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pirfenidone of within a range of about 1 ng/g tissue to about 100 mg/g tissue.

151. The composition of claim 147, wherein the anti-fibrotic agent comprises pirfenidone and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pirfenidone of within a range of about 10 ng/g tissue to about 100 mg/g tissue.

152. The composition of claim 148-151, wherein the therapeutically effective dose is sufficient to maintain a tissue concentration of pirfenidone of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

153. The composition of claim 147, wherein the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a blood concentration of nintedanib of less than about 100 ng/ml, 50 ng/ml, 40 ng/ml, or 30 ng/ml.

154. The composition of claim 147, wherein the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of nintedanib of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

155. The composition of claim 147, wherein the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of nintedanib of within a range of about 1 ng/g tissue to about 100 mg/g tissue/

156. The composition of claim 147, wherein the anti-fibrotic agent comprises nintedanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of nintedanib of within a range of about 10 ng/mg tissue to about 100 mg/g tissue.

157. The composition of claim 153 to 156, wherein the therapeutically effective dose is sufficient to maintain a tissue concentration of nintedanib of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

158. The composition of claim 147, wherein the anti-fibrotic agent comprises nintedanib at a dosage within a range of about 0.1 mg to about 5 mg.

159. The composition of claim 147, anti-viral agent or anti-diabetic agent comprises metformin or its salt and is present in the therapeutic composition at a concentration sufficient to generate a blood concentration of metformin or its salt of less than about 1300 ng/ml, 1000 ng/ml, or 800 ng/ml.

160. The composition of claim 147, wherein the anti-viral agent or anti-diabetic agent comprises metformin or its salt and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of metformin or its salt of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

161. The composition of claim 147, wherein the anti-viral agent or anti-diabetic agent comprises metformin or its salt and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of metformin or its salt of within a range of about 1 ng/g tissue to about 100 mg/g tissue.

162. The composition of claim 159 to 161, wherein the therapeutically effective dose is sufficient to maintain a tissue concentration of metformin or its salt of about 0.1 ng/mg tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

163. The composition of claim 147, wherein the anti-angiogenic agent comprises an anti-VEGF agent.

164. The composition of claim 163, wherein the anti-VEGF agent comprises ranibizumab, aflibercept, pegaptanib sodium, bevacizumab, brolucizumab, lapatinib, sorafenib, axitinib, pazopanib, sunitinib, cabozantinib, lenvatinib, ponatinib, ramucirumab, regorafenib, vandetanib, verteporfin, cediranib, thiazolidinediones or RGX-314.

165. The composition of claim 164, wherein the anti-VEGF agent comprises ranibizumab and is present in the therapeutic composition at a concentration within a range of about 6 mg/ml to about 40 mg/ml.

166. The composition of claim 164, wherein the anti-VEGF agent comprises ranibizumab and a therapeutically effective dose of ranibizumab comprises ranibizumab within a range of about 0.3 mg to about 2 mg.

167. The composition of claim 164, wherein the anti-VEGF agent comprises ranibizumab and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of ranibizumab of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

168. The composition of claim 164, wherein the anti-VEGF agent comprises ranibizumab and is present in the therapeutic composition at a concentration sufficient to

generate a tissue concentration of ranibizumab of within a range of about 1 ng/g tissue to about 100 mg/g tissue.

169. The composition of claim 164, wherein the therapeutically effective dose is sufficient to maintain a tissue concentration of ranibizumab within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

170. The method of claim 164, wherein the anti-VEGF agent comprises aflibercept and is present in the therapeutic composition at a concentration within a range of about 10 mg/ml to about 100 mg/ml.

171. The composition of claim 164, wherein the anti-VEGF agent comprises aflibercept and the therapeutically effective dose comprises aflibercept within a range of about 0.5 mg to about 5 mg.

172. The composition of claim 164, wherein the anti-VEGF agent comprises aflibercept and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of aflibercept of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

173. The composition of claim 164, wherein the anti-VEGF agent comprises aflibercept and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of aflibercept of within a range of about 1 ng/g tissue to about 100 mg/g tissue.

174. The composition of claim 164, wherein the therapeutically effective dose is sufficient to maintain a tissue concentration of aflibercept within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

175. The method of claim 164, wherein the anti-VEGF agent comprises pegaptanib and is present in the therapeutic composition at a concentration within a range of about 1 mg/ml to about 10 mg/ml.

176. The composition of claim 164, wherein the anti-VEGF agent comprises pegaptanib and the therapeutically effective dose comprises pegaptanib within a range of about 0.1 mg to about 1 mg.

177. The composition of claim 164, wherein the anti-VEGF agent comprises pegaptanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pegaptanib of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

178. The composition of claim 164, wherein the anti-VEGF agent comprises pegaptanib and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of pegaptanib of within a range of about 1 ng/g tissue to about 100 mg/g tissue.

179. The composition of claim 164, wherein the therapeutically effective dose is sufficient to maintain a tissue concentration of pegaptanib within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

180. The composition of claim 147, wherein the therapeutic composition comprises an anti-proliferative agent comprising an mTOR inhibitor.

181. The composition of claim 180, wherein the mTOR inhibitor comprises rapamycin, everolimus, biolimus, temsirolimus, ridaforolimus, zotarolimus, myolimus, or novolimus.

182. The composition of claim 180, wherein the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration within a range of 1 mg/ml to about 250 mg/ml.

183. The composition of claim 180, wherein the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration a range of about 50 µg to about 5 mg, about 0.1 mg to about 5 mg, or about 0.5 mg to about 3 mg.

184. The composition of claim 180, wherein the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of sirolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

185. The composition of claim 180, wherein the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of sirolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 1 day.

186. The composition of claim 180, wherein the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of sirolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 72 days.

187. The composition of claim 180, wherein the anti-proliferative agent comprises sirolimus and is present in the therapeutic composition at a concentration sufficient to maintain a tissue concentration of sirolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

188. The composition of claim 181, wherein the anti-proliferative agent comprises novolimus.

189. The composition of claim 188, wherein the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration within a range of 1 mg/ml to about 250 mg/ml.

190. The composition of claim 188, wherein the anti-proliferative agent comprises novolimus and a therapeutically effective dose comprises novolimus within a range of about 50 µg to about 5 mg, about 0.1 mg to about 5 mg, or about 0.5 mg to about 3 mg.

191. The composition of claim 188, wherein the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of novolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

192. The composition of claim 188, wherein the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of novolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 1 day.

193. The composition of claim 188, wherein the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of novolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 72 days.

194. The composition of claim 188, wherein the anti-proliferative agent comprises novolimus and is present in the therapeutic composition at a concentration sufficient

to maintain a tissue concentration of novolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

195. The composition of claim 181, wherein the anti-proliferative agent comprises myolimus.

196. The composition of claim 195, wherein the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration within a range of 1 mg/ml to about 250 mg/ml.

197. The composition of claim 195, wherein the anti-proliferative agent comprises myolimus and a therapeutically effective dose comprises myolimus within a range of about 50 µg to about 5 mg, about 0.1 mg to about 5 mg, or about 0.5 mg to about 3 mg.

198. The composition of claim 195, wherein the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of myolimus of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue.

199. The composition of claim 195, wherein the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of myolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 1 day.

200. The composition of claim 195, wherein the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to generate a tissue concentration of myolimus in a sclera of the eye of within a range of about 0.1 ng/g tissue to about 100 mg/g tissue within about 72 days.

201. The composition of claim 195, wherein the anti-proliferative agent comprises myolimus and is present in the therapeutic composition at a concentration sufficient to maintain a tissue concentration of myolimus of within a range of about 0.1ng/g tissue to about 100 mg/g tissue for about 1 day to about 1 year, 30 days to about 1 year, 3 months to about 1 year, or 6 months to about 1 year.

202. The composition of claim 180 to 201, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the anti-proliferative agent which is smaller than a median maximum serum concentration (C_{max}) of the anti-proliferative agent

generated by systemic delivery of the anti-proliferative agent to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease.

203. The composition of claim 180 to 201, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the anti-proliferative agent which does not exceed a median maximum serum concentration (C_{max}) of the anti-proliferative agent generated by systemic delivery of the anti-proliferative agent to achieve the same tissue concentration at the site of the inflammatory ophthalmic condition or disease for more than about 6 hours to about 7 days.

204. The composition of claim 180 to 201, wherein the therapeutically effective dose is sufficient to generate a blood concentration of the anti-proliferative agent which is less than about 10 ng/ml

205. The composition of any one of claims 105 to 204, wherein the therapeutic composition comprises one or more of a pharmaceutically acceptable carrier, a diluent, an adjuvant, an excipient, a vehicle, a preserving agent, a filler, a polymer, a disintegrating agent, a glidant, a wetting agent, an emulsifying agent, a suspending agent, a sweetening agent, a flavoring agent, a perfuming agent, a lubricating agent, an acidifying agent, and a dispensing agent.

206. The composition of any one of claims 105 to 205, wherein delivery of a therapeutically effective dose of the therapeutic composition is effective to inhibit thrombosis, inhibit clot formation, or inhibit fibrin formation in the patient's eye.

207. The composition any one of claims 105 to 206, wherein the therapeutic composition is formulated to suppress or prevent initiation, progression, or relapse of disease, including the progression of established disease.

208. The method of claim 46, wherein the anti-angiogenic agent comprises a complement activation inhibitor selected from a group consisting of compstatin, Eculizumab, Ravulizumab or analogues thereof.

209. The method of claim 46, wherein anti-angiogenic agent comprises a serine protease such as tissue plasminogen activator.

210. The composition of claim 147, wherein the anti-angiogenic agent comprises a complement inhibitor selected from a group consisting of compstatin, Eculizumab, Ravulizumab, and analogues thereof.

211. The composition of claim 147, wherein the anti-angiogenic agent comprises a serine protease such as tissue plasminogen activator.

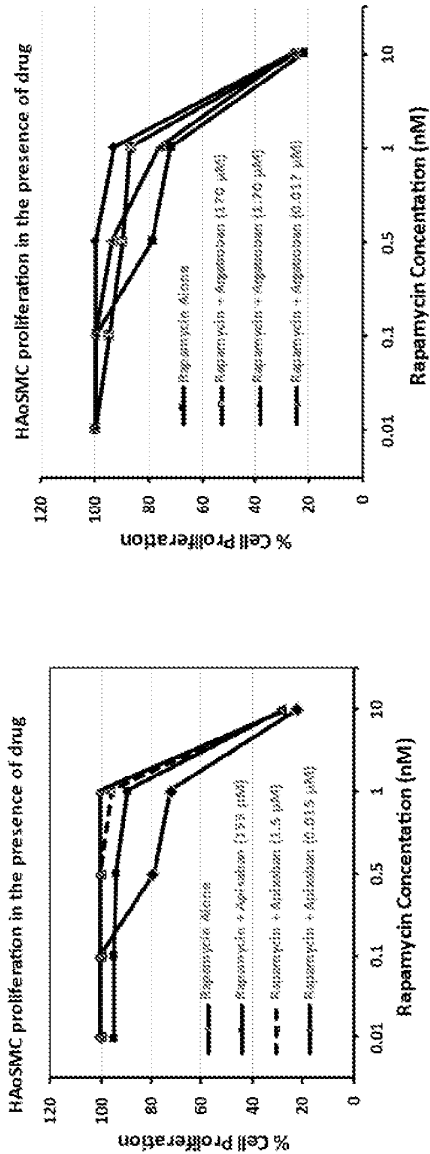


FIG. 1A

FIG. 1B

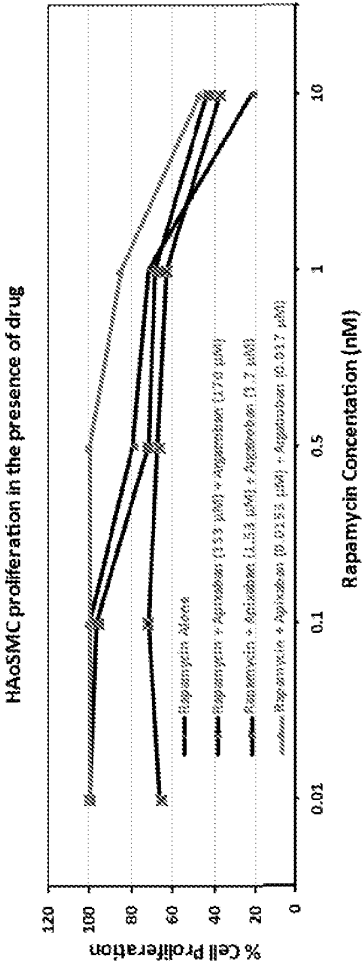
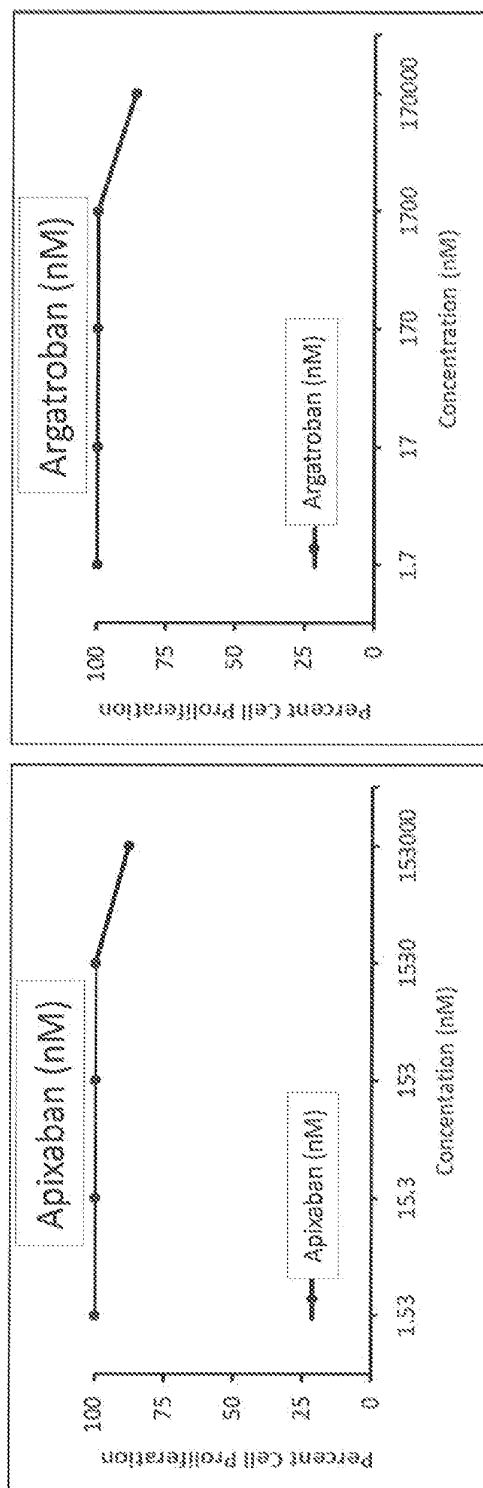


FIG. 1C



LEO.1E

FIG. 1D

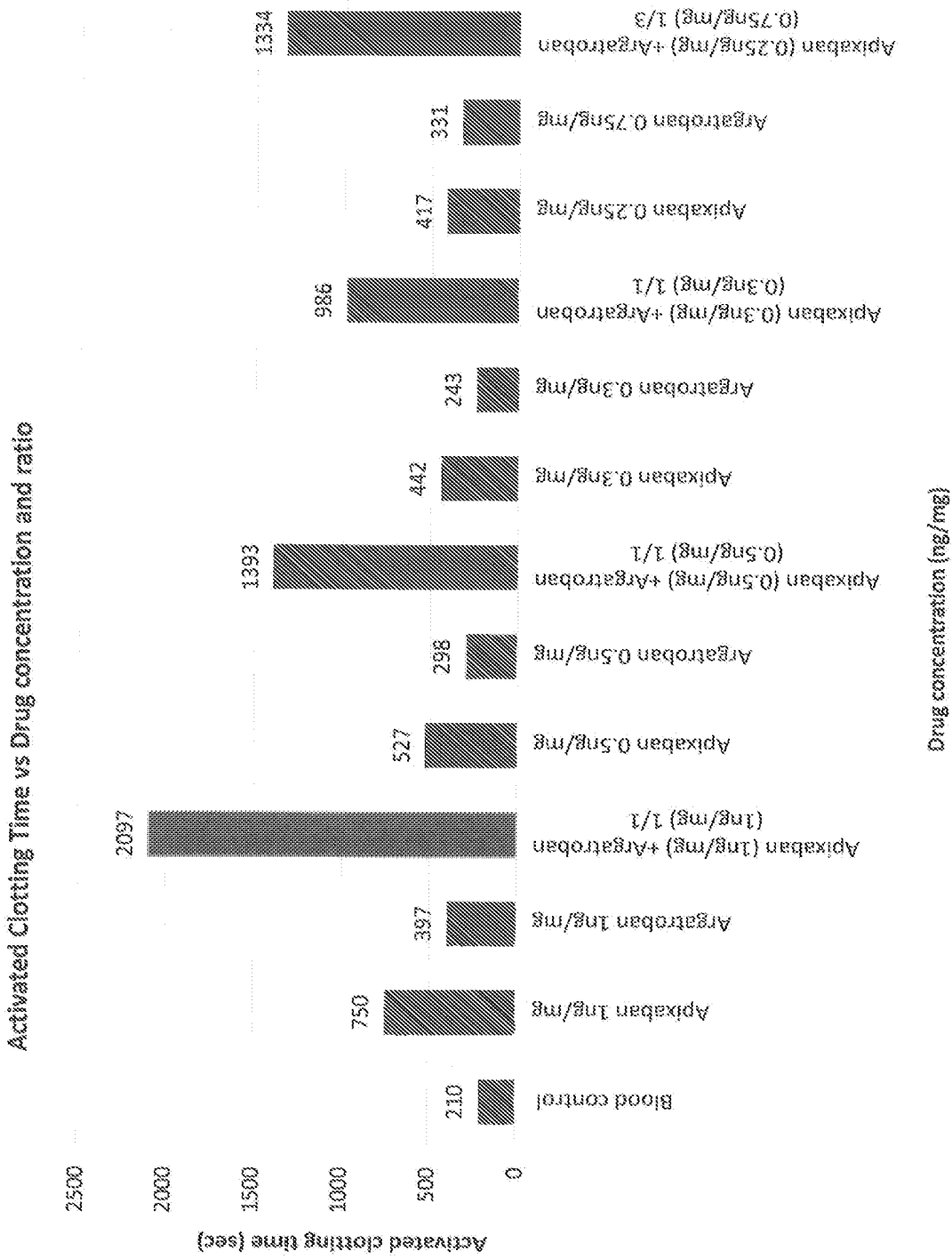


FIG. 2A

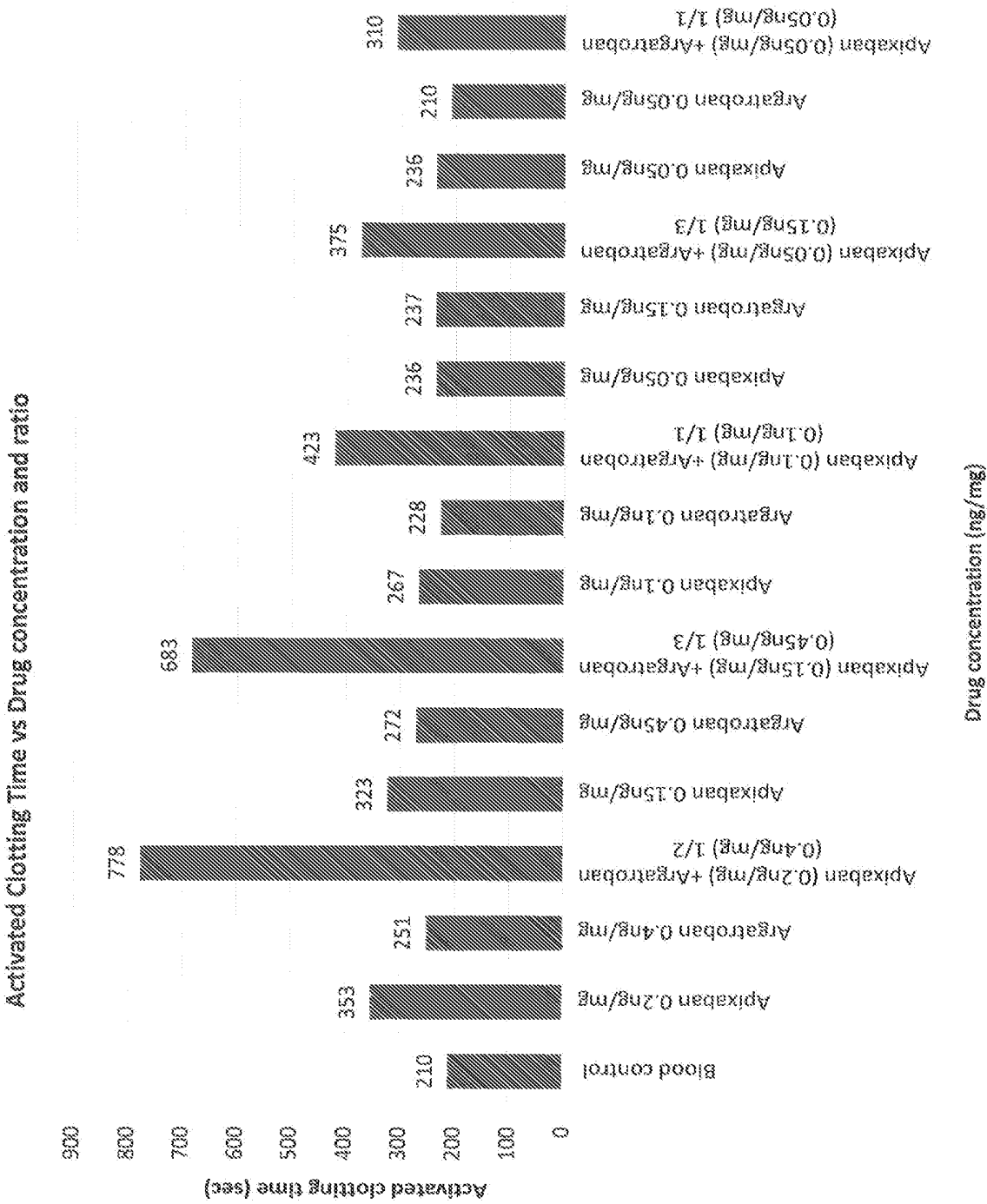


FIG. 2B

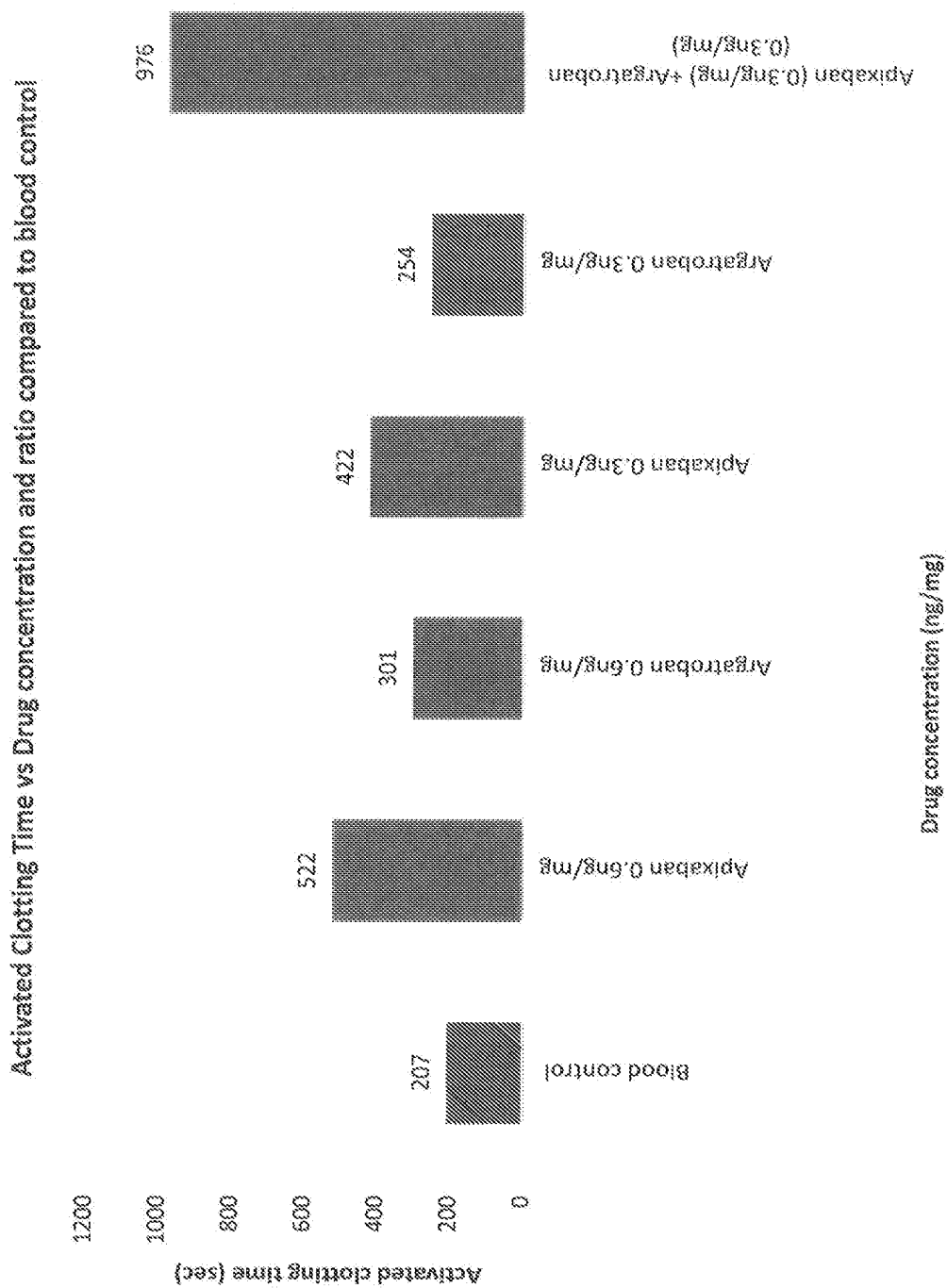


FIG. 2C

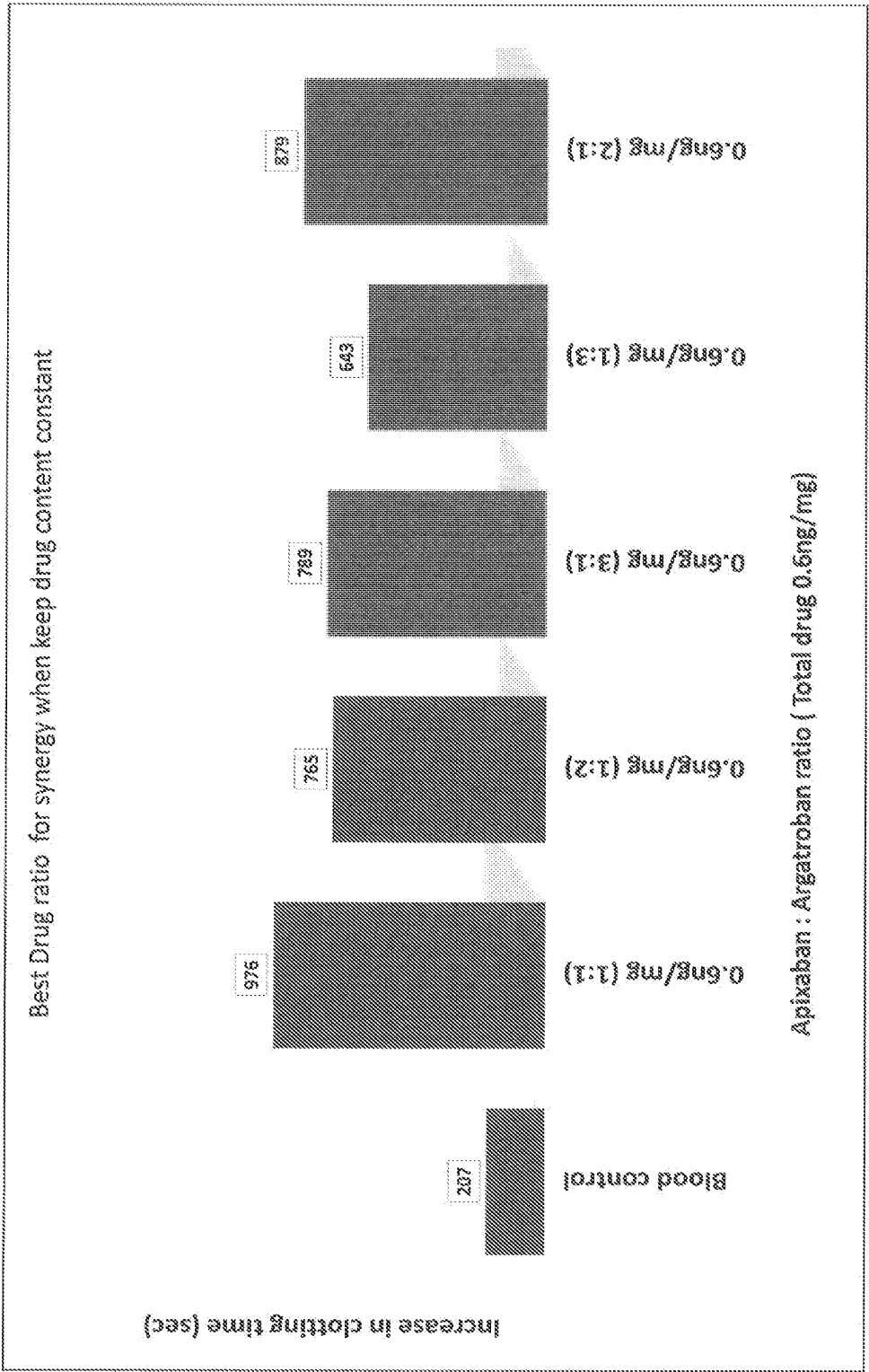


FIG. 2D

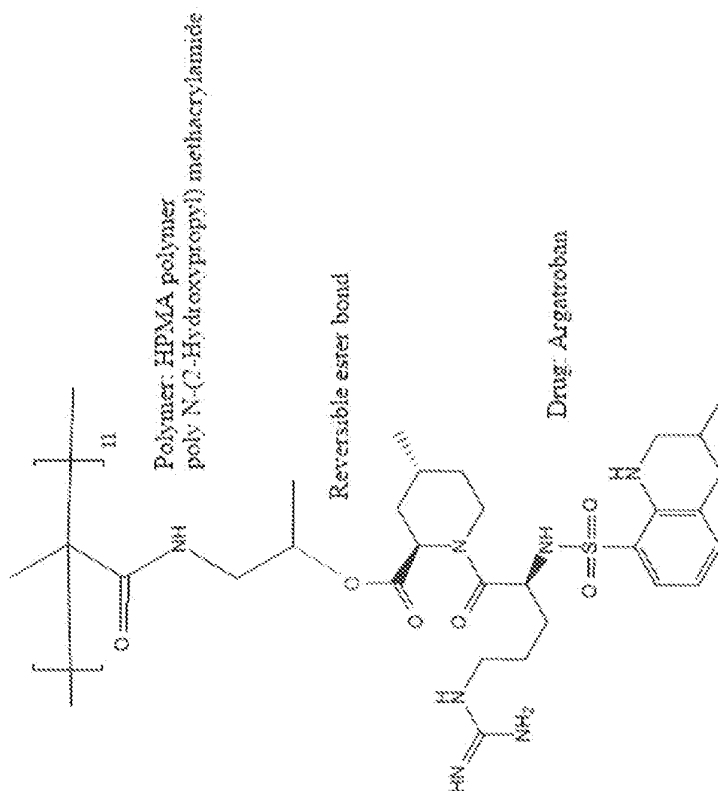


FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/49964

A. CLASSIFICATION OF SUBJECT MATTER IPC - A61K 9/00, A61K 31/47, A61K 31/727, A61P 7/02 (2021.01) CPC - A61K 9/0048, A61K 31/47, A61K 31/727, A61P 7/02 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) See Search History document Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched See Search History document Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) See Search History document		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,506,241 A (Mano et al.) 09 April 1996 (09.04.1996); entire document, especially abstract, col 2 lines 33-35, col 2 lines 53-55, col 2 lines 56-64	1-3, 105-107
X	US 2017/0291892 A1 (BAYER PHARMA AKTIENGESELLSCHAFT) 12 October 2017 (12.10.2017); entire document	1-3, 105-107
A	US 2012/0202232 A1 (Braun et al.) 09 August 2012 (09.08.2012); entire document	1-3, 105-107
A	US 2017/0056518 A1 (Immunwork Inc.) 02 March 2017 (02.03.2017); entire document	1-3, 105-107
A	US 2015/0056604 A1 (BIOVEC TRANSFUSION, LLC) 26 February 2015 (26.02.2015); entire document	1-3, 105-107
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "D" document cited by the applicant in the international application "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 16 November 2021		Date of mailing of the international search report DEC 20 2021
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300		Authorized officer Kari Rodriguez Telephone No. PCT Helpdesk: 571-272-4300

Form PCT/ISA/210 (second sheet) (July 2019)

PGR2026-00039
 Biocon Ex.1002
 Page 4044 of 4945

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/49964

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-104, 108-211
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Electronic Acknowledgement Receipt	
EFS ID:	45726674
Application Number:	17384070
International Application Number:	
Confirmation Number:	1061
Title of Invention:	High Concentration VEGF Receptor Fusion Protein Containing Formulations
First Named Inventor/Applicant Name:	Kenneth S. Graham
Customer Number:	147803
Filer:	Lance A. Termes/Brandi Henley
Filer Authorized By:	Lance A. Termes
Attorney Docket Number:	125984-266215
Receipt Date:	16-MAY-2022
Filing Date:	23-JUL-2021
Time Stamp:	19:01:58
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	10430US02_IDS-Transmittal.pdf	86937	no	3
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Warnings:

Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	10430US02_IDS-SB08.pdf	127108	no	3
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Information:					
This is not an USPTO supplied IDS fillable form					
3	Foreign Reference	434_FR-EP3640643B1.pdf	559346	no	30
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Information:					
4	Foreign Reference	435_FR-WO2021252647A1.pdf	4001357	no	97
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Warnings:					
Information:					
5	Foreign Reference	436_FR-WO2022008699A1.pdf	9863075	no	140
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Warnings:					
Information:					
6	Foreign Reference	437_FR-WO2022011323A1.pdf	15196986	no	287
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Warnings:					
Information:					
7	Foreign Reference	438_FR-WO2022013171A1.pdf	5213398	no	96
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Warnings:					
Information:					
8	Foreign Reference	439_FR-WO2022026510A1.pdf	5938963	no	127
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Warnings:					
Information:					

9	Foreign Reference	440_FR-WO2022038038A2.pdf	2801950	no	39
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Information:					
10	Foreign Reference	441_FR-WO2022056326A1.pdf	10865543	no	185
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Warnings:					
Information:					
Total Files Size (in bytes):			54654663		
<p>This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.</p> <p><u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.</p> <p><u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.</p> <p><u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.</p>					

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Kenneth S. Graham, et al. Application No.: 17/384,070 Filed: Jul-23-2021 For: HIGH CONCENTRATION VEGF RECEPTOR FUSION PROTEIN CONTAINING FORMULATIONS Customer No.: 147803	Confirmation No.: 1061 Examiner: Not Yet Assigned Art Unit: Not Yet Assigned SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR §1.97 and §1.98 Date of Transmission: May 16, 2022
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Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

The references cited on the attached IDS are being called to the attention of the Examiner. Copies of the references in compliance with the requirements of 37 CFR §1.98(a)(2) are submitted herewith.

It is respectfully requested that the cited references be expressly considered during the prosecution of this application, and the references be made of record therein and appear among the “references cited” on any patent to issue therefrom.

The cited references may include office actions from related cases. The Examiner is encouraged to review the full prosecution history from related cases. Applicant understands

that the prosecution history can be viewed electronically on PALM. Nevertheless, if the Examiner wishes Applicant to provide copies of any documents from related applications to the extent not already provided, he or she is requested to contact the undersigned.

Applicant cites the following applications, which are directed to related subject matter and are commonly owned or under an assignment obligation to be commonly owned:

Attorney Docket No.	Application No.	Filing Date	Publication No.	Status
125984-248591	62/669,506	05-10-2018	N/A	Expired
125984-248592	62/752,127	10-29-2018	N/A	Expired
125984-248593	62/769,876	11-20-2018	N/A	Expired
125984-248594	62/813,882	03-05-2019	N/A	Expired
125984-248595	16/409,631	05-10-2019	US 11,103,552	Issued
125984-248596	PCT/US2019/031879	05-10-2019	WO 2019/217927	Expired

As provided for by 37 CFR §1.97(g) and (h), no inference should be made that the information and references cited are prior art merely because they are in this statement and no representation is being made that a search has been conducted or that this statement encompasses all the possible relevant information.

Applicant believes that no fee is required for submission of this statement.

However, if a fee is required in connection with this paper, the Commissioner is authorized to deduct such fee from the undersigned's Deposit Account No. 500393.

Respectfully submitted,

/Lance A. Termes/

Lance A. Termes
Reg. No. 43184

Schwabe Williamson & Wyatt, PC
1211 SW 5th Avenue, Suite 1900
Portland, OR 97204
Tel: 503-222-9981

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (not for submission under 37 CFR 1.99)	Application Number	17/384,070
	Filing Date	Jul-23-2021
	First Named Inventor	Kenneth S. Graham
	Art Unit	Not Yet Assigned
	Confirmation Number	1061
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	125984-266215 / 10430US02

U.S. PATENTS						
Examiner Initial*	Cite No	Patent Number	Kind Code ¹	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear

U.S. PATENT APPLICATION PUBLICATIONS						
Examiner Initial*	Cite No	Publication Number	Kind Code ¹	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS								
Examiner Initial*	Cite No	Foreign Document Number ³	Country Code ²	Kind Code ⁴	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear	T ⁵

NON-PATENT LITERATURE DOCUMENTS				
Examiner Initial*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, pages(s), volume-issue number(s), publisher, city and/or country where published		T 5
	411	AVERY, "What is the evidence for systemic effects of intravitreal anti-VEGF agents, and should we be concerned?," Open Access, Br J Ophthalmol, Volume (98) i7–i10, (2014). [doi:10.1136/bjophthalmol-2013-303844].		
	412	HANNA et al., "Three patients with injection of intravitreal vascular endothelial growth factor inhibitors and subsequent exacerbation of chronic proteinuria and hypertension," Clinical Kidney Journal, 2019, vol. 12, no. 1, 92–100. [doi: 10.1093/ckj/sfy060].		
	413	HAYMAN et al., "VEGF Inhibition, Hypertension, and Renal Toxicity," Curr Oncol Rep. 2012 August ; 14(4): 285–294. [doi:10.1007/s11912-012-0242-z].		
	414	RAISER et al., "The effect of intravitreal bevacizumab (avastin) administration on systemic hypertension," Macmillan Publishers Limited, Eye (2009) 23, 1714–1718. [doi:10.1038/eye.2008.360; published online 12 December 2008].		

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (not for submission under 37 CFR 1.99)	Application Number	17/384,070
	Filing Date	Jul-23-2021
	First Named Inventor	Kenneth S. Graham
	Art Unit	Not Yet Assigned
	Confirmation Number	1061
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	125984-266215 / 10430US02

EXAMINER SIGNATURE			
Examiner Signature		Date Considered	
<p>*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.</p>			
<p>¹ See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. ² Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). ³ For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. ⁴ Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. ⁵ Applicant is to place a check mark here if English language translation is attached.</p>			

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <small>(not for submission under 37 CFR 1.99)</small>	Application Number	17/384,070
	Filing Date	Jul-23-2021
	First Named Inventor	Kenneth S. Graham
	Art Unit	Not Yet Assigned
	Confirmation Number	1061
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	125984-266215 / 10430US02

CERTIFICATION STATEMENT			
Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):			
☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).			
OR			
☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).			
☐ See attached certification statement.			
☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.			
☒ None			
SIGNATURE			
A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.			
Signature	/Lance A. Termes/	Date (YYYY-MM-DD)	2022-04-11
Name/Print	Lance A. Termes	Registration Number	43184

Electronic Acknowledgement Receipt	
EFS ID:	45446752
Application Number:	17384070
International Application Number:	
Confirmation Number:	1061
Title of Invention:	High Concentration VEGF Receptor Fusion Protein Containing Formulations
First Named Inventor/Applicant Name:	Kenneth S. Graham
Customer Number:	147803
Filer:	Lance A. Termes/Brandi Henley
Filer Authorized By:	Lance A. Termes
Attorney Docket Number:	125984-266215
Receipt Date:	11-APR-2022
Filing Date:	23-JUL-2021
Time Stamp:	17:29:56
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	10430US02_IDS-Transmittal.pdf	86944 dd5eeaa4724e8b1ccf4412aeb0f4f35afed07786	no	3

Warnings:

Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	10430US02_IDS-SB08.pdf	90508	no	3
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Information:					
This is not an USPTO supplied IDS fillable form					
3	Non Patent Literature	411_NPL-Avery2013.pdf	171717	no	4
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Warnings:					
Information:					
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Warnings:					
Information:					
6	Non Patent Literature	414_NPL-Raiser.pdf	99172	no	5
			35235afe211e4ef4fe818e08a2ece9329f5667a2		
Warnings:					
Information:					
Total Files Size (in bytes):			1895256		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Kenneth S. Graham, et al. Application No.: 17/384,070 Filed: Jul-23-2021 For: HIGH CONCENTRATION VEGF RECEPTOR FUSION PROTEIN CONTAINING FORMULATIONS Customer No.: 147803	Confirmation No.: 1061 Examiner: Not Yet Assigned Art Unit: Not Yet Assigned SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR §1.97 and §1.98 Date of Transmission: April 11, 2022
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Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

The references cited on the attached IDS are being called to the attention of the Examiner. Copies of the references in compliance with the requirements of 37 CFR §1.98(a)(2) are submitted herewith.

It is respectfully requested that the cited references be expressly considered during the prosecution of this application, and the references be made of record therein and appear among the “references cited” on any patent to issue therefrom.

The cited references may include office actions from related cases. The Examiner is encouraged to review the full prosecution history from related cases. Applicant understands

that the prosecution history can be viewed electronically on PALM. Nevertheless, if the Examiner wishes Applicant to provide copies of any documents from related applications to the extent not already provided, he or she is requested to contact the undersigned.

Applicant cites the following applications, which are directed to related subject matter and are commonly owned or under an assignment obligation to be commonly owned:

Attorney Docket No.	Application No.	Filing Date	Publication No.	Status
125984-248591	62/669,506	05-10-2018	N/A	Expired
125984-248592	62/752,127	10-29-2018	N/A	Expired
125984-248593	62/769,876	11-20-2018	N/A	Expired
125984-248594	62/813,882	03-05-2019	N/A	Expired
125984-248595	16/409,631	05-10-2019	US 11,103,552	Issued
125984-248596	PCT/US2019/031879	05-10-2019	WO 2019/217927	Expired

As provided for by 37 CFR §1.97(g) and (h), no inference should be made that the information and references cited are prior art merely because they are in this statement and no representation is being made that a search has been conducted or that this statement encompasses all the possible relevant information.

Applicant believes that no fee is required for submission of this statement.

However, if a fee is required in connection with this paper, the Commissioner is authorized to deduct such fee from the undersigned's Deposit Account No. 500393.

Respectfully submitted,

/Lance A. Termes/

Lance A. Termes
Reg. No. 43184

Schwabe Williamson & Wyatt, PC
1211 SW 5th Avenue, Suite 1900
Portland, OR 97204
Tel: 503-222-9981

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (not for submission under 37 CFR 1.99)	Application Number	17/384,070
	Filing Date	Jul-23-2021
	First Named Inventor	Kenneth S. Graham
	Art Unit	Not Yet Assigned
	Confirmation Number	1061
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	125984-266215 / 10430US02

U.S. PATENTS						
Examiner Initial*	Cite No	Patent Number	Kind Code ¹	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear

U.S. PATENT APPLICATION PUBLICATIONS						
Examiner Initial*	Cite No	Publication Number	Kind Code ¹	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS								
Examiner Initial*	Cite No	Foreign Document Number ³	Country Code ²	Kind Code ⁴	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear	T ⁵

NON-PATENT LITERATURE DOCUMENTS				
Examiner Initial*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, pages(s), volume-issue number(s), publisher, city and/or country where published		T 5
	407	COORS et al., "Polysorbate 80 in medical products and nonimmunologic anaphylactoid reactions," Ann Allergy Asthma Immunol, Volume (95): 593-599, (2005).		
	408	EMA et al., "Evaluation of Developmental neurotoxicity of polysorbate 80 in rats," Science Direct, Reproductive Toxicology, Volume (25): 89-99, (2008). [DOI: 10.1016/j.reprotox.2007.08.003]		
	409	GAJDOVA et al., "Delayed Effects of neonatal exposure to tween 80 on female reproductive organs in rats," Fd Chem.Toxic, Volume (31) No. (3): 183-190, (1993).		

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (not for submission under 37 CFR 1.99)	Application Number	17/384,070
	Filing Date	Jul-23-2021
	First Named Inventor	Kenneth S. Graham
	Art Unit	Not Yet Assigned
	Confirmation Number	1061
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	125984-266215 / 10430US02

NON-PATENT LITERATURE DOCUMENTS			
Examiner Initial*	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, pages(s), volume-issue number(s), publisher, city and/or country where published	T 5
	410	THACKABERRY et al., "Comprehensive Investigation of Hydroxypropyl Methylcellulose, Propylene Glycol, Polysorbate 80, and Hydroxypropyl-Beta-Cyclodextrin for use in General Toxicology Studies," TOXICOLOGICAL SCIENCES, Volume (117) No. (2): 485-492, (2010). [Downloaded from https://academic.oup.com/toxsci/article/117/2/485/1641380 by Williams Connolly user on 17 December 2021].	

EXAMINER SIGNATURE	
Examiner Signature	Date Considered
<p>*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.</p> <p>¹ See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. ² Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). ³ For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. ⁴ Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. ⁵ Applicant is to place a check mark here if English language translation is attached.</p>	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (not for submission under 37 CFR 1.99)	Application Number	17/384,070
	Filing Date	Jul-23-2021
	First Named Inventor	Kenneth S. Graham
	Art Unit	Not Yet Assigned
	Confirmation Number	1061
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	125984-266215 / 10430US02

CERTIFICATION STATEMENT			
Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):			
☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).			
OR			
☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).			
☐ See attached certification statement.			
☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.			
☒ None			
SIGNATURE			
A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.			
Signature	/Lance A. Termes/	Date (YYYY-MM-DD)	2022-01-04
Name/Print	Lance A. Termes	Registration Number	43184

Electronic Acknowledgement Receipt	
EFS ID:	44667468
Application Number:	17384070
International Application Number:	
Confirmation Number:	1061
Title of Invention:	High Concentration VEGF Receptor Fusion Protein Containing Formulations
First Named Inventor/Applicant Name:	Kenneth S. Graham
Customer Number:	147803
Filer:	Lance A. Termes/Brandi Henley
Filer Authorized By:	Lance A. Termes
Attorney Docket Number:	125984-266215
Receipt Date:	04-JAN-2022
Filing Date:	23-JUL-2021
Time Stamp:	18:33:01
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	10430US02_IDS-Transmittal.pdf	86914 b69fa9c9295bf0103a87825d7c4dea67d91fe0176	no	3

Warnings:

Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	10430US02_IDS-SB08.pdf	91069	no	3
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Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Non Patent Literature	407_NPL_COORS-2005.pdf	3391512	no	7
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Information:					
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Information:					
5	Non Patent Literature	409_NPL_GAJODVA-1993.pdf	11175464	no	8
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Information:					
6	Non Patent Literature	410_NPL_THACKABERRY-2010.pdf	145697	no	8
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Warnings:					
Information:					
Total Files Size (in bytes):			20870472		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

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New International Application Filed with the USPTO as a Receiving Office

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Kenneth S. Graham, et al. Application No.: 17/384,070 Filed: Jul-23-2021 For: HIGH CONCENTRATION VEGF RECEPTOR FUSION PROTEIN CONTAINING FORMULATIONS Customer No.: 147803	Confirmation No.: 1061 Examiner: Not Yet Assigned Art Unit: Not Yet Assigned SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR §1.97 and §1.98 Date of Transmission: January 4, 2022
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Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

The references cited on the attached IDS are being called to the attention of the Examiner. Copies of the references in compliance with the requirements of 37 CFR §1.98(a)(2) are submitted herewith.

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125984-248593	62/769,876	11-20-2018	N/A	Expired
125984-248594	62/813,882	03-05-2019	N/A	Expired
125984-248595	16/409,631	05-10-2019	US 11,103,552	Issued
125984-248596	PCT/US2019/031879	05-10-2019	WO 2019/217927	Published

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Applicant believes that no fee is required for submission of this statement.

However, if a fee is required in connection with this paper, the Commissioner is authorized to deduct such fee from the undersigned's Deposit Account No. 500393.

Respectfully submitted,

/Lance A. Termes/

Lance A. Termes
Reg. No. 43184

Schwabe Williamson & Wyatt, PC
1211 SW 5th Avenue, Suite 1900
Portland, OR 97204
Tel: 503-222-9981

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (not for submission under 37 CFR 1.99)	Application Number	17/384,070
	Filing Date	Jul-23-2021
	First Named Inventor	Kenneth S. Graham
	Art Unit	Not Yet Assigned
	Confirmation Number	1061
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	125984-266215 / 10430US02

U.S. PATENTS						
Examiner Initial*	Cite No	Patent Number	Kind Code ¹	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear

U.S. PATENT APPLICATION PUBLICATIONS						
Examiner Initial*	Cite No	Publication Number	Kind Code ¹	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS								
Examiner Initial*	Cite No	Foreign Document Number ³	Country Code ²	Kind Code ⁴	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear	T ⁵

NON-PATENT LITERATURE DOCUMENTS				
Examiner Initial*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, pages(s), volume-issue number(s), publisher, city and/or country where published	T 5	
	406	CHANG et al., "Ziv-Aflibercept as a Possible Alternative to Aflibercept," Medical Retina, Retina Today, Pages 67-68, (2014).		

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <small>(not for submission under 37 CFR 1.99)</small>	Application Number	17/384,070
	Filing Date	Jul-23-2021
	First Named Inventor	Kenneth S. Graham
	Art Unit	Not Yet Assigned
	Confirmation Number	1061
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	125984-266215 / 10430US02

EXAMINER SIGNATURE			
Examiner Signature		Date Considered	
<p>*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.</p>			
<p>¹ See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. ² Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). ³ For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. ⁴ Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. ⁵ Applicant is to place a check mark here if English language translation is attached.</p>			

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <small>(not for submission under 37 CFR 1.99)</small>	Application Number	17/384,070
	Filing Date	Jul-23-2021
	First Named Inventor	Kenneth S. Graham
	Art Unit	Not Yet Assigned
	Confirmation Number	1061
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	125984-266215 / 10430US02

CERTIFICATION STATEMENT			
<p>Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):</p> <p>☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).</p> <p>OR</p> <p>☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).</p> <p>☐ See attached certification statement.</p> <p>☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.</p> <p>☒ None</p>			
SIGNATURE			
<p>A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.</p>			
Signature	/Lance A. Termes/	Date (YYYY-MM-DD)	2021-12-17
Name/Print	Lance A. Termes	Registration Number	43184

Electronic Acknowledgement Receipt	
EFS ID:	44547156
Application Number:	17384070
International Application Number:	
Confirmation Number:	1061
Title of Invention:	High Concentration VEGF Receptor Fusion Protein Containing Formulations
First Named Inventor/Applicant Name:	Kenneth S. Graham
Customer Number:	147803
Filer:	Lance A. Termes/Brandi Henley
Filer Authorized By:	Lance A. Termes
Attorney Docket Number:	125984-266215
Receipt Date:	17-DEC-2021
Filing Date:	23-JUL-2021
Time Stamp:	12:54:06
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	10430US02_IDS-Transmittal.pdf	86915	no	3
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Warnings:

Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	10430US02_IDS-SB08.pdf	88825	no	3
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Information:					
This is not an USPTO supplied IDS fillable form					
3	Non Patent Literature	406_NPL-Chang_2014.pdf	182584	no	2
			dee9db6328bf2cd6c9956422bcd27cfca5636c7		
Warnings:					
Information:					
Total Files Size (in bytes):			358324		
<p>This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.</p> <p><u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.</p> <p><u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.</p> <p><u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.</p>					

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Kenneth S. Graham, et al. Application No.: 17/384,070 Filed: Jul-23-2021 For: HIGH CONCENTRATION VEGF RECEPTOR FUSION PROTEIN CONTAINING FORMULATIONS Customer No.: 147803	Confirmation No.: 1061 Examiner: Not Yet Assigned Art Unit: Not Yet Assigned SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR §1.97 and §1.98 <u>Date of Transmission: December 17, 2021</u>
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Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

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125984-248593	62/769,876	11-20-2018	N/A	Expired
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125984-248595	16/409,631	05-10-2019	US 11,103,552	Issued
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Respectfully submitted,

/Lance A. Termes/

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Reg. No. 43184

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Tel: 503-222-9981

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (not for submission under 37 CFR 1.99)	Application Number	17/384,070
	Filing Date	Jul-23-2021
	First Named Inventor	Kenneth S. Graham
	Art Unit	Not Yet Assigned
	Confirmation Number	1061
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	125984-266215 / 10430US02

U.S. PATENTS						
Examiner Initial*	Cite No	Patent Number	Kind Code ¹	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear

U.S. PATENT APPLICATION PUBLICATIONS						
Examiner Initial*	Cite No	Publication Number	Kind Code ¹	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear

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Examiner Initial*	Cite No	Foreign Document Number ³	Country Code ²	Kind Code ⁴	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear	T ⁵

NON-PATENT LITERATURE DOCUMENTS				
Examiner Initial*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, pages(s), volume-issue number(s), publisher, city and/or country where published		T 5
	402	Fauser S, Schwabecker V, Muether PS. Suppression of intraocular vascular endothelial growth factor during aflibercept treatment of age-related macular degeneration. Am J Ophthalmol. 2014;158:532-536.		
	403	Framme C, Eter N, Hamacher T, et al. Aflibercept for patients with neovascular age-related macular degeneration in routine clinical practice in Germany: Twelve month outcomes of PERSEUS. Ophthalmology Retina. 2018;2:539-549.		
	404	Khurana RN, Rahimy E, Joseph WA, et al. Extended (every 12 weeks or longer) dosing interval with intravitreal aflibercept and ranibizumab in neovascular age-related macular degeneration: post hoc analysis of VIEW trials. Am J Ophthalmol. 2019;200:161-168.		

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	Filing Date	Jul-23-2021
	First Named Inventor	Kenneth S. Graham
	Art Unit	Not Yet Assigned
	Confirmation Number	1061
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	125984-266215 / 10430US02

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Examiner Initial*	Cite Number	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, pages(s), volume-issue number(s), publisher, city and/or country where published	T 5
	405	Krohne TU, Liu Z, Holz FG, Meyer CH. Intraocular pharmacokinetics of ranibizumab following a single intravitreal injection in humans. Am J Ophthalmol. 2012;154:682-686	

EXAMINER SIGNATURE			
Examiner Signature		Date Considered	
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<p>¹ See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. ² Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). ³ For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. ⁴ Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. ⁵ Applicant is to place a check mark here if English language translation is attached.</p>			

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☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.			
☒ None			
SIGNATURE			
A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.			
Signature	/Lance A. Termes/	Date (YYYY-MM-DD)	2021-12-06
Name/Print	Lance A. Termes	Registration Number	43184

Electronic Acknowledgement Receipt	
EFS ID:	44445142
Application Number:	17384070
International Application Number:	
Confirmation Number:	1061
Title of Invention:	High Concentration VEGF Receptor Fusion Protein Containing Formulations
First Named Inventor/Applicant Name:	Kenneth S. Graham
Customer Number:	147803
Filer:	Lance A. Termes/Brandi Henley
Filer Authorized By:	Lance A. Termes
Attorney Docket Number:	125984-266215
Receipt Date:	06-DEC-2021
Filing Date:	23-JUL-2021
Time Stamp:	17:22:24
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	10430US02_IDS-Transmittal.pdf	86943 9c7420eb828b9eaa4f8ff3e67881e6f2259956	no	3

Warnings:

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This is not an USPTO supplied IDS fillable form					
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Warnings:					
Information:					
6	Non Patent Literature	405_NPL-Krohne-2012.pdf	710140	no	7
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Warnings:					
Information:					
Total Files Size (in bytes):			3403995		

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Kenneth S. Graham, et al. Application No.: 17/384,070 Filed: Jul-23-2021 For: HIGH CONCENTRATION VEGF RECEPTOR FUSION PROTEIN CONTAINING FORMULATIONS Customer No.: 147803	Confirmation No.: 1061 Examiner: Not Yet Assigned Art Unit: Not Yet Assigned SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR §1.97 and §1.98 <u>Date of Transmission: December 6, 2021</u>
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Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

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125984-248593	62/769,876	11-20-2018	N/A	Expired
125984-248594	62/813,882	03-05-2019	N/A	Expired
125984-248595	16/409,631	05-10-2019	US 11,103,552	Issued
125984-248596	PCT/US2019/031879	05-10-2019	WO 2019/217927	Published

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Reg. No. 43184

Schwabe Williamson & Wyatt, PC
1211 SW 5th Avenue, Suite 1900
Portland, OR 97204
Tel: 503-222-9981

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (not for submission under 37 CFR 1.99)	Application Number	17/384,070
	Filing Date	Jul-23-2021
	First Named Inventor	Kenneth S. Graham
	Art Unit	Not Yet Assigned
	Confirmation Number	1061
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	125984-266215 / 10430US02

U.S. PATENTS						
Examiner Initial*	Cite No	Patent Number	Kind Code ¹	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear
	231	5,580,856		12-03-1996	Prestrelski et al.	
	232	6,136,346		10-24-2000	Eljamal et al.	
	101	6,171,586		01-09-2001	Lam et al.	
	102	6,267,958		07-31-2001	Andya et al.	
	233	6,358,530		03-19-2002	Eljamal et al.	
	234	6,582,729		06-24-2003	Eljamal et al.	
	235	6,586,574		07-01-2003	Hansen	
	236	6,652,875		11-25-2003	Bannister	
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EXAMINER SIGNATURE			
Examiner Signature		Date Considered	
<p>*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.</p>			
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CERTIFICATION STATEMENT			
Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):			
☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).			
OR			
☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).			
☐ See attached certification statement.			
☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.			
☒ None			
SIGNATURE			
A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.			
Signature	/Lance A. Termes/	Date (YYYY-MM-DD)	2021-12-03
Name/Print	Lance A. Termes	Registration Number	43184

1. WO2021125852 - NOVEL INJECTABLE FORMULATION


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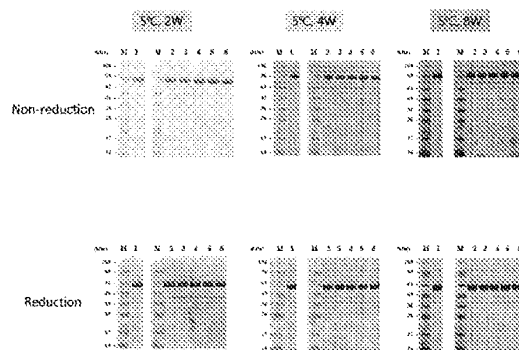
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Title

[EN] NOVEL INJECTABLE FORMULATION
[FR] NOUVELLE FORMULATION INJECTABLE
[KO] 신규 주사제 제형



Abstract

[EN] The present invention relates to a novel injectable composition, and provides an injectable composition containing a VEGFR extracellular domain-IgG Fc domain fusion protein, the injectable composition comprising, in an aqueous medium, a therapeutically effective amount of the VEGFR extracellular domain-IgG Fc domain fusion protein, a citric acid-sodium citrate buffer, sucrose, and two or more amino acids.

[FR] La présente invention concerne une nouvelle composition injectable, et fournit une composition injectable contenant une protéine de fusion domaine extracellulaire du VEGFR-domaine Fc de l'IgG, la composition injectable comprenant, dans un milieu aqueux, une quantité thérapeutiquement efficace de la protéine de fusion domaine extracellulaire du VEGFR-domaine Fc de l'IgG, un tampon acide citrique-citrate de sodium, du saccharose et au moins deux acides aminés.

[KO] 본 발명은 신규 주사제 조성물에 관한 것으로서, 수성 매질 중에 치료적으로 유효한 양의 VEGFR 수용체 세포외 도메인-IgG Fc 도메인 융합단백질, 구연산-소듐 시트레이트 완충제, 수크로오스, 둘 이상의 아미노산을 포함하는, VEGFR 수용체 세포외 도메인-IgG Fc 도메인 융합단백질 함유 주사제 조성물을 제공한다.

Related patent documents

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SPECIFICATION

NAME OF THE INVENTION

TECHNICAL FIELD

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 A 16B 16C 16D 16E 17A 17B 17C 17D 18 19 20 21 22A 22B 22C 22D 23A 23B 23C

SPECIFICATION



NOVEL INJECTION AGENT FORMULATION

TECHNICAL FIELD

- [1] The present invention relates to a novel injection formulation and, more specifically, to a syringe formulation containing a VEGFR receptor extracellular domain-bound GFC-domain fusion protein.

BACKGROUND ART

- [illegible]

TABLE 1. Detection Criteria of Microscopic Observable Particles

ITEM	NUMBER OF PARTICLES	
	$\geq 10 \mu\text{m}$	$\geq 25 \mu\text{m}$
SMALL AMOUNT OF INJECTION ($< 100 \text{ MZ}$)	$\leq 6,000/\text{container}$	$< 600/\text{container}$
Mass Injection ($> 100 \text{ meters}$)	$\leq 25/\text{M}$	$\leq 3/\text{M} \times 3$
OPHTHALMIC FORMULATION	$\leq 50/\text{M}$	$\leq 5/\text{M}$

- [4] In this regard, Korean Patent Laid-Open Patent No. 146811 discloses a formulation of an ophthalmic formulation and a lyophilized formulation having a specific content of an ionic isotonic agent selected from an organic co-solvent, sodium chloride or potassium chloride, a sodium phosphate buffer, sucrose, and the like, and a stabilizer such as sodium phosphate buffer and sucrose

DETAILED DESCRIPTION OF THE INVENTION

TECHNICAL PROBLEM

- [5] It is an object of the present invention to provide an injectable formulation comprising a more efficient and stable VEGFR receptor extracellular domain-conjugate Fc domain fusion protein, but the scope of the present invention is not limited by the purpose.

PROBLEM SOLVING MEANS

- [6] According to one aspect of the present invention, provided is an injectable composition containing a VEGFR receptor extracellular domain-conjugate domain fusion protein, comprising a therapeutically effective amount of a VEGFR receptor extracellular domain-KAPGC protein domain fusion protein, a citric acid-sodium citrate buffer, sucrose, and two or more amino acids in an aqueous medium.

EFFECT OF THE PRESENT INVENTION

- [7] The injection composition according to an embodiment of the present invention exhibits better stability compared to when a phosphate buffer is used, thereby having excellent long-term storage. Of course, the scope of the present invention is not limited by the effects.

BRIEF DESCRIPTION OF THE DRAWINGS

- [8] FIG. 1 is a graph showing results of analyzing 12 weeks through SEC-HPLC analysis of low-temperature long-term stability of an injection formulation candidate and a control (phosphate formulation) containing an applique prepared according to an embodiment of the present invention.
- [9] FIG. 2 is a series of gel photographs showing an analysis result of 12 weeks through SDS-PAGE specification analysis of a control (top) and reduction (bottom) by SDS-PAGE specification analysis of a control (top) and reduction condition (bottom), which are prepared according to an embodiment of the present invention.
- [10] FIG. 3 is a graph showing the results of two weeks analysis through SEC-HPLC analysis of storage stability at an acceleration condition (25° C.) of an injection formulation candidate and a control (phosphate formulation) containing an applique prepared according to an embodiment of the present invention.
- [11] FIG. 4 is a series of gel photographs showing a result of analyzing storage stability at an acceleration condition (25° C.) of an injection formulation candidate and a control (phosphate formulation) containing an applique prepared according to an embodiment of the present invention, through SDS-PAGE specification analysis of reduction (top) and

reduction conditions (bottom).

- [12] FIG. 5 is a graph showing the results of two weeks analysis through SEC-HPLC analysis of storage stability at a severe condition (40° C.) of a control (phosphate formulation) and an injection formulation candidate including an applique prepared according to an embodiment of the present invention.
- [13] FIG. 6 is a series of gel photographs showing the results of two-week analysis through SDS-PAGE specification analysis of reduction (top) and reduction (bottom) of storage stability at a severe condition (40° C.) of a control (upper) and a reduction condition (lower end), which are prepared according to an embodiment of the present invention.
- [14] FIG. 7 is a graph showing results of analyzing 12 weeks through SEC-HPLC analysis of low-temperature long-term stability of a control (phosphate formulation) candidate and a control (phosphate formulation) containing an applique prepared according to an embodiment of the present invention.
- [15] FIG. 8 is a series of gel photographs showing the results of analysis of the low-temperature long-term stability of an injectable formulation candidate and a control (phosphate formulation), which are prepared according to an embodiment of the present invention, through SDS-PAGE specification analysis of non-reduction (top) and reduction conditions (bottom).
- [16] FIG. 9 is a graph showing results of analyzing four weeks through SEC-HPLC analysis of storage stability at an acceleration condition (25° C.) of an injection formulation candidate and a control (phosphate formulation) containing an applique prepared according to an embodiment of the present invention.
- [17] FIG. 10 is a series of gel photographs showing results of analysis of storage stability at an acceleration condition (25° C.) of an adjuvant-containing injectable formulation candidate and a control (phosphate formulation) prepared according to an embodiment of the present invention, through SDS-PAGE specification analysis of reduction (top) and reduction conditions (bottom).
- [18] FIG. 11 is a graph showing the results of two weeks analysis through SEC-HPLC analysis of storage stability at a severe condition (40° C.) of a control (phosphate formulation) and an injection formulation candidate including an applique prepared according to an embodiment of the present invention.
- [19] FIG. 12 is a series of graphs showing results of analyzing the results of the results of analyzing the results of the results obtained by analyzing the results of the results obtained by analyzing the results of the results analyzed for three months in the conditions of freezing (Control, left ear), Long-term, right ear, Accelerated condition (left ear), and severe conditions of a Control (phosphate formulation, # 5), which are prepared according to an embodiment of the present invention.
- [20] FIG. 13 is a series of graphs showing results obtained by analyzing an osmotic concentration in a Control (left ear), a low-temperature condition (left ear), a Long-term (right ear), an Accelerated condition (left ear), and a harsh condition of a Control (phosphate formulation, # 5), which are prepared according to an embodiment of the present invention.
- [21] FIG. 14 is a series of graphs showing results analyzed for six months with UV spectroscopy analysis at which a protein content in a Control (left ear), a low temperature condition (left ear), an Accelerated condition (left ear), a cold condition (left ear), an Accelerated condition (left ear), and a severe condition (streamer, right ear), which are prepared according to an embodiment of the present invention, is quantified by using 280 nm absorbance.
- [22] FIG. 15 is a series of graphs showing results of analyzing protein content at 6 months using SEC-HPLC.
- [23] FIG. 16A is a series of gel photographs showing the results analyzed through SDS-PAGE specification analysis of non-reduction (left) and reduction conditions (right) after the formulation initial stability of the formulation candidates (# 1 to # 4) and the Control (phosphate formulation, # 5) prepared according to an embodiment of the present invention is analyzed through SDS-PAGE specification analysis of reduction (left) and reduction conditions (right), and FIG. 16C is a series of gel photographs showing results analyzed by SDS-PAGE specification analysis of a reduction (left) and a reduction (right). In addition, FIG. 16D is a series of gel photographs showing results analyzed through SDS-PAGE specification analysis of non-reduction (left) and reduction conditions (right) after storing three months in an Accelerated condition and a severe condition, and FIG. 16E is a series of gel photographs showing results analyzed through SDS-PAGE specification analysis of non-reduction (left) and reduction conditions (right) after storing a Control (phosphate formulation, # 5) in an Accelerated condition for six months in an Accelerated condition, and FIG. 16F is a series of gel photographs showing a result analyzed by SDS-PAGE specification analysis of reduction (left) and reduction conditions (right).
- [24] FIG. 17A is a gel photograph showing a result of analyzing stability of a formulation after three months storage in a refrigerator condition and a Long-term condition, and FIG. 17C is a gel photograph showing a result of analyzing stability of a formulation after three months storage in an Accelerated condition and a severe condition, in addition, FIG. 17D is a series of gel photographs showing the results of analyzing the stability of the dosage form after six months of storage at a freezing condition (left), a low temperature condition (a center), and an Accelerated condition (right) in a refrigerator condition (left), a low temperature condition (a center), and an Accelerated condition (right) according to an embodiment of the present invention.
- [25] FIG. 18 is a series of graphs showing the results of analyzing the results of the results of analyzing the results of the results obtained by analyzing the results of the results obtained by analyzing the type of surfactant-containing injectable formulation candidates (A1-DS-2007-D) and A1-DS-200 D-200 D-1 and the control (phosphate formulation, A1-DS-2006-D) according to the type of the surfactant prepared in accordance with an embodiment of the present invention, and the results of analyzing the results of the results of analyzing the results of the results of the results analyzed for up to 6 months in the refrigeration conditions of the control (phosphate formulation, A1-DS-2006-D) and the control (phosphate formulation, A1-DS-2006-D).
- [26] FIG. 19 is a series of graphs showing results of analyzing an osmotic concentration at a maximum of 6 months in a refrigerant formulation candidate (A1-DS-2007D-1 and A1-DS-2008-D) and a control (phosphate formulation, A1-DS-200D-2008D) and a control (phosphate formulation, A1-DS-2006-D) according to the type of surfactant produced according to an embodiment of the present invention.
- [27] FIG. 20 is a series of graphs showing the results analyzed for up to 6 months by UV spectroscopy analysis at which a protein content in a Control (left ear), a Long-term, right ear, an Accelerated condition, a left ear, and a severe condition is quantified by using 280 nm absorbance according to the type of surfactant manufactured according to an embodiment of the present invention.
- [28] FIG. 21 is a series of graphs showing results of analyzing A protein content at A maximum of 6 months using SEC-HPLC.
- [29] FIG. 22A is a series of gel photographs showing the results analyzed through SDS-PAGE specification analysis of A Control (left) and A reduction condition (right) according to the type of surfactant prepared according to an embodiment of the present invention, and FIG. 22B is a series of gel photographs showing the results of analysis through SDS-PAGE specification analysis of A non-reduction (left) and A reduction condition (right) according to the type of surfactant manufactured according to an embodiment of the present invention. According to an embodiment of the present invention, FIG. 22C is a series of gel photographs showing an analysis result through SDS-PAGE specification analysis of a non-reduction (left) and a reduction condition (right) after storing a formulation candidate (A1-DS-2007D) and a control (phosphate formulation, A1-DS-200D-2008-D) and a control (phosphate formulation, A1-DS-2006-D) according to the type of a surfactant manufactured according to an embodiment of the present invention in an accelerated condition and an accelerated condition.
- [30] FIG. 23A is a gel photograph showing a result of analyzing the stability of formulation initial stability of a formulation candidate (A1-DS-200D-2007D) and a control (phosphate formulation, A1-DS-200D-2008-D) according to the type of a surfactant manufactured according to an embodiment of the present invention. In addition, FIG. 23C is a series of gel photographs showing the results of analyzing the stability of the formulation after six months of storage at a freezing condition, a long term-term and an accelerated condition, and analyzing the stability of the formulation after six months of storage in a refrigeration condition, a long term-term, and an accelerated condition according to the type of surfactant manufactured according to an embodiment of the present invention.

BEST FORM FOR THE PRACTICE OF THE INVENTION

[31] DEFINITION OF TERM

[32] The term used in the present document is defined as follows.

[33] The term "VEGFR extracellular domain-IgG Fc domain fusion protein" used in the present document is one of a VEGF antagonist which binds to VEGF as a fusion protein in the form of a VEGF receptor, VEGFR1 or VEGF binding site of VEGFR2, which is a VEGF binding site of VEGFR1 or VEGFR2, thereby inhibiting the function thereof. Representative examples of the present invention have been developed and sold under the development of ophthalmic injection agents for the treatment of age-related macular degeneration, such as "aflibercept".

[34] As used in the present document, the term "aflibercept" as used in the present document refers to a fusion protein comprising an extracellular domain of VEGFR1 protein or VEGFR2 protein fused to an Fc domain region of human Gcγ1, by name of an active ingredient contained in an ophthalmic injection agent which is developed by Regeneron Pharmaceuticals Corporation and sold as a macular degeneration therapeutic agent.

[35] As used in the present document, the term "bio-miller" refers to a copy of a bio-drug which can be produced by a third party which is not a patent right after the patent has expired, and is also referred to as a bio-drug, a biogenic, and the like. In general, it is possible to chemically synthesize the same component as a low molecular compound as a chemical synthetic agent. Accordingly, the medicine is referred to as a Generic drug. However, since the bio-drug is not chemically synthesized, it is possible to prepare a protein product of a polymer by using animal cells, yeast, *Escherichia coli*, etc. so that the same product cannot be duplicated. Therefore, the present invention has to be manufactured through living cells when producing such a bio-drug, and is not considered to simply produce a product which is completely identical to each other, and only a similar product can be produced. Therefore, the term "bio-miller" used in the present document has the same amino acid sequence information as the received biopharmaceutical product but is not prepared by the same process, but is recognized as a bio-syringe when the same effect as a blood test result is obtained through a clinical trial, and is not the same but is also referred to as a bio-syringe.

[36] As used in the present document, the term "surfactant" refers to a compound having both a hydrophilic portion that is easily dissolved in water and a hydrophobic portion that is easily dissolved in oil. Due to these properties, the present invention is used in soap or detergent, and is used for cosmetics or medicines. The surfactant includes an ionic surfactant and a nonionic surfactant, and the ionic surfactant is divided into a cationic surfactant and an anionic surfactant. The surfactant induces the emulsification of the lipid and the aqueous solution in the aspect of the purpose, and is also referred to as an emulsifier.

[37] As used in this document, the term "buffering agent" refers to a weak acid or a weak base used to constantly maintain the pH of the aqueous solution even after adding other acids or bases. Phosphate salts having a pKa value of 7.2, which are close to physiological pH, are widely used for biological and medical purposes.

[38] As used in this document, the term "isotonic agent" refers to the solute added to the pharmaceutical formulation to tailor the osmotic concentration similar to the osmotic pressure of the body fluid in vivo. The isotonic agent includes a non-ionic isotonic agent including an ionic isotonic agent such as sodium chloride and a saccharide such as sorbitol.

[39] DETAILED DESCRIPTION OF THE INVENTION

[40] According to one aspect of the present invention, provided is an injectable composition containing a VEGFR receptor extracellular domain-conjugate domain fusion protein, comprising a therapeutically effective amount of a VEGFR receptor extracellular domain-KAPGFC protein domain fusion protein, a citric acid-sodium citrate buffer, sucrose, and two or more amino acids in an aqueous medium.

[41] In the injection composition, the VEGFR knot may be VEGFR_2 or VEGFR2_3.

[42] In the injection composition,

[43] The fusion protein may be an applicator or a bio-syringe thereof.

[44] In the injection composition, the amino acid can be arginine and glycine.

[45] The injection composition may include 30-50 mg/ml of the fusion protein, 5-15 mmol of citric acid-sodium citrate buffer, 3-7 wt % of sucrose, 20-70 mmol of arginine, and 20-70 mmol of glycine.

[46] The pH of the injectable composition can be adjusted to 5.8 to 6.6.

[47] The injectable composition may or may not include an isotonic agent.

[48] In the injection composition, when the isotonic agent is further included, the isotonic agent may be sorbitol.

[49] The injection composition may include or may not include a surfactant.

[50] When the injection composition includes the surfactant, the surfactant may be included in 0.01-0.2 wt %/v %, and the surfactant may be Polysorbate 20, Polysorbate 80, or Poloxamer 188.

[51] The injectable composition can be used for ophthalmic applications, in which case the angiogenesis-related ophthalmic diseases can be diabetic macular edema, diabetic retinopathy, macular edema, or macular degeneration.

[52] The injectable compositions of the present invention may further comprise suitable carriers, excipients, and diluents commonly used in the preparation of pharmaceutical compositions. In addition, an additive for a liquid preparation can be used in the preparation of the injection composition. The additive for the formulation may be any of organic or inorganic.

[53] The therapeutically effective amount of the fusion protein can vary depending on the disease to be treated. In the case of an intraocular injection composition for treating macular degeneration, the therapeutically effective amount can be provided at a concentration of 40 mg/ml in the injection formulation, and in the case of a syringe for treating colorectal cancer, the injection formulation can be provided at a concentration of 25 mg/ml in a 4 mg/kg body.

[54] The dosage of the injectable composition of the present invention can be administered once or at a proper interval on day 1, or in two to three times at a proper interval, and can be administered at an interval of several days.

[55] The present inventors have completed the use of citric acid-sodium citrate as a buffer, arginine and glycine as a stabilizer, and having improved stability in low-temperature storage and acceleration conditions without an isotonic agent or a surfactant when sucrose is used as a stabilizer. The present inventors confirmed that the injection composition according to an embodiment of the present invention has a significantly improved storage stability at a low temperature condition (5° C.) and an acceleration condition (25° C.) as well as a low temperature condition (5° C.), as well as an acceleration condition (25° C.). Therefore, according to an embodiment of the present invention, the injection composition containing an applicator or a bio-miller containing the same can be used as an economical and efficient bio-miller formulation.



FORM FOR THE PRACTICE OF THE INVENTION

- [56] According to one aspect of the present invention, provided is an injectable composition containing a VEGFR receptor extracellular domain-conjugate domain fusion protein, comprising a therapeutically effective amount of a VEGFR receptor extracellular domain-KAPGFC protein domain fusion protein, a citric acid-sodium citrate buffer, sucrose, and two or more amino acids in an aqueous medium.
- [57] In the injection composition, the VEGFR knot may be VEGFR_2 or VEGFR2_3.
- [58] In the injection composition, the fusion protein may be an appliqué or a bio-synthetic thereof.
- [59] In the injection composition, the amino acid can be arginine and glycine.
- [60] The injection composition may include 30-50 mg/ml of the fusion protein, 5-15 mmol of citric acid-sodium citrate buffer, 3-7 wt % of sucrose, 20-70 mmol of arginine, and 20-70 mmol of glycine.
- [61] The pH of the injectable composition can be adjusted to 5.8 to 6.6.
- [62] The injectable composition may or may not include an isotonic agent.
- [63] In the injection composition, when the isotonic agent is further included, the isotonic agent may be sorbitol.
- [64] The injection composition may include or may not include a surfactant.
- [65] When the injection composition includes the surfactant, the surfactant may be included in 0.01-0.2 wt %/v %, and the surfactant may be Polysorbate 20, Polysorbate 80, or Poloxamer 188.
- [66] The injectable composition can be used for ophthalmic applications, in which case the angiogenesis-related ophthalmic diseases can be diabetic macular edema, diabetic retinopathy, macular edema, or macular degeneration.
- [67] The injectable compositions of the present invention may further comprise suitable carriers, excipients, and diluents commonly used in the preparation of pharmaceutical compositions. In addition, an additive for a liquid preparation can be used in the preparation of the injection composition. The additive for the formulation may be any of organic or inorganic.
- [68] The therapeutically effective amount of the fusion protein can vary depending on the disease to be treated. In the case of an intraocular injection composition for treating macular degeneration, the therapeutically effective amount can be provided at a concentration of 40 mg/ml in the injection formulation, and in the case of a syringe for treating colorectal cancer, the injection formulation can be provided at a concentration of 25 mg/ml to a 4 mg/kg body.
- [69] The dosage of the injectable composition of the present invention can be administered once or at a proper interval on day 1, or in two to three times at a proper interval, and can be administered at an interval of several days.
- [70] The present inventors have completed the use of citric acid-sodium citrate as a buffer, arginine and glycine as a stabilizer, and having improved stability in low-temperature storage and acceleration conditions without an isotonic agent or a surfactant when sucrose is used as a stabilizer when sucrose is used as a stabilizer. The present inventors confirmed that the injection composition according to an embodiment of the present invention has a significantly improved storage stability at a low temperature condition (5° C.) and an acceleration condition (25° C) as well as a low temperature condition (5° C.), as well as an acceleration condition (25° C). Therefore, according to an embodiment of the present invention, the injection composition containing an appliqué or a bio-mimetic containing the same can be used as an economical and efficient bio-mimetic formulation.
- [71] The present invention will now be described in more detail through an embodiment. However, the present invention is not limited to the embodiments disclosed below but can be implemented in a variety of different forms. The following examples are provided to complete the disclosure of the present invention and to fully inform a person skilled in the art.
- [72] EMBODIMENT
- [73] GENERAL METHOD
- [74] 1. PQI ANALYSIS
- [75] A method for measuring the potential generated in the ion exchange reaction of hydrogen ions generated on the surface of a glass electrode is provided to measure and confirm the result of initial and long-term, acceleration, and ad-hoc stability by using a PQI measuring device.
- [76] 2. Osmolality analysis
- [77] An osmotic pressure measuring device using an osmotic pressure measuring device is provided to measure and confirm the result of an early stage, an organ, an acceleration, and an ad-hoc stability by using an osmotic pressure measuring device using a method for obtaining a mass osmium concentration by using a phenomenon of dropping a coagulation point of a solution in which solute is dissolved in a solvent.
- [78] 3. UV SPECTROSCOPY
- [79] The protein content measurement using the UV-Vis-Vis spectrometer was measured using the principle that a proportional is established between the concentration of a unique amino acid sequence of the protein and the UV light absorbance. The absorbance was measured at 280 nm, and a background, such as purity, can be measured at 320,325,330, 335,340,345, and 350 nm after measuring the absorbance at 320,325,330, 335,340,345, and 350 nm.
- [80] 4. SIZE EXCLUSION HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS
- [81] When the primary peak position is determined to be different from the protein molecular weight difference, a TSK kappa-gel G3000SWXL (7.8 × 300 M Ω) column (TOSHO) was used to flow at a flow rate of 0.5 M/min at 0.2M sodium phosphate, 0.2 M-arginine chloride, P37.2 mobile phase conditions, and the absorbance at 280 nm was confirmed. The SEC-HPLC mathematical analysis value confirmed the % Area value of the main peak of the appliqué and compared with the result of the early stage, the long-term, the acceleration, and the ad-hoc stability.
- [82] 5. SDS-PAGE SPECIFICATION ANALYSIS
- [83] The results are compared by confirming SDS-PAGE, which is a method using the principle of being separated according to the size of the molecular weight on the polyacrylamide gel. In the analysis condition, electrophoresis was applied at 120 V using 4-12% Bis-Tris-Gel to confirm separation according to the molecular weight of the protein. At this time, the specimen is pre-treated with reduction and non-reduction conditions to compare the results of the initial, long-term, acceleration, and ad-hoc stability at the same time.

[84] 6 GEL-TYPE REF ASSAY

[85] A protein mixture is loaded on a gel-loaded gel and an electric field is applied to check isoelectric focusing (IEF), which is a principle in which the protein becomes NET CHARGE, and the result is compared. According to the analysis condition, PETA 3-10 IEF Protein Matrigel was used, and electrophoresis is applied for each step at 100 V, 200 V, and 500 V, and compared with the PQI range having the protein at the time of early, long-term, acceleration, and ad-hoc stability.

[86] METHOD FOR SEARCHING A FORMULATION OF AN INJECTION AGENT FOR AN APPLICATION OF AN APPLICATION

[87] The present inventors have to confirm a difference according to their concentration/ratio by adding 10 mM citric acid-sodium citrate to a basic buffer, and adding sugar (sucrose and mannitol), amino acid (arginine, glycine, histidine, methionine, proline, alanine, and glutamic acid), isotonic agent (sorbitol, mannitol, sodium chloride), and Polysorbate (80) as an excipient to confirm an excipient combination which effectively contributes to the stability of an applier (SEQ ID NO: 1) in a citric acid-sodium citrate buffer. In the case of concentration/proportion, the present invention has been found to have a difference in two numerical values for the purpose of screening, and the corresponding numerical values were randomly selected, and finally a corresponding excipient is replaced in the sample to confirm the corresponding PQI and osmolality (Table 2). A comparative analysis is performed through the results of PQI, osmotic concentration, differential scanning fluorescence measurement (DSF) analysis, with respect to a control (10 M citric acid-sodium citrate) 1 species and 28 type vehicle combinations (Table 2). In an analysis method used for initial screening purposes during formulation study, experiments were performed according to the manufacturer's instructions by using PROTEOSTAT ® Thermal Quick Shift Stability Assay Law (Engzo, Life, Sciences USA). When the protein is unfolded to form a polymer impurity aggregate, the PROTEOSTAT dye is bound to a polymer impurity aggregate, and the temperature at which the polymer impurity aggregate is detected is expressed as a degree (T_{AGG}) showing that the protein is structurally stable. Therefore, it is possible to analyze that T_{AGG} is higher than the control sample, which is structurally stable when a corresponding excipient is added to the control sample, and T_{AGG} is utilized as a relative comparison value. Through the above experiment, a control agent (10 M citric acid-sodium citrate buffer without an excipient) was confirmed to be 64.36, and an excipient having a T-AGG value higher than the control group in each combination was selected as two types. As a result, in an amino acid group, sucrose and trehalose are selected as candidate materials such as sodium chloride and sorbitol in an amino acid group, sodium chloride and sorbitol in an isotonic group.

[88] METHOD FOR SCREENING AN EXCIPIENT AND A RESULT THEREOF

ITEM	EXCIPIENT	SAMPLE NUMBER	CONCENTRATION / RATIO	PADDY TUNNEL	OSMOTIC CONCENTRATION (PCT Rule 43bis.1/kg)	DSF ANALYSIS (Tagg tar)
CONTROL GROUP	N/A HINGE	1	-	6.35	32	
	SUCROSE	2	5% MACHINE * FOR IMPROVING THE DURABILITY OF A	6.40	186	65.80
		3	2%	6.35	90	64.65
		4	5%	6.37	63	64.36
	MANNITOL	5	2%	6.33	46	62.29
		6	5%	6.35	100	64.73
	TREHALOSE	7	2%	6.33	57	64.17
SURFACTANT	Polysorbate 20	8	0.05%	6.33	36	64.67
		9	0.03%	6.34	33	64.67
	Polysorbate ® 80	10	0.05%	6.35	33	65.55
		11	0.03%	6.32	32	65.36
AMINO ACID	NON-POLAR AMINO ACID	14	50 MDIO	6.00	83	64.67
		15	150 MDIO	6.29	177	64.61
		22	50 MDIO	6.31	83	64.86
		23	150 MDIO	5.97	185	64.61
		18	50 MDIO	5.76	85	65.11
		19	150 MDIO	6.28	188	64.42
		20	50 MDIO	6.18	82	64.92
		21	10 MDIO	6.31	46	64.48
	CHARGED AMINO ACID	16	50 MDIO	6.18	100	63.29
		17	150 MDIO	6.26	227	64.17
		12	50 MDIO	6.23	123	65.24
		13	150 MDIO	6.20	289	65.24
		24	10 MDIO	6.32	50	63.98
		25	2 MDIO	6.15	41	64.04
		26	50 MDIO	5.82	85	65.11
		27	150 MDIO	6.03	181	65.43
ISOTONIC AGENT	SODIUM CHLORIDE	28	50 MDIO	6.00	126	65.11
		29	150 MDIO	6.10	298	65.17

[89] w/v %

[90] As a result, as shown in Table 2, when sucrose and trehalose are used, a better numerical value can be confirmed in the result of DSF analysis, and in case of a surfactant, glycine and arginine are found to be effective, and in the case of an isotonic agent, glycine and arginine are found to be effective. In the case of an isotonic agent, sorbitol and sodium chloride are all suitable.

[91] COMBINATION OF VEHICLE AND VEHICLE

[92] From the results of Example 1, the present inventors designed 11 combinations by varying the composition of an excipient (Table 3). According to the present invention, an analysis is performed by comprising a control three species (10 M citric acid-sodium citrate buffer phosphate formulation and a histidine formulation) in a formulation composed like the design. The 11 combination forms an excipient concentration range of 2.5-20% sucrose, 5% trehalose, 40-100 mM sodium chloride, 50-100 M-arginine/Arg⁺-pepper, 40 m@sorbitol, 20-80 m@glycine, 0.03-0.1% Polysorbate-20, 0.03% Polysorbate ® 80. The concentration of the applierate was prepared by the following composition at 37-44 mg/ml, p205.9-6.2, and the results of Example 1 were arbitrarily adjusted with reference to the results of Example 1 to confirm Osmolality and DSF analysis results.

[93] COMBINATION OF VARIOUS EXCIPIENTS AND RESULTS THEREOF

SAMPL E	FORMULATION INFORMATION				OSMOTIC CONCENTR ATION (PCT Rule 43bis.1/kg)	DSF ANALYSIS (Tagg tar)
	Na ⁺ drop	PADD Y TUNNEL	CONCENTR ATION	ANAL YSIS		
		EL				

NUMBER		(Mg/Min)			
1	10 M-citric acid-sodium citrate, 5% sucrose, 40 M sodium chloride, 0.03% Polysorbate 20	38.24	6.11	300	65.33
2	10 M-citric acid-sodium citrate, 29% sucrose, 100 mM sodium chloride, 0.1% Polysorbate 20	37.03	5.92	1035	67.47
3	10 M-citric acid-sodium citrate, 2.5% sucrose, 0.01% Polysorbate 20	39.07	6.19	142	64.25
4	10 M-citric acid-sodium citrate, 5% sucrose, 100 mM sodium chloride, 0.03% Polysorbate @ 80	40.30	6.13	373	65.39
5	10 M-citric acid-sodium citrate, 5% sucrose, 50 M-arginine, 50 mmol glycine, 0.03% Polysorbate @ 80	42.73	6.06	321	66.08
6	10 M-citric acid-sodium citrate, 2.5% sucrose, 40 mM sorbitol, 40 mmol glycine, 0.03% Polysorbate @ 80	43.68	6.02	371	65.26
7	10 M-citric acid-sodium citrate, 5% sucrose, 100 M-arginine, 0.03% Polysorbate 20	38.83	6.22	551	64.95
8	10 M-citric acid-sodium citrate, 5% trehalose, 100 mM sodium chloride, 0.03% Polysorbate 20	42.31	6.04	362	65.45
9	10 The method comprises the steps of: mixing citric acid-sodium citrate, 5% trehalose, 40 mmol sorbitol, 20 mmol glycine, 0.03% Polysorbate 20	38.25	6.18	380	66.21
10	10 M-citric acid-sodium citrate, 5% sucrose, 40 mM sodium chloride, 50 M-arginine, 0.03% Polysorbate @ 80	37.99	6.07	351	66.09
11	10 M-citric acid-sodium citrate, 5% sucrose, 40 M sorbitol, 0.03% Polysorbate 20	40.30	6.17	423	66.28
CONTR OL GROUP 1	10 SODIUM CITRATE-SODIUM CITRATE	38.50	6.21	37	64.35
CONTR OL GROUP 2	10 Sodium phosphate, 5% sucrose, 40 mM sodium chloride, 0.03% Polysorbate 20 (so called phosphate formulation)	41.09	6.04	278	65.83
CONTR OL GROUP 3	10 The present invention also relates to a method for manufacturing the same, wherein the method comprises the steps of: (a) preparing a steel sheet having a structure selected from the group consisting of MNU His/His VII-Dowanol, 2.5% sucrose, 0.01% Polysorbate D20 (SO-Bar, Histidine Formulation)	38.28	6.06	102	65.33

[94] As a result of the analysis, a candidate formulation combination having a high T-AGG result value compared to the control (Control 1 and Control 2) was identified as Sample No. 5,9,10, and 11. In the case of Sample No. 2, it has been found that T-AGG is the highest value, but in general, it has been found that the osmotic concentration is not suitable for use as an eye-type formulation, thereby being excluded from a future consideration. That is, the combination of the four excipients as follows was confirmed to have excellent control compared to the control group when the DSF analysis is analyzed.

[95] 2) trehalose, sorbitol, glycine, Polysorbate 20

[96] 3) sucrose, arginine/Arg-toluidine, sodium chloride, Polysorbate 80

[97] 4) sucrose, sorbitol, Polysorbate 20

[98] STORAGE STABILITY ANALYSIS OF CANDIDATE FORMULATIONS

[99] As shown in Table 3, the present inventors investigated storage stability at low temperature conditions (5°C and 12 weeks), acceleration conditions (25°C, 12 weeks), and severe conditions (40°C and 2 weeks) by selecting six candidate formulations as shown in Table 3.

[100] COMPOSITION OF FORMULA CANDIDATE GROUP

	SAMPLE NUMBER	SELECTED EXCIPIENT COMPOSITION
CONTROL GROUP	1	10 Sodium phosphate, 5% sucrose, 40 mM sodium chloride, 0.03% Polysorbate 20 (Phosphate formulation)
FORMULATION CANDIDATE 1	2	10 M-citric acid-sodium citrate, 7% sucrose, 40 M sodium chloride, 0.1% Polysorbate 20
FORMULATION CANDIDATE 2	3	10 M-citric acid-sodium citrate, 5% sucrose, 50 M-Arg /Arg'-Technol, 50 mmol glycine, 0.03% Polysorbate @ 80
FORMULATION CANDIDATE 3	4	10 M-citric acid-sodium citrate, 5% trehalose, 100 mM sodium chloride, 0.03% Polysorbate 20
FORMULATION CANDIDATE 4	5	10 The method comprises the steps of: mixing citric acid-sodium citrate, 5% trehalose, 40 mmol sorbitol, 20 mmol glycine, 0.03% Polysorbate 20
FORMULATION CANDIDATE 5	6	10 M-citric acid-sodium citrate, 5% sucrose, 40 M-sodium chloride, 50 M-arginine/Arg-toluidine, 0.03% Polysorbate 80
FORMULATION CANDIDATE 6	7	10 M-citric acid-sodium citrate, 5% sucrose, 40 M sorbitol, 0.03% Polysorbate 20

[101] When the primary peak position is determined to be different from the protein molecular weight difference, 0.1 M potassium phosphate, 0.3 M sodium chloride, 10% acetonitrile, 0.1 M potassium phosphate, 0.3 M potassium chloride, 10% acetonitrile, and P36.8 movement phase conditions were used to confirm the effective peak of the applicant at 214 nm. The SEC-HPLC mathematical analysis value confirmed the % Area value of the main peak of the applicant and compared with the result of the early, long-term,



acceleration, and ad-hoc stability. An SDS-PAGE winning result is confirmed using a moving speed difference on a polyacrylamide gel by a protein molecular weight. In the analysis condition, electrophoresis is applied at 120 V using 4-12% Bis-Tris-Gel to confirm separation according to the molecular weight of the protein. As a result, as shown in FIG. 1, in a low-temperature storage condition, as shown in FIG. 1, in the SEC-HPLC analysis, the purity of the control group and all experimental groups was maintained to be 1% or less, and more particularly, three samples (Formulation Candidate 2) and 6 samples (Formulation Candidate 5) have the lowest reduction in purity. In addition, in the case of SDS-PAGE specification analysis, it is difficult to confirm a significant difference.

[102] Meanwhile, as shown in FIG. 3, the oil phase difference according to the formulation group has been confirmed, as shown in FIG. 3. In the case of the control group, the purity is maintained until the first main elapsed time, but the purity reduction is rapidly increased at the time of 2 weeks, while the test formulation candidate 2 (# 3), 3 (# 4), and 6 (# 5) of Table 3 of the present invention have significantly lower purity reduction compared to the control group and another formulation candidate group. On the other hand, as shown in FIG. 4, as shown in FIG. 4, the tendency of the cut form (low molecular weight, impurity) below the main band is significantly increased in the case of a control (phosphate formulation) or a first formulation candidate. In particular, in the case of a control group after 12 weeks, a band is almost eliminated in an initial main band position and a lower cut shape is confirmed. However, the formulation candidates according to one embodiment of the present invention have been confirmed to be little or almost no such phenomenon. As a result of the analysis, it can be seen that the formulation candidates according to one embodiment of the present invention have significantly higher room temperature stability compared to existing phosphate formulations.

[103] Finally, as a result of analysis at severe conditions (40° C.), as shown in FIG. 5, SEC-HPLC analysis showed that the degree of loss at 2 weeks is maintained within ± 30%. In this case, a significant difference according to the formulation group was confirmed, and a decrease in purity in the third sample (formulation candidate 2) can be confirmed to be the lowest. On the other hand, as shown in FIG. 6, as shown in FIG. 6, as shown in FIG. 6, in the case of a control sample as shown in FIG. 6, a low molecular weight cut shape is most severe, and the purity reduction of candidate formulations according to an embodiment of the present invention is lower than that of a control group.

[104] ANALYSIS OF CANDIDATE FORMULATIONS

[105] As a result of Example 3, since the total stability of the formulation candidate 2 (10 M-citric acid-sodium citrate, 5% sucrose, 50 M-arginine/Arg-threitol, 50 mmol glycine, 0.03% Polysorbate 80) has been confirmed to be highest, the present inventors designed 5% sucrose, 50 mM arginine, 50 mmol glycine as an amino acid, and 5% of a formulation candidate (5) in which the type and pH of the surfactant are changed (Table 5).

[106] COMPOSITION OF FORMULATION CANDIDATES

SAMPLE	SAMPLE NUMBER	FORMULATION INFORMATION	BIDET CAPABLE OF PREVENTING THE DAMAGE OF a
CONTROL GROUP	1	10 Sodium phosphate, 5% sucrose, 40 mM sodium chloride, 0.03% Polysorbate 2020 (PWH6.2)	PHOSPHATE FORMULATION
FORMULATION CANDIDATE 1	2	10 M-citric acid-sodium citrate, 5% sucrose, 50 M-arginine/Arg-toluidine, 50 m@glycine (PWH6.2)	The method comprises the steps of:
FORMULATION CANDIDATE 2	3	10 M-citric acid-sodium citrate, 5% sucrose, 50 M-arginine/Arg-toluidine, 50 mmol glycine, 0.03% Polysorbate @ 80 (PWH6.2)	Polysorbate @ 80
FORMULATION CANDIDATE 3	4	10 M-citric acid-sodium citrate, 5% sucrose, 50 M-arginine/Arg-toluidine, 50 M@glycine, 0.1% Polox-Amer 188 (PWH6.2)	Polox Amer 188
FORMULATION CANDIDATE 4	5	10 M-citric acid-sodium citrate, 5% sucrose, 50 M-arginine/Arg-toluidine, 50 mmol glycine, 0.03% Polysorbate @ 80 (P206.5)	Polysorbate @ 80, P206.5
FORMULATION CANDIDATE 5	6	10 M-citric acid-sodium citrate, 5% sucrose, 50 M-arginine/Arg-toluidine, 50 mmol glycine, 0.03% Polysorbate @ 80 (P205.9)	Polysorbate @ 80, P. 5.9

[107] The 5 kinds of formulation candidates designed as in Table 4 and the control (phosphate formulation) were irradiated with SEC-HPLC and SDS-PAGE specification analysis at a low temperature long-term stability between 12 weeks and an acceleration condition of 4 weeks (25° C.) and a severe condition (40° C.) of 2 weeks. As a result, as shown in FIGS. 7-11, significantly higher stability is exhibited in both the low temperature condition, the acceleration condition, and the severe conditions compared to the control group having the candidate formulation 5 of the present invention. Particularly, it has been confirmed that the stability of the formulation candidate 1, which is a surfactant, is highest, and it is possible to stably form the applicator even if the surfactant and the isotonic agent are not included. In particular, in the case of a phosphate formulation, stability in an acceleration condition has been confirmed to be significantly lower than that of the present invention, and thus excellent storage stability having the formulation of the present invention has been demonstrated.

[108] ANALYSIS OF LONG-TERM STORAGE STABILITY FOR ADDITIONAL CANDIDATE FORMULATIONS

[109] From the result of Example 3, it has been confirmed that the overall stability of formulation candidate 2 (10 M-citric acid-sodium citrate, 5% sucrose, 50 mM arginine/Arg-toluidine, 50 m@glycine, 0.03% Polysorbate 80) is highest. In consideration of the high osmolarity of formulation candidate 2, the present inventors have further prepared additional candidate formulations # 2 to # 4, which have a low concentration of arginine and glycine, which are amino acids, and then analyze various items such as stability of these additional candidate formulations (Table 6).

[110] COMPOSITION OF CANDIDATE FORMULATIONS

SAMPLE	SAMPLE NUMBER	FORMULATION INFORMATION	BIDET CAPABLE OF PREVENTING THE DAMAGE OF a
ADDITIONAL CANDIDATE FORMULATION # 1	1	10 M-citric acid-sodium citrate, 5% sucrose, 50 M-arginine/Arg-toluenediamine, 50 m@glycine (PWH6.2), 0.03% Polysorbate D20 (PWH6.2)	Polysorbate 20
ADDITIONAL CANDIDATE FORMULATION # 2	2	10 M-citric acid-sodium citrate, 5% sucrose, 30 M-arginine/Arg-toluidine, 30 m@glycine, 0.03% Polysorbate 2020 (PWH6.2)	Polysorbate 20
ADDITIONAL CANDIDATE FORMULATION # 3	3	10 M-citric acid-sodium citrate, 5% sucrose, 10 M-arginine/Arg-toluidine, 50 mmol glycine, 0.03% Polysorbate D20 (PWH6.2)	Polysorbate 20
ADDITIONAL CANDIDATE FORMULATION # 4	4	10 M-citric acid-sodium citrate, 5% sucrose, 50 M-arginine/Arg-toluidine, 10 m@glycine, 0.03% Polysorbate D20 (P206.2)	Polysorbate 20
ETHYLENE-A GUM FORMULATION	5	10 Sodium phosphate, 5% sucrose, 40 mM sodium chloride, 0.03% Polysorbate 2020 (PWH6.2)	Sodium PHOSPHATE FORMULATION

[111] As shown in Table 6, after preparing four types of candidate formulations and control (Sodium phosphate formulations), 278 µL of 0.5 M microtubes were sorted into 0.5 M microtubes, followed by cooling at a freezing condition (-20° C.), low temperature conditions (-5° C.), room temperature conditions (acceleration conditions, 25° C.), and severe conditions (40° C.) to a maximum of 6 months, followed by PDI, osmotic concentration, SDS-PAGE VII, Gel CHEMIEF ®, UV spectroscopy, and SEC-HPLC over time. As a result, as shown in FIG. 12, candidate formulations according to an embodiment of the present invention showed no significant variation of PDI from various temperature conditions to three months. In addition, as shown in FIG. 13, the osmotic concentration at various temperature conditions shows only a difference within 5% of the initial concentration, and thus it is possible to confirm that there is stability even in long-term storage between three months. In addition, as shown in FIG. 14, the analysis result of protein content through UV spectroscopy analysis has been confirmed to show differences in the range of 7% and 5%, respectively, compared to the initial content when stored.

for a long period of 6 months in the case of freezing conditions and low temperature conditions, and shows differences in the range of 6% and 14%, respectively, compared to the initial content in case of acceleration and severe conditions. In addition, as shown in FIG. 15, the initial comparison loss rate is confirmed to be less than 1% at the time of 6 months when the SEC-HPLC analysis result is stored, and the loss rate compared to the initial comparison is confirmed to be less than 2% even after six months of storage at low temperature conditions. On the other hand, the present invention has been found to have a high purity of candidate formulations # 2 and # 4 of the present invention than sample 5 (not shown) in the case of an accelerating condition, and the purity of all the samples tends to decrease overall in severe conditions, and the purity of the sample 5 (Sodium phosphate formulation) is slightly higher than that of the present invention.

[112] As shown in FIGS. 16A and 16B, as shown in FIGS. 16A and 16B, the candidate formulations and the Sodium phosphate formulation according to an embodiment of the present invention have been found to be different for each formulation after three months after storage and three months. However, as shown in FIG. 16C, in case of severe conditions, the cut-shaped bands have been confirmed somewhat, and the difference in formulation was not observed. This trend has been similarly shown for six months long-term storage, as identified in FIGS. 16D and 16E.

[113] The storage stability of the candidate formulations according to one embodiment of the present invention is confirmed to have an isoelectric point between P206.9 and 7.8 after formulation, as seen in FIG. 17A, and the difference in the pattern between formulations was not confirmed, and as confirmed in FIGS. 17B and 17C, the same amount of phase is observed under refrigeration conditions, low temperature conditions, and acceleration conditions, but in the case of severe conditions, the movement of the PQI value toward the acidic condition was observed. In addition, as shown in FIG. 17D, there is no significant difference in the freezing condition, the low temperature condition, and the band pattern in the acceleration condition, and no significant difference is confirmed by the formulation as shown in FIG. 17D.

[114] METHOD FOR ANALYZING EFFECT ACCORDING TO TYPE OF SURFACTANT

[115] The present inventors have confirmed that the concentration of arginine and glycine is slightly lowered through the results of Example 5, so that even if the osmotic concentration of the formulation is dropped, it has been confirmed that the long-term storage stability of the formulation does not have a great problem. Accordingly, the present inventors have to confirm the impact on stability when a Polysorbate @ 80 is used instead of the surfactant which is a surfactant used in Example 4.

[116] To this end, a long-term storage stability analysis is performed in the same manner as in Example 5 after being formulated in a configuration as in Table 7 below.

[117] COMPOSITION ACCORDING TO VARIATION OF SURFACTANT IN FORMULATION

FORMULATION NAME	FORMULATION COMPOSITION
A1-DS-200-D HINGE	10 Sodium phosphate, 5% sucrose, 40 mM sodium chloride, 0.03% Polysorbate 2020 (PWH6.2)
A1-DS-2007-D HINGE	10 M-citric acid-sodium citrate, 5% sucrose, 30 M-arginine/Arg-toluidine, 30 m@glycine, 0.03% Polysorbate 2020 (PWH6.2)
A1-DS-2008-D HINGE	10 M-citric acid-sodium citrate, 5% sucrose, 30 M-arginine/Arg-toluidine, 30 m@glycine, 0.03% Polysorbate @ 80 (PWH6.2)

[118] The formulations having the composition of Table 7 were also subjected to PQI, osmotic concentration, UV spectroscopy analysis, SEC-HPLC interpolation, SDS-PAGE analysis, and Geological analysis at 3 months and 6 months while keeping the samples in the same manner as in Example 5 for 6 months. First, as shown in FIG. 18, a Long-term stability of 6 months is confirmed in a range of 6.2 ± 0.2 , which is a target PQI, in the case of a freezing condition and an Accelerated condition. However, the Long-term stability is similar to the low temperature condition and the acceleration condition. In severe conditions, storage stability of 2 months is confirmed in the range of 6.2 ± 0.1 . The difference according to the formulation is not large.

[119] In addition, as a result of performing an osmotic concentration analysis, as shown in FIG. 19, it is possible to confirm that stability is maintained in a range of 13% of the initial comparison of the osmotic concentration after 6 months when the freezing condition is stored, and stability is maintained in a range of 11% compared to an initial value when storing a low temperature condition, and stability is maintained in a range of 13% compared to an initial value when the acceleration condition is stored. However, when a severe condition is stored, stability is maintained in a range of 15% compared to an initial period after two months, and stability is slightly lowered under severe conditions. According to the present invention, it is confirmed that there is no difference due to the formulation.

[120] In addition, as a result of the UV spectroscopy analysis using a 280 nm absorption wavelength, as shown in FIG. 20, the protein content in the freezing condition, the low temperature condition, and the acceleration condition is stored at a storage stability of 6 months in the 5% range of the initial comparison. However, in the case of severe condition storage like the osmotic concentration analysis, three months storage stability was confirmed in a range of 12% compared to the initial comparison.

[121] As a result, as shown in FIG. 21, all three formulations have similar results.

[122] As a result of performing SDS-ON-PAGE analysis, there was no difference in formulation initialization (FIG. 22A), which was true even in freezing conditions or low-temperature condition storage (FIG. 22B). Although there is no significant difference in stability even during long-term storage performed up to 3 months under acceleration conditions, the cut pieces have been confirmed after three months when storing severe conditions. However, the difference according to the formulation is not confirmed. As shown in FIG. 22C after six months, the cut pieces are not confirmed in the freezing condition and the low temperature condition storage, but the cut band is observed under the acceleration condition, and the difference due to the formulation is not confirmed.

[123] As a result of Gel Chemief assay, three months of freezing condition, low temperature condition, and acceleration condition storage are almost similar to the initial band pattern (FIG. 23A) (FIG. 23B), whereas in severe conditions, the band pattern tends to be moved to an acidic direction, and in this case, the difference is not confirmed. On the other hand, when storing six months, the freezing condition and the low temperature condition have the same pattern, and, in the case of an acceleration condition, the band pattern tends to move to an acidic direction in a very weak manner. Similarly, the difference caused by the formulation is not confirmed (FIG. 23C).

[124] As described above, according to an embodiment of the present invention, the injection formulation comprising an applique or a biocides thereof according to an embodiment of the present invention uses a citrate buffer instead of a phosphate buffer which has been conventionally used, and shows improvement in storage stability by oil under various conditions despite the addition of an isotonic agent or a surfactant. Therefore, according to an embodiment of the present invention, the injection formulation containing an applique can be used as a therapeutic agent for angiogenesis-related ocular diseases such as age-related macular degeneration.

INDUSTRIAL APPLICABILITY

[125] The injectable composition, according to one embodiment of the present invention, can be used as a therapeutic agent for treatment of angiogenesis-related ophthalmic diseases, such as ophthalmology retinopathy, age-related macular degeneration, and the like.

[126] SEQ ID NO: 1 is an amino acid sequence of an applique.

SCOPE OF THE CLAIMS

NON-ESTABLISHMENT OF OPINION WITH REGARD TO NOVELTY, INVENTIVE SA injectable composition containing a VEGFR receptor extracellular domain-dependent GFFFC protein domain fusion protein comprising a therapeutically effective amount of a VEGFR receptor extracellular domain-lavitrigen Fc domain fusion protein, a citric acid-sodium citrate buffer, sucrose, and two or more amino acids in an aqueous medium.

NON-ESTABLISHMENT OF OPINION WITH REGARD TO NOVELTY, INVENTIVE SThe injection composition of claim 1, wherein the VEGFR knot is VEGFR1 or VEGFR2.

NON-ESTABLISHMENT OF OPINION WITH REGARD TO NOVELTY, INVENTIVE SThe injection composition of claim 1, wherein the fusion protein is an applique or a bio-syringe thereof.

NON-ESTABLISHMENT OF OPINION WITH REGARD TO NOVELTY, INVENTIVE SThe injection composition of any one of claims 1 to 3, wherein the amino acid is arginine and glycine.

NON-ESTABLISHMENT OF OPINION WITH REGARD TO NOVELTY, INVENTIVE SThe injection composition of claim 4, wherein the injection composition comprises 30-50 mg/ml of the fusion protein, 5-15 mmol of citric acid-sodium citrate buffer, 3-7 wt % of sucrose, 20-70 mmol of arginine, and 20-70 mmol of glycine.

NON-ESTABLISHMENT OF OPINION WITH REGARD TO NOVELTY, INVENTIVE SThe injectable composition of claim 5, wherein the pH is adjusted to 5.8 to 6.6.

NON-ESTABLISHMENT OF OPINION WITH REGARD TO NOVELTY, INVENTIVE SThe injection composition of any one of claims 1 to 3, further comprising an isotonic agent.

NON-ESTABLISHMENT OF OPINION WITH REGARD TO NOVELTY, INVENTIVE SThe injectable composition of claim 7, wherein the isotonic agent is sorbitol.

NON-ESTABLISHMENT OF OPINION WITH REGARD TO NOVELTY, INVENTIVE SThe injection composition of any one of claims 1 to 3, wherein the injection composition does not include a surfactant.

NON-ESTABLISHMENT OF OPINION WITH REGARD TO NOVELTY, INVENTIVE SThe injection composition of claim 9, wherein the surfactant is contained in an amount of 0.01 to 0.2 wt/v %.

NON-ESTABLISHMENT OF OPINION WITH REGARD TO NOVELTY, INVENTIVE SThe injection composition of claim 9, wherein the surfactant is polysorbate 20, polysorbate 80, or poloxamer 188.

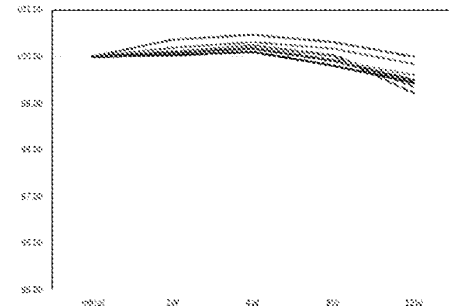
NON-ESTABLISHMENT OF OPINION WITH REGARD TO NOVELTY, INVENTIVE SThe injectable composition of any one of claims 1 to 3, wherein the injectable composition is used for ophthalmic treatment.

NON-ESTABLISHMENT OF OPINION WITH REGARD TO NOVELTY, INVENTIVE SA injectable composition according to claim 12, used in the treatment of angiogenesis-related ophthalmic diseases.

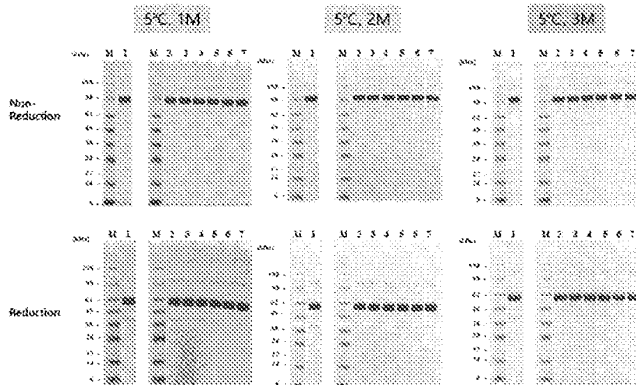
NON-ESTABLISHMENT OF OPINION WITH REGARD TO NOVELTY, INVENTIVE SThe composition of claim 13, wherein the angiogenesis-related ophthalmic disease is diabetic macular edema, diabetic retinopathy, macular edema, or macular degeneration.

DRAWING

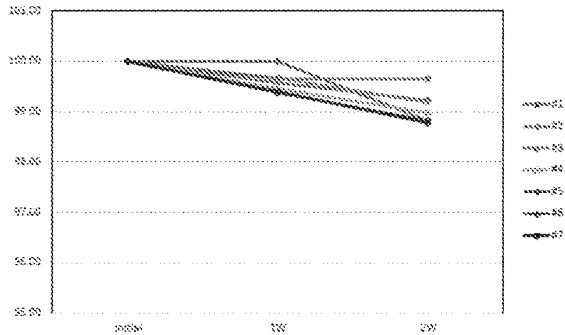
[도 1]



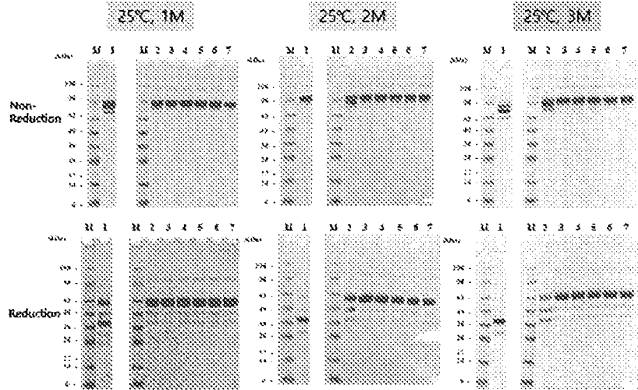
[도 2]



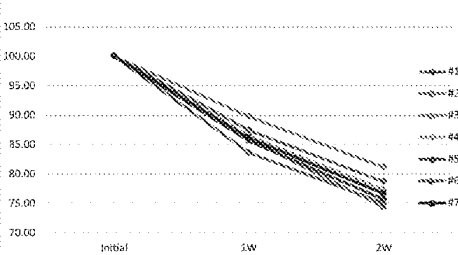
[E3]



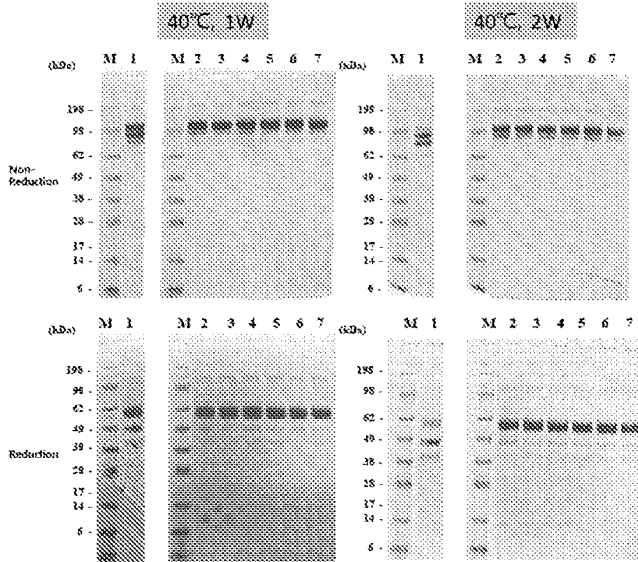
[E4]

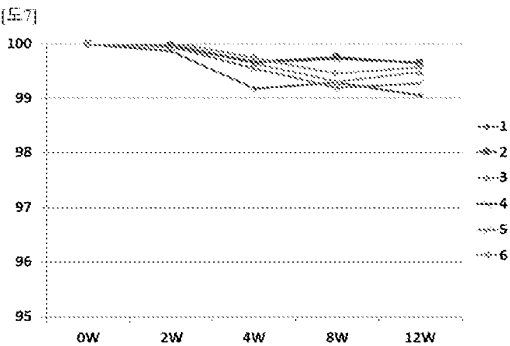
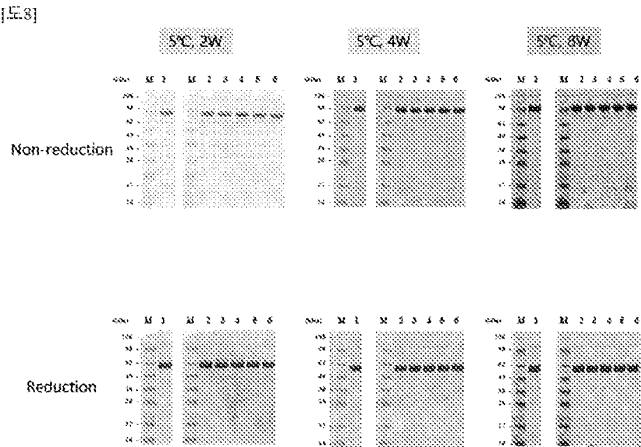
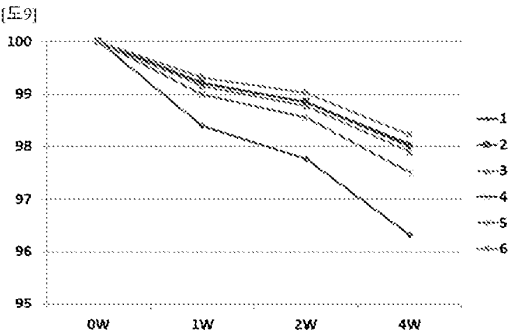
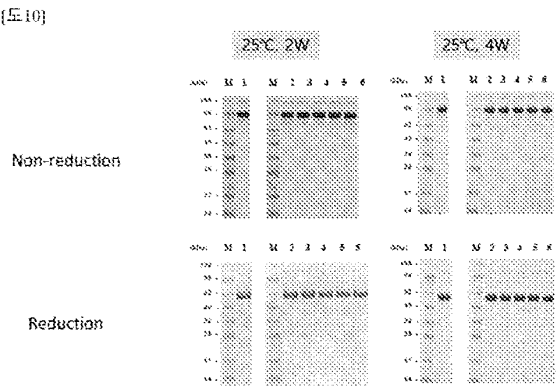


[E5]

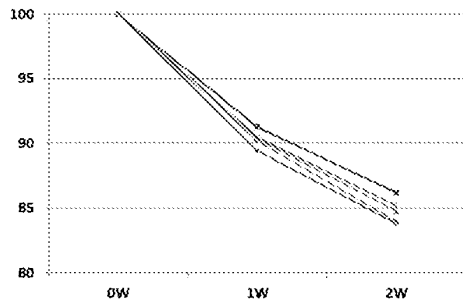


[E6]

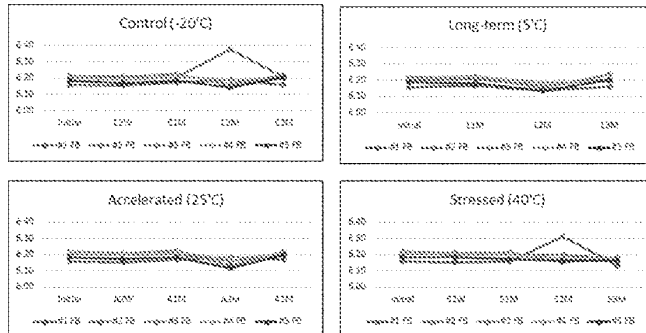




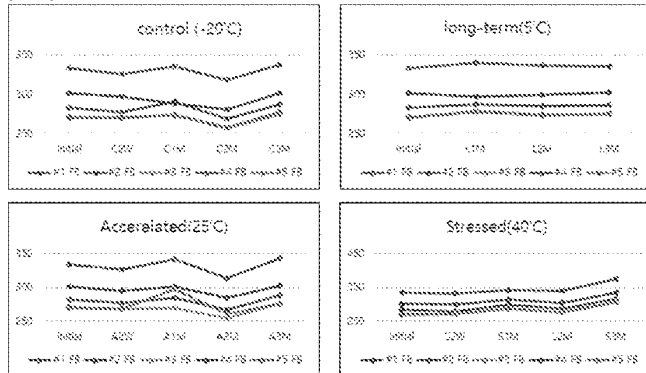
[도 11]



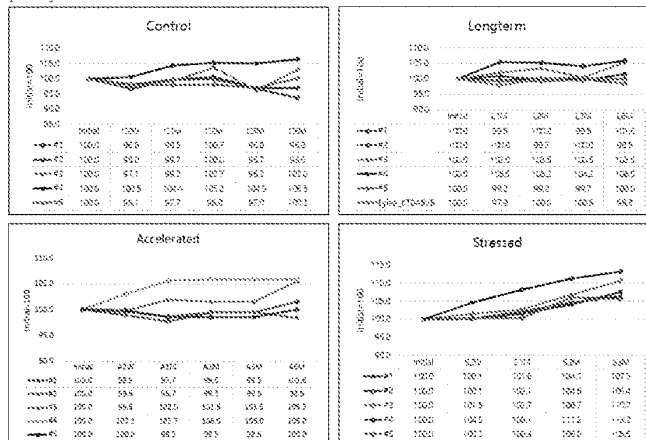
[도 12]



[도 13]



[도 14]



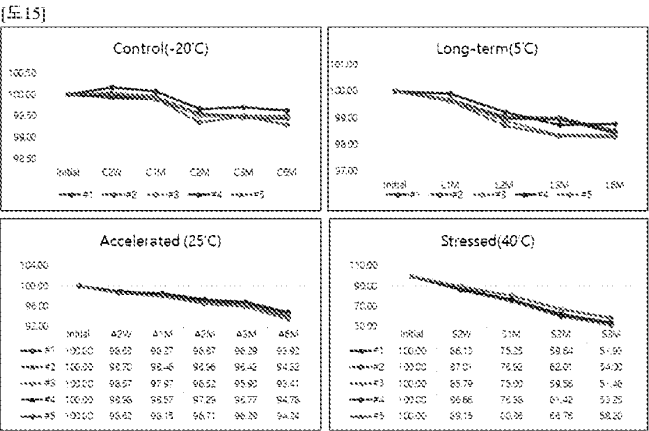
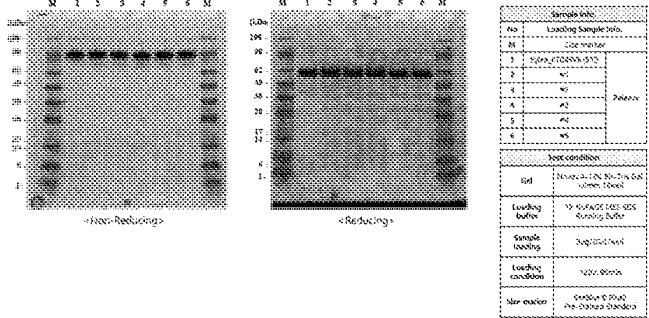
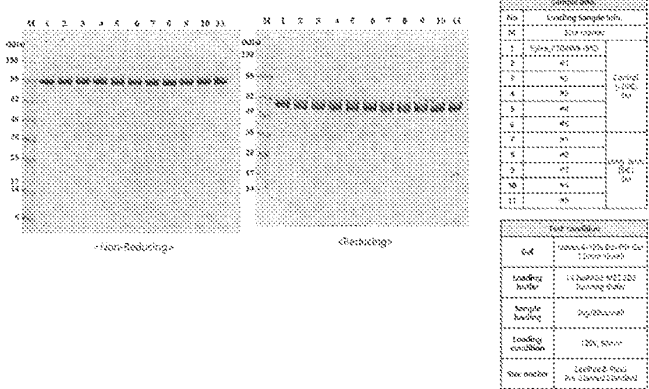


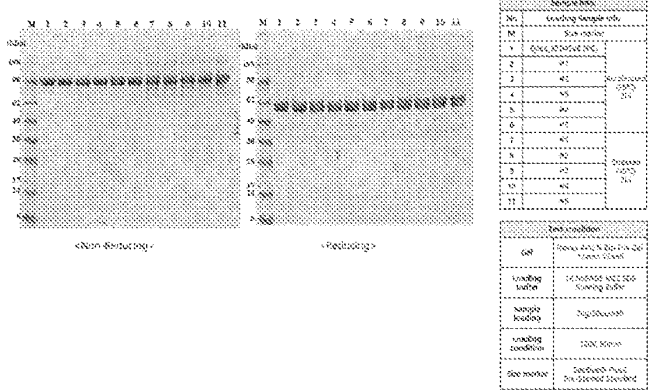
FIG. 16A



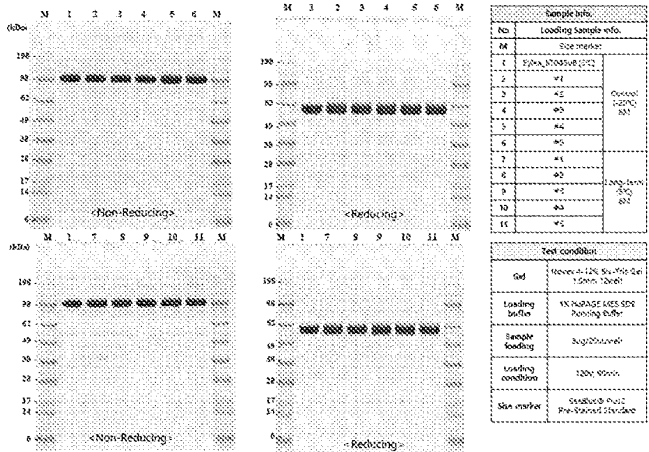
[FIG. 16B]



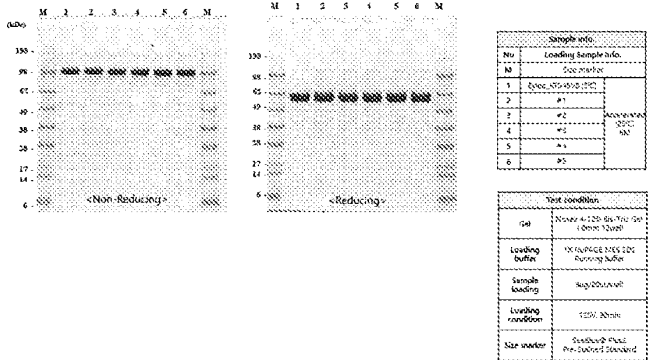
[FIG. 16C]



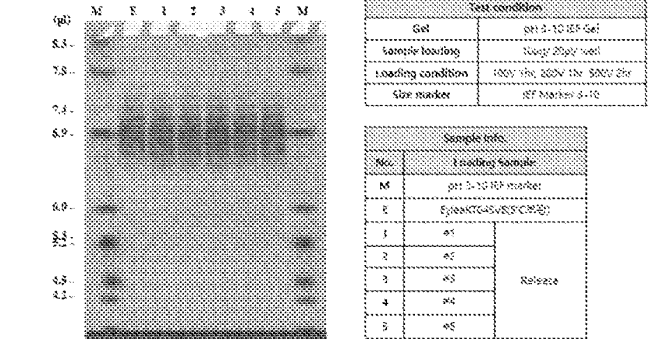
[FIG. 16D]



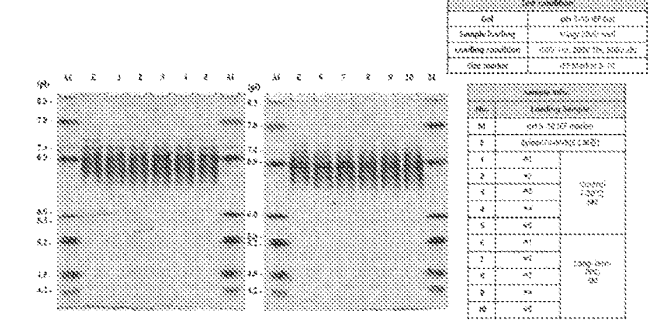
[FIG. 16E]



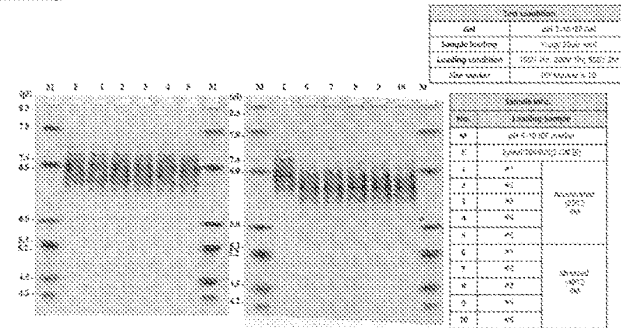
[FIG. 17A]



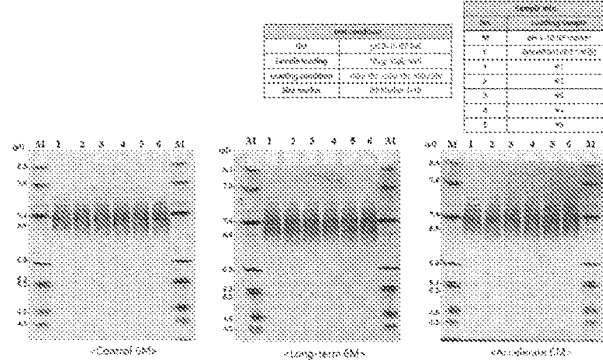
[FIG. 17B]



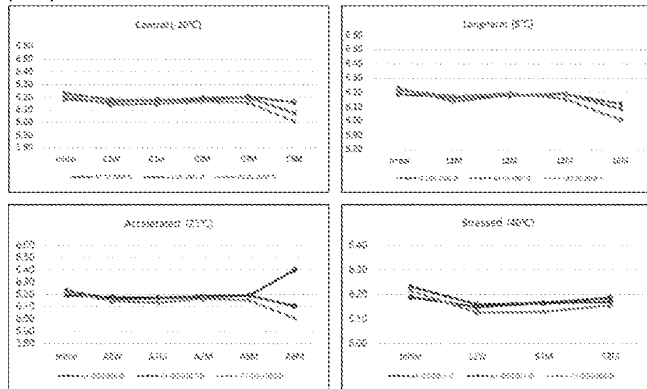
[FIG. 17C]



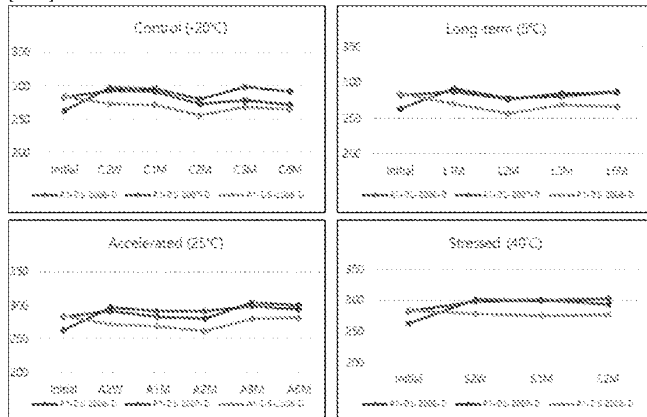
[FIG. 17D]



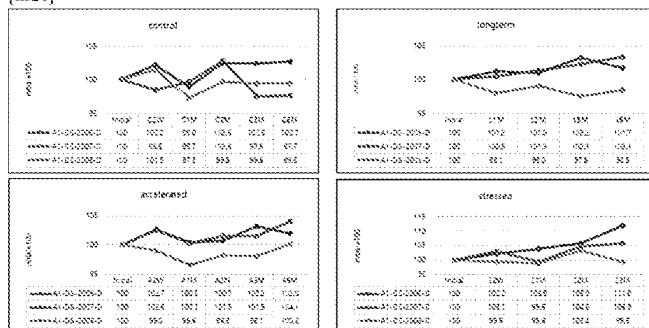
[FIG. 18]



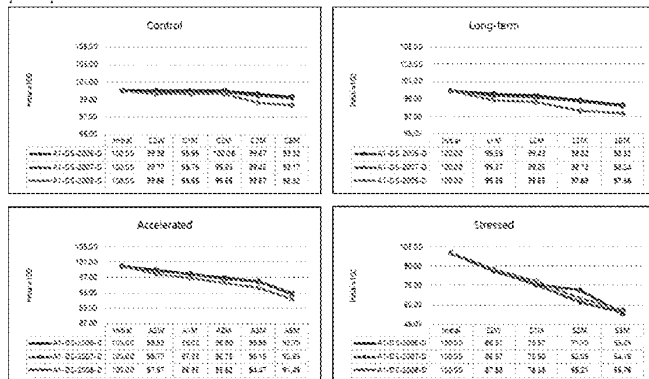
[FIG. 19]



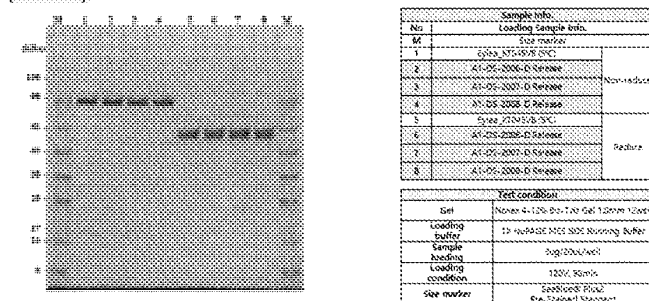
[520]



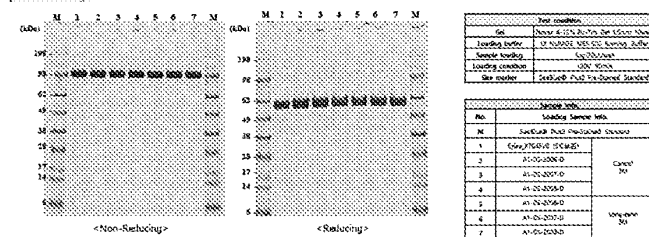
[52]



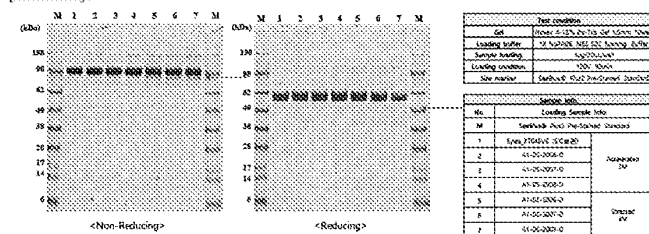
[FIG. 22A]



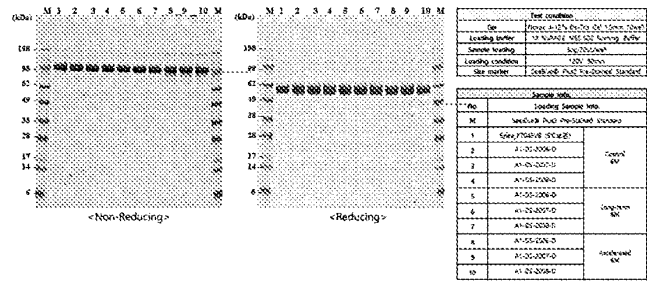
[FIG. 22B]



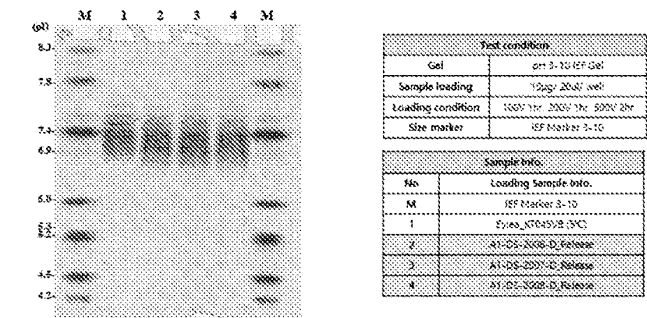
[FIG. 22C]



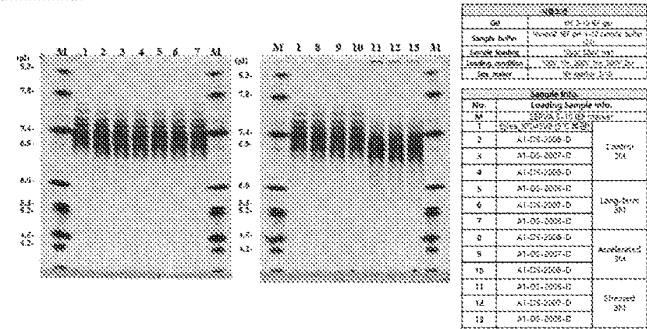
[FIG. 22D]



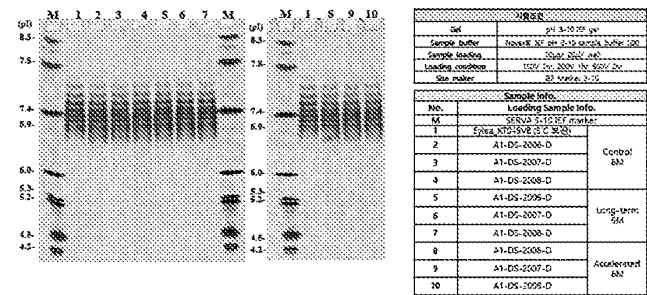
[FIG. 23A]



[FIG. 23B]



[FIG. 23C]



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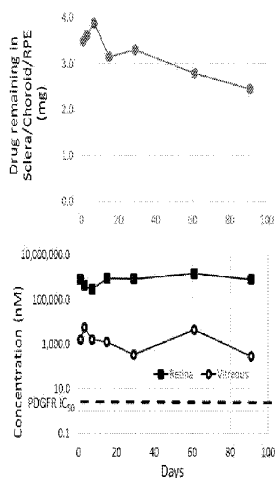
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DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
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MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

(54) Title: COMPOSITIONS COMPRISING AXITINIB AND METHODS OF TREATING OCULAR DISORDERS

FIG. 1A



(57) Abstract: This disclosure is generally related to ophthalmic therapies, and more particularly to methods and devices that allow for infusion of a fluid drug formulation into posterior ocular tissues for targeted, localized treatment, for example, for the treatment of wet AMD in human patients. The drug formulation may be injected into the suprachoroidal space (SCS) or subretinal space of an eye. The drug formulation may include a tyrosine kinase inhibitor, such as axitinib.

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[Continued on next page]

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

- *with international search report (Art. 21(3))*

COMPOSITIONS COMPRISING AXITINIB AND METHODS OF TREATING OCULAR DISORDERS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U. S. Provisional Application No. 62/978,540, filed February 19, 2020; which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] Neovascular age-related macular degeneration (nAMD), also known as “wet” AMD, is the leading cause of vision loss in the industrialized world. It is caused by abnormal blood vessel growth in the choriocapillaris, a layer of capillaries situated immediately below Bruch’s membrane. Choroidal neovascularization leads to the leakage of blood, lipids, and serum into the retinal layers, which can result in permanent damage to light-sensitive retinal cells and irreversible central vision distortions.

[0003] Although not fully elucidated, much is known regarding the pathogenesis of nAMD. The vascular endothelial growth factor (VEGF) signaling pathway has been shown to be centrally involved in the 10 to 15% of AMD diagnoses classified as the neovascular type (nAMD). In this pathway, VEGF signaling ligands bind to different isoforms of VEGF receptors (VEGFR) to activate cellular processes that promote growth of new vasculature. Specifically, VEGF-A, which acts at VEGFR-1 and -2, has been shown to promote abnormal blood vessel growth and is therefore an optimal target for treatment. Currently, anti-VEGF-A drugs are the standard of care for the treatment of nAMD; however, an unmet need remains for significantly improving and maintaining visual acuity in patients

[0004] Choroidal neovascularization (CNV) is the creation of new blood vessels in the choroid layer of the eye. Choroidal neovascularization is a common cause of, and/or can be associated with, nAMD. CNV can occur rapidly in individuals with defects in Bruch's membrane, the innermost layer of the choroid. It is also associated with excessive amounts of VEGF. CNV can also occur frequently with the rare genetic disease pseudoxanthoma elasticum and rarely with the more common optic disc drusen. CNV has also been associated with extreme myopia or malignant

myopic degeneration, where in choroidal neovascularization occurs primarily in the presence of cracks within the retinal macular tissue known as lacquer cracks. CNV can create a sudden deterioration of central vision, noticeable within a few weeks. Other symptoms which can occur include color disturbances, and metamorphopsia (distortions in which straight lines appears wavy). Hemorrhaging of the new blood vessels can accelerate the onset of symptoms of CNV.

[0005] Currently, even the most successful treatments of nAMD and CNV do not preclude reoccurrence, making multiple treatments likely. In addition, currently available treatments do not restore vision that has already been lost. Therefore, there is a need in the art for treatment breakthroughs, in order to maintain vision for a longer period of time without repeated laser use.

SUMMARY OF THE INVENTION

[0006] Methods and compositions for the treatment of neovascular age-related macular degeneration (nAMD; also referred to herein as wet AMD), choroidal neovascularization (CNV), nAMD associated with CNV, retinopathy, diabetic retinopathy, diabetic macular edema (DME), and related diseases are provided. The compositions comprise one or more tyrosine kinase inhibitors and are delivered to the suprachoroidal space of the eye via a non-surgical means. In some embodiments the tyrosine kinase inhibitor has activity against vascular endothelial growth factor (VEGF) and/or platelet derived growth factor (PDGF). In some embodiments, the tyrosine kinase inhibitor is axitinib. In some embodiments, the present disclosure provides an axitinib formulation, CLS-AX.

[0007] In some embodiments, the present disclosure provides methods for treating nAMD, choroidal neovascularization (CNV), nAMD associated with CNV, and/or DME comprising administering a formulation comprising axitinib to the suprachoroidal space of an eye of a subject in need thereof. In some embodiments, the method comprises administering about 0.01 mg to about 3.0 mg of axitinib to the eye. In some embodiments, the method comprises administering about 0.01 mg to about 0.5 mg of axitinib to the eye. In some embodiments, the method comprises administering about 0.01 mg to about 0.3 mg of axitinib to the eye. In further embodiments, the method comprises administering about 0.03 to about 0.1 mg of axitinib to the eye. In some embodiments, the method comprises administering about 0.01, about 0.02, about 0.03, about 0.04, about 0.05, about 0.06, about 0.07, about 0.08, about 0.09, about 0.10, about 0.2, about 0.3, about

0.4, about 0.5, about 1.0, about 2.0, or about 3.0 mg of axitinib to the eye. In some embodiments, the method comprises administering about 0.03 mg of axitinib to the eye. In some embodiments, the method comprises administering about 0.06 mg of axitinib to the eye. In some embodiments, the method comprises administering about 0.1 mg of axitinib to the eye. In some embodiments, the method comprises administering about 0.3 mg of axitinib to the eye. In some embodiments, the axitinib is administered in a volume of about 100 μ L. Thus, in some embodiments, the method comprises administering an axitinib formulation in a concentration of about 0.1 mg/mL, to about 1 mg/mL, or about 0.1 mg/mL, about 0.3 mg/mL, about 0.6 mg/mL, or about 1.0 mg/mL. In some embodiments, the method comprises administering the axitinib to the eye non-surgically. For example, in embodiments, the method comprises administering the axitinib to the eye non-surgical suprachoroidal injection. In embodiments, the non-surgical suprachoroidal injection is achieved by administering the axitinib using an injection device comprising a needle, wherein the needle has an effective length of about 500 to about 2000 microns.

[0008] In some embodiments, the method comprises administering axitinib to the eye in at least one dose. In some embodiments, the method comprises administering axitinib to the eye in two doses, wherein the two doses are spaced apart by at least about 1, at least about 2, at least about 3, at least about 4, at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 11, or at least about 12 months.

[0009] In some embodiments, following administration of axitinib to the eye of the subject, the subject experiences no loss in visual acuity as measured by best corrected visual acuity (BCVA). In some embodiments, following administration of axitinib to the eye of the subject, the subject experiences minimal loss in visual acuity as measured by BCVA. In embodiments, the minimal loss in visual acuity is a loss of no more than 2 letters. In further embodiments, the subject experiences an improvement in BCVA of ≥ 10 letters, ≥ 15 letters or ≥ 25 letters, as compared to the subject's visual acuity prior to the administration of the axitinib formulation. In embodiments, the subject experiences no loss in visual acuity as measured by BCVA for 2 months, 3, months, 4 months, 5 months, 6 months, or longer after administration of the axitinib formulation. In embodiments, the subject experiences minimal loss in visual acuity as measured by BCVA for 2 months, 3, months, 4 months, 5 months, 6 months, or longer after administration of the axitinib formulation. In embodiments, the subject experiences an improvement in visual acuity as

measured by BCVA for 2 months, 3, months, 4 months, 5 months, 6 months, or longer after administration of the axitinib formulation.

[0010] In some embodiments, following administration of axitinib to the eye of the subject, the subject experiences a decrease in retinal thickness from baseline (*e.g.*, retinal thickness such as central subfield thickness (CST) prior to treatment), at any given time point after administration of a drug provided herein, *e.g.*, a decrease of least about 20 μm , or at least about 40 μm , or at least about 50 μm , or at least about 100 μm , or at least about 150 μm or at least about 200 μm , or from about 50-100 μm , and all values in between. In another embodiment, the patient experiences a $\geq 5\%$, $\geq 10\%$, $\geq 15\%$, $\geq 20\%$, $\geq 25\%$ decrease in retinal thickness (*e.g.*, CST) subsequent to administration of axitinib. In one embodiment, change in retinal thickness from baseline is measured as a change in CST, for example, by spectral domain optical coherence tomography (SD-OCT). In embodiments, the subject experiences the decrease in retinal thickness for 2 months, 3, months, 4 months, 5 months, 6 months, or longer after administration of the axitinib formulation. In embodiments, the subject experiences no increase in retinal thickness for 2 months, 3, months, 4 months, 5 months, 6 months, or longer after administration of the axitinib formulation.

[0011] In some embodiments, the axitinib is Form IX axitinib. In some embodiments, the axitinib is present in the formulation in an amount of about 0.5 mg/mL to about 100 mg/mL, *e.g.*, about 1 mg/mL to about 10 mg/mL or about 20 mg/mL to about 80 mg/mL. In embodiments, the axitinib is present in the formulation in an amount of about 1 mg/mL or about 40 mg/mL. In some embodiments, the present disclosure provides formulations comprising axitinib and Polysorbate 80. In further embodiments, the present disclosure provides formulations comprising carboxymethylcellulose sodium, Polysorbate 80, sodium chloride, and sodium phosphate. In further embodiments, the formulation comprises Polysorbate 80 at a w/v of about 0.025% to about 0.2%. In some embodiments, the Polysorbate 80 is present in the formulation at an amount of about 0.05% to about 0.1%. In some embodiments, the Polysorbate 80 is present in the formulation at an amount of about 0.05% or about 0.1%. In some embodiments, formulation comprises about 0.04% w/v to about 0.07% w/v sodium phosphate (monobasic monohydrate). In further embodiments, the formulation comprises about 0.059% w/v sodium phosphate (monobasic monohydrate). In some embodiments, the formulation comprises about 0.05% w/v to about 0.09% w/v sodium phosphate (dibasic, anhydrous). In some embodiments, the formulation comprises about 0.079%

w/v sodium phosphate (dibasic, anhydrous). In some embodiments, the formulation comprises about 0.5% w/v to about 1.0% w/v sodium chloride. In further embodiments, the formulation comprises about 0.7% w/v to about 0.9% w/v sodium chloride. In some embodiments, the formulation comprises about 0.79% w/v sodium chloride. In some embodiments, the formulation comprises about 0.25% w/v to about 0.75% w/v carboxymethylcellulose sodium. In further embodiments, the formulation comprises about 0.3% w/v to about 0.7% w/v carboxymethylcellulose sodium. In some embodiments, the formulation comprises about 0.5% w/v carboxymethylcellulose sodium. In some embodiments, the axitinib formulation comprises microparticles comprising axitinib. In some embodiments, the microparticles are about 1-20 microns in size for the D₉₀ distribution. In some embodiments, the microparticles are about 1-10 microns in size for the D₅₀ distribution. In some embodiments, the microparticles are about 1-4 microns in size for the D₁₀ distribution. In some embodiments, the microparticles are about 3-5 microns in size for the D₉₀ distribution. In some embodiments, the microparticles are about 3 microns in size for the D₉₀ distribution. In some embodiments, the microparticles are about 10 microns in size for the D₉₀ distribution. In some embodiments, the clinical formulation of CLS-AX comprises axitinib microparticles of about 10 microns in size for the D₉₀ distribution. In some embodiments, the clinical formulation of CLS-AX comprises axitinib microparticles of about 2 microns in size for the D₁₀ distribution. In some embodiments, the clinical formulation of CLS-AX comprises axitinib microparticles of about 5 microns in size for the D₅₀ distribution. In some embodiments, the clinical formulation of CLS-AX comprises axitinib microparticles of about 10 microns for the D₉₀ distribution.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] FIGS. 1A and 1B show the mean ($\pm$ SD) CLS-AX concentrations in the indicated tissues following suprachoroidal injection with CLS-AX into the eyes of male Dutch Belted rabbits. FIG. 1A shows the drug remaining (mg) in sclera/choroid/RPE (top panel) and the concentration (in nM) of drug in the retina and vitreous, at the indicated time points up to Day 91 (bottom panel). FIG. 1B shows the concentration of axitinib (ng/g or ng/mL) in the vitreous humor, sclera/choroid/RPE, or retina at the lower (0.03 mg/eye) or higher (0.1 mg/eye) dose.

[0013] FIG. 2A shows the fluorescein angiography (FA) grading scale for the laser CNV model in rats. FIG. 2B shows the % of Grade IV lesions at Day 21 in control animals vs. CLS-AX recipient animals. *, Fischer's Exact p-value = 0.0002.

[0014] FIGS. 3A and 3B show the results of the vascular leakage studies in the CNV model in pigs. FIG. 3A is a bar graph showing the area of vascular leakage 7 days or 14 days after laser treatment to induce retinal lesions. OS = left eye; OD = right eye; BSS = balanced salt solution; SCS = suprachoroidal space. The OS-BSS treatment is shown as the left bar at both time points and the OD-CLS-AX treatment is shown as the right bar at both time points. FIG. 3B provides representative images from BSS-treated eyes and CLS-AX treated eyes.

[0015] FIGS. 4A and 4B show the mean ($\pm$ SEM) isolectin IB4 on retinal flat mount. FIG. 4A provides representative images. Scale bar = 100 μ m. FIG. 4B shows the mean $\pm$ SEM Isolectin IB4 signal. Signal in the CLS-AX group was significantly less than that of the BSS treated group ($p=0.0297$; t-test [27 sites measured OS; 354 sites measured OD]).

DETAILED DESCRIPTION OF THE INVENTION

[0016] This disclosure is generally related to ophthalmic therapies, and more particularly to methods and devices that allow for infusion of a fluid drug formulation into posterior ocular tissues for targeted, localized treatment, for example, for the treatment of diseases and disorders of the eye associated with neovascularization. For example, the diseases and disorders include neovascular age-related macular degeneration (nAMD; also referred to herein as wet AMD), nonexudative AMD, choroidal neovascularization (CNV), retinal vein occlusion (RVO), nAMD associated with RVO, nAMD associated with CNV, retinopathy, diabetic retinopathy, and diabetic macular edema (DME).

[0017] In some embodiments, the formulations comprise one or more tyrosine kinase inhibitors and are administered to the suprachoroidal space (SCS) of the eye via a non-surgical means, for example via a hollow microneedle, and/or an injection device comprising a hollow needle wherein the needle has an effective length of about 500 to about 2000 microns. The methods and

formulations provided herein allow for effective posterior segment drug delivery, and generally embody the following characteristics: (1) the methods are non-surgical and thus minimally invasive and safe; (2) the drug formulations are administered in such a way that they are well targeted to the posterior segment of the eye and/or the suprachoroidal space (SCS) of the eye and/or the supraciliary space of the eye and/or the suprachoroidal space of the eye and/or the subretinal space of the eye, while simultaneously limiting drug exposure to the anterior segment or other regions of the eye; (3) the methods and formulations are capable of delivering drug in a sustained and/or controlled manner; (4) the methods and devices are user-friendly. The non-surgical SCS delivery methods and drug formulations for SCS delivery set forth herein achieve these desired characteristics.

[0018] Axitinib is a tyrosine kinase inhibitor (TKI) that antagonizes the vascular endothelial growth factor receptors VEGFR-1, VEGFR-2, and VEGFR-3, as well as of the platelet-derived growth factor receptors (PDGFR) and c-Kit receptors. Axitinib was initially approved in 2012 as an oral tablet formulation (INLYTA[®]) at a dose of 5 mg given twice daily for the treatment of advanced renal cell carcinoma after failure of one prior systemic therapy. An axitinib formulation suitable for delivery to the eye is provided herein. In some embodiments, the axitinib formulation suitable for delivery to the eye provided herein is “CLS-AX.” Exemplary CLS-AX formulations are provided in Table 1A and Table 1B.

[0019] The term “suprachoroidal space,” is used interchangeably herein with suprachoroidal, SCS, suprachoroid, suprachoroidia, and the like; and describes the potential space in the region of the eye disposed between the sclera and choroid. This region primarily is composed of closely packed layers of long pigmented processes derived from each of the two adjacent tissues; however, a space can develop in this region as a result of fluid or other material buildup in the suprachoroidal space and the adjacent tissues. Those skilled in the art will appreciate that the suprachoroidal space frequently is expanded by fluid buildup because of some disease state in the eye or as a result of some trauma or surgical intervention. In the present description, however, in some embodiments, the fluid buildup is intentionally created by infusion of a drug formulation into the suprachoroid to create the suprachoroidal space (which is filled with drug formulation). Not wishing to be bound by theory, it is believed that the SCS region serves as a pathway for uveoscleral outflow (i.e., a

natural process of the eye moving fluid from one region of the eye to the other through) and becomes a real space in instances of choroidal detachment from the sclera.

[0020] As used herein, “non-surgical” ocular drug delivery devices and methods refer to methods and devices for drug delivery that do not require general anesthesia and/or retrobulbar anesthesia (also referred to as a retrobulbar block). Alternatively or additionally, a “non-surgical” ocular drug delivery method is performed with an instrument having a needle with an effective length of less than 2000 microns; and or an instrument having a needle with a diameter of 28 gauge or smaller. Alternatively or additionally, “non-surgical” ocular drug delivery methods do not require a guidance mechanism that is typically required for ocular drug delivery via a shunt or cannula. Non-surgical ocular drug delivery methods provided herein may be used in a clinic or out-patient setting and do not require a hospital setting. As used herein, “surgical” ocular drug delivery includes insertion of devices or administration of drugs by surgical means, for example, via incision to expose and provide access to regions of the eye including the posterior region, and/or via insertion of a stent, shunt, or cannula and/or via a method that requires anesthesia (e.g., general or retrobulbar anesthesia).

[0021] The surgical and non-surgical posterior ocular disorder treatment methods and devices described herein are particularly useful for the local delivery of drugs to the posterior region of the eye, for example the retinochoroidal tissue, macula, retinal pigment epithelium (RPE) and optic nerve in the posterior segment of the eye. In one embodiment, the non-surgical methods provided herein can be used to target drug delivery to specific posterior ocular tissues or regions within the eye or in neighboring tissue. In one embodiment, the methods described herein deliver drug specifically to the sclera, the choroid, the Brach’s membrane, the retinal pigment epithelium, the subretinal space, the retina, the macula, the optic disk, the optic nerve, the ciliary body, the trabecular meshwork, the aqueous humor, the vitreous humor, and/or other ocular tissue or neighboring tissue in the eye of a human subject in need of treatment. The methods provided herein, in one embodiment, can be used to target drug delivery to specific posterior ocular tissues or regions within the eye or in neighboring tissue.

[0022] As provided throughout, in one embodiment, the methods described herein are carried out with a puncture member, which may comprise a hollow or solid microneedle, for example, a rigid

microneedle. As used herein, the term “microneedle” refers to a conduit body having a base, a shaft, and a tip end suitable for insertion into the sclera and other ocular tissue and has dimensions suitable for minimally invasive insertion and drug formulation infusion as described herein. Both the “length” and “effective length” of the microneedle encompass the length of the shaft of the microneedle and the bevel height of the microneedle. In some embodiments, the needles used herein are microneedles in that they have an effective length of less than 2000 microns. For example, in some embodiments, the needles useful in the methods described herein are microneedles in that they have an effective length of about 50 microns to about 2000 microns, or about 500 microns to about 1800 microns, or about 700 microns to about 1500 microns, or about 900 microns to about 1200 microns. In some embodiments, the needles useful in the methods described herein are microneedles in that they have an effective length of about 800 microns, about 900 microns, about 1000 microns, about 1100 microns, or about 1200 microns. In some embodiments, the device used to carry out the methods described herein comprises one of the devices disclosed in U.S. Patent No. 9,539,139, issued January 10, 2017 or International Patent Application Publication No. WO2014/179698 (Application No. PCT/US2014/036590), filed May 2, 2014 and entitled “Apparatus and Method for Ocular Injection,” each of which is incorporated by reference herein in its entirety for all purposes. In some embodiments, the device used to carry out the methods described herein comprises one of the devices disclosed in International Patent Application Publication No. WO2014/036009 (Application No. PCT/US2013/056863), filed August 27, 2013 and entitled “Apparatus and Method for Drug Delivery Using Microneedles,” incorporated by reference herein in its entirety for all purposes. In some embodiments, the microneedle is an SCS microinjector as described herein.

[0023] As used herein, the term “hollow” includes a single, straight bore through the center of the microneedle, as well as multiple bores, bores that follow complex paths through the microneedles, multiple entry and exit points from the bore(s), and intersecting or networks of bores. That is, a hollow microneedle has a structure that includes one or more continuous pathways from the base of the microneedle to an exit point (opening) in the shaft and/or tip portion of the microneedle distal to the base.

[0024] The microneedle device in one embodiment, comprises a fluid reservoir for containing the therapeutic formulation (*e.g.*, drug or cell formulation), *e.g.*, as a solution or suspension, and the

drug reservoir (which can include any therapeutic formulation) being in operable communication with the bore of the microneedle at a location distal to the tip end of the microneedle. The fluid reservoir may be integral with the microneedle, integral with the elongated body, or separate from both the microneedle and elongated body.

[0025] In some embodiments, features of the devices, formulations, and methods are provided in U.S. Patent No. 9,636,332, U.S. Patent Application Publication No. 2018-0042765, International Patent Application Publication Nos. WO2014/074823 (Application No. PCT/US2013/069156), WO2015/195842 (Application No. PCT/US2015/036299), U.S. Patent Publication No. 2019-0269702, WO 2017/120600 (Application No. PCT/US2017/012755), and/or WO2017/120601 (Application No. PCT/US2017/012757), each of which is hereby incorporated by reference in its entirety for all purposes.

[0026] In one embodiment, the device used to carry out one of the methods described herein comprises the device described in U.S. Design Patent Application Serial No. 29/506,275 entitled, “Medical Injector for Ocular Injection,” filed October 14, 2014, the disclosure of which is incorporated herein by reference in its entirety for all purposes. In one embodiment, the device used to carry out one of the methods described herein comprises the device described in U.S. Patent Publication No. 2015/0051581 or U.S. Patent Publication No. 2017/0095339, which are each incorporated herein by reference in their entireties for all purposes. In some embodiments, such a device is an SCS microinjector as described herein.

[0027] As used herein, the terms “about” and “approximately” generally mean plus or minus 10% of the value stated. For example, about 0.5 would include 0.45 and 0.55, about 10 would include 9 to 11, about 1000 would include 900 to 1100.

[0028] Further details of possible manufacturing techniques for the microneedles and/or microinjectors provided herein are described, for example, in U.S. Patent Application Publication No. 2006/0086689, U.S. Patent Application Publication No. 2006/0084942, U.S. Patent Application Publication No. 2005/0209565, U.S. Patent Application Publication No. 2002/0082543, U.S. Patent No. 6,334,856, U.S. Patent No. 6,611,707, U.S. Patent No. 6,743,211 and PCT/US2014/36590, filed May 2, 2014, all of which are incorporated herein by reference in their entireties for all purposes.

[0029] Any of the methods described herein can be performed use any suitable injector of the types shown and described herein. In some embodiments, in accordance with the methods described herein, the dose of drug has a delivered volume of at least about 20 μL , at least about 50 μL , at least about 100 μL , at least about 200 μL or at least about 500 μL . In one embodiment, the amount of therapeutic formulation delivered into the suprachoroidal space from the devices described herein is from about 10 μL to about 200 μL , e.g., from about 50 μL to about 150 μL . In another embodiment, from about 10 μL to about 500 μL , e.g., from about 50 μL to about 250 μL , is non-surgically administered to the suprachoroidal space. In some embodiments, about 100 μL of a drug formulation is non-surgically administered to the suprachoroidal space. In some embodiments, the drug formulation comprises 100 μL of an axitinib formulation, e.g., CLS-AX.

[0030] The SCS drug delivery methods provided herein allow for the delivery of drug formulation over a larger tissue area and to more difficult to target tissue in a single administration as compared to previously known needle devices. Not wishing to be bound by theory, it is believed that upon entering the SCS the drug formulation flows circumferentially from the insertion site toward the retinochoroidal tissue, macula, and optic nerve in the posterior segment of the eye as well as anteriorly toward the uvea and ciliary body. In addition, a portion of the infused drug formulation may remain in the SCS as a depot, or remain in tissue overlying the SCS, for example the sclera, near the microneedle insertion site, serving as additional depot of the drug formulation that subsequently can diffuse into the SCS and into other adjacent posterior tissues.

[0031] The term “subject” is used interchangeably herein with the term “patient.” The subject may be any mammal. Preferably, the subject is a human subject. The human subject treated with the methods and devices provided herein may be an adult or a child. In one embodiment, the subject presents with a retinal thickness of greater than 300 μm (e.g., central subfield thickness as measured by optical coherence tomography). In another embodiment, the subject in need of treatment has a BCVA score of ≥ 20 letters read in each eye (e.g., 20/400 Snellen approximate). In yet another embodiment, the subject in need of treatment has a BCVA score of ≥ 20 letters read in each eye (e.g., 20/400 Snellen approximate), but ≤ 70 letters read in the eye in need of treatment.

[0032] Therapeutic response, in one embodiment, is assessed via a visual acuity measurement at one and/or two months post treatment (e.g., by measuring the mean change in best corrected visual

acuity (BCVA) from baseline, *i.e.*, prior to treatment). In one embodiment, a patient treated by one or more of the methods provided herein experiences an improvement in BCVA from baseline, at any given time point (*e.g.*, 2 weeks after administration, 4 weeks after administration, 2 months after administration, 3 months after administration), of at least 2 letters, at least 3 letters, at least 5 letters, at least 8 letters, at least 12 letters, at least 13 letters, at least 15 letters, at least 20 letters, and all values in between, as compared to the patient's BCVA prior to administration of axitinib.

[0033] In one embodiment, the patient gains about 5 letters or more, about 10 letters or more, 15 letters or more, about 20 letters or more, about 25 letters or more in a BCVA measurement after administration of axitinib, compared to the patient's BCVA measurement prior to undergoing treatment. In even a further embodiment, the patient gains from about 5 to about 30 letters, 10 to about 30 letters, from about 15 letters to about 25 letters or from about 15 letters to about 20 letters in a BCVA measurement, compared to the patient's BCVA measurement prior to treatment with axitinib. In one embodiment, the BCVA gain is about 2 weeks, about 1 month, about 2 months, about 3 months or about 6 months after administration of axitinib. In another embodiment, the BCVA is measured at least about 2 weeks, at least about 1 month, at least about 2 months, at least about 3 months or at least about 6 months after administration of axitinib.

[0034] In one embodiment, the BCVA is based on the Early Treatment of Diabetic Retinopathy Study (ETDRS) visual acuity charts and is assessed at a starting distance of 4 meters.

[0035] In another embodiment, the patient subjected to a treatment method, *e.g.*, with one of the devices provided herein substantially maintains his or her vision subsequent to the treatment (*e.g.*, a single administration or multiple administrations of axitinib to the suprachoroidal space of the eye), as measured by losing fewer than 15 letters in a best-corrected visual acuity (BCVA) measurement, compared to the patient's BCVA measurement prior to undergoing treatment. In a further embodiment, the patient loses fewer than 10 letters, fewer than 8 letters, fewer than 6 letters, fewer than 5 letters, or fewer than 2 letters in a BCVA measurement, compared to the patient's BCVA measurement prior to undergoing treatment. In some embodiments, the patient experiences no further loss of vision subsequent to treatment with axitinib (*e.g.*, the patient experiences a loss of 0 letter as measured by BCVA). In some embodiments, the patient experiences a gain in vision subsequent to treatment (*e.g.*, a single administration or multiple

administrations of axitinib to the suprachoroidal space of the eye). For example, in some embodiments, the patient experiences a gain of at least 2, at least 5, at least 10, at least 15, at least 20, or more letters. In some embodiments, “minimal loss of vision” as used herein means losing no more than 1 letter, no more than 2 letters, no more than 3 letters, no more than 4 letters, no more than 5 letters, no more than 6 letters, no more than 7 letters, no more than 8 letters, no more than 9, letters, no more than 10 letters, no more than 12 letters, or no more than 15 letters. In some embodiments, the patient experiences a minimal loss of vision, relative to the patient’s baseline vision prior to treatment, over 2, 6, 12, 18, or 24 months. In some embodiments, the patient experiences a gain in vision, relative to the patient’s baseline vision prior to treatment, over 2, 6, 12, 18, or 24 month.

[0036] A decrease in retina thickness and/or macula thickness is one measurement of treatment efficacy of the methods provided herein. For example, in one embodiment, patient suffering from nAMD treated by one of the methods provided herein (e.g., administration of a drug (e.g., axitinib) to the suprachoroidal space of an eye) experiences a decrease in retinal thickness from baseline. For example, in one embodiment, the patient experiences a decrease in central subfield thickness (CST) at any given time point after administration of the drug, e.g., a decrease of least about 20 μm , or at least about 40 μm , or at least about 50 μm , or at least about 100 μm , or at least about 150 μm or at least about 200 μm , or from about 50-100 μm , and all values in between. In another embodiment, the patient experiences a $\geq 5\%$, $\geq 10\%$, $\geq 15\%$, $\geq 20\%$, $\geq 25\%$ decrease in retinal thickness (e.g., CST) subsequent to administration of the drug. In some embodiments, the patient experiences a decrease in CST, relative to the patient’s baseline CST prior to treatment, for at least 2, 6, 12, 18, or 24 months.

[0037] In one embodiment, a patient treated by the methods provided herein experiences a decrease in retinal thickness from baseline (*i.e.*, retinal thickness prior to treatment), at any given time point, of from about 20 μm to about 200 μm , at from about 40 μm to about 200 μm , of from about 50 μm to about 200 μm , of from about 100 μm to about 200 μm , or from about 150 μm to about 200 μm . In one embodiment, change in retinal thickness from baseline is measured as a change in CST, for example, by spectral domain optical coherence tomography (SD-OCT).

[0038] In one embodiment, the decrease in retinal thickness is measured about 2 weeks, about 1 month, about 2 months, about 3 months, about 6 months, about 12 months, about 18 months, or about 24 months after administration of the drug. In another embodiment, the decrease in retinal thickness is measured at least about 2 weeks, at least about 1 month, at least about 2 months, at least about 3 months, at least about 6 months, at least about 12 months, at least about 18 months, or at least about 24 months after administration of the drug. In one embodiment, where multiple dosing sessions are employed, a decrease in retinal thickness is sustained by the patient for at least about 2 weeks, at least about 1 month, at least about 2 months, at least about 3 months or at least about 6 months after each drug administration.

[0039] A reduction in the frequency and/or severity in ocular lesions within the eye is also one measurement of treatment efficacy of the methods provided herein.

[0040] In one embodiment, the suprachoroidal drug dose sufficient to achieve a therapeutic response in a human subject treated with the non-surgical SCS drug delivery method is less than the intravitreal, parenteral, intracameral, topical, or oral drug dose sufficient to elicit the identical or substantially identical therapeutic response. In a further embodiment, the suprachoroidal drug dose is at least 10 percent less than the oral, parenteral or intravitreal dose sufficient to achieve the identical or substantially identical therapeutic response. In a further embodiment, the suprachoroidal dose is about 10 percent to about 25 percent less, or about 10 percent to about 50 percent less than the oral, parenteral, intracameral, topical, or intravitreal dose sufficient to achieve the identical or substantially identical therapeutic response.

[0041] In some embodiments, the non-surgical administration of axitinib according to the methods described herein reduces the number and/or frequency of administration of a VEGF modulator to the subject. Thus, in some embodiments, the administration of the axitinib increases the effectiveness and/or durability of the VEGF modulator treatment. For example, in some embodiments, the SCS administration of axitinib results in a need for fewer administrations of a VEGF modulator, and/or results in a longer period of time between administrations of a VEGF modulator.

[0042] In some embodiments, the non-surgical administration of axitinib according to the methods described herein results in maintenance of the improved BCVA and/or the improved CST in the

subject for at least 12 weeks, at least 16 weeks, at least 20 weeks, at least 24 weeks, at least 30 weeks, at least 36 weeks, at least 40 weeks, at least 44 weeks, at least 48 weeks, or longer after the initial dose of axitinib. In some embodiments, the non-surgical administration of axitinib according to the methods described herein results in maintenance of the improved BCVA and/or the improved CST in the subject for at least 12 weeks, at least 16 weeks, at least 20 weeks, at least 24 weeks, at least 30 weeks, at least 36 weeks, at least 40 weeks, at least 44 weeks, at least 48 weeks, at least 52 weeks, or longer after a second dose of axitinib.

[0043] In one embodiment, the non-surgical administration of axitinib to the eye according to the methods provided herein results in a decreased number of deleterious side effects or clinical manifestations in the treated patient as compared to the number of side effects or clinical manifestations caused by the same drug dose administered intravitreally, intracamerally, orally or parenterally; or results in a decreased number of deleterious side effects or clinical manifestations in the treated patient as compared to those caused by administration of a drug previously used to treat the disease.

[0044] Examples of side effects and clinical manifestations that can be reduced or ameliorated include, but are not limited to, inflammation, gastrointestinal side effects (e.g., diarrhea, nausea, gastroenteritis, vomiting, gastrointestinal, rectal, and duodenal hemorrhage, hemorrhagic pancreatitis, large intestine perforation black or bloody stools, and/or coughing up blood); hematologic side effects (e.g., leucopenia, anemia, pancytopenia and agranulocytosis, thrombocytopenia, neutropenia, pure red cell aplasia (PRCA), deep venous thrombosis easy bruising, and /or unusual bleeding from the nose, mouth, vagina, or rectum); immunologic side effects/clinical manifestations (e.g., immunosuppression, immunosuppression resulting in sepsis, opportunistic infections (herpes simplex virus, herpes zoster, and invasive candidal infections), and/or increased infection); oncologic side effects/clinical manifestations (e.g., lymphoma, lymphoproliferative disease and/or non-melanoma skin carcinoma); renal side effects/clinical manifestations (e.g. dysuria, urgency, urinary tract infections, hematuria, kidney tubular necrosis, and/or BK virus-associated nephropathy); metabolic side effects/clinical manifestations (e.g. edema, hyperphosphatemia, hypokalemia, hyperglycemia, hyperkalemia, swelling, rapid weight gain, and/or enlarged thyroid); respiratory side effects/clinical manifestations (e.g., respiratory infection, dyspnea, increased cough, primary tuberculosis dry cough, wheezing, and/or stuffy

nose); dermatologic side effects/clinical manifestations (*e.g.*, acne, rash, dyshidrotic eczema, papulosquamous psoriatic-like skin eruption rash, blisters, oozing, mouth sores, and/or hair loss); musculoskeletal side effects/clinical manifestations (*e.g.* myopathy and/or muscle pain), hepatic side effects/clinical manifestations (*e.g.* hepatotoxicity and/or jaundice), abdominal pain, increased incidence of first trimester pregnancy loss, missed menstrual periods, severe headache, confusion, change in mental status, vision loss, seizure (convulsions), increased sensitivity to light, dry eye, red eye, itchy eye, and/or high blood pressure.

[0045] As provided throughout, the compositions administered herein in one embodiment, the methods described herein comprise administering a tyrosine kinase inhibitor. Exemplary tyrosine kinase inhibitors for use in the methods described herein include, but are not limited to, Alectinib (Alecensa®); angiokinase inhibitors such as Nintedanib (Vargatev®), Afatinib (Gilotrif®), and Motesanib; Apatinib; Axitinib; Cabozantinib (Cometriq®); Canertinib; Crenolanib; Damnacanthal; Foretinib; Fostamatinib; growth factor receptor inhibitor; Ibrutinib (Imbruvica®); Icotinib; Imatinib (Gleevec®); Linifanib; Mubritinib; Radotinib; T790M; V600E; Vatalanib; Vemurafenib (Zelboraf®); AEE788 (TKI, VEGFR-2, EGFR: Novartis); ZD6474 (TKI, VEGFR-1, -2, -3, EGFR: Zactima: AstraZeneca); AZD2171 (TKI, VEGFR-1, -2: AstraZeneca); SU 11248 (TKI, VEGFR-1, -2, PDGFR: Sunitinib: Pfizer); AG13925 (TKI, VEGFR-1, -2: Pfizer); AG013736 (TKI, VEGFR-1, -2: Pfizer); CEP-7055 (TKI, VEGFR-1, -2, -3: Cephalon); CP-547,632 (TKI, VEGFR-1, -2: Pfizer); GW756024 (TKL VEGFR-1, -2, -3: GlaxoSmithKline); GW786034 (TKI, VEGFR-1, -2, -3: GlaxoSmithKline); sorafenib (TKI, Bay 43-9006, VEGFR-1, -2, PDGFR: Bayer/Onyx); SU4312 (TKI, VEGFR-2, PDGFR: Pfizer); AMG706 (TKI, VEGFR-1, -2, -3: Amgen); XL647 (TKI, EGFR, HER2, VEGFR, ErbB4: Exelixis); XL999 (TKI, FGFR, VEGFR, PDGFR, FII-3: Exelixis); PKC412 (TKI, KIT, PDGFR, PKC, FLT3, VEGFR-2: Novartis); AEE788 (TKI, EGFR, VEGFR2, VEGFR-1: Novartis); OSI-030 (TKI, c-kit, VEGFR: OSI Pharmaceuticals); OSI-817 (TKI c-kit, VEGFR: OSI Pharmaceuticals); DMPQ (TKI, ERGF, PDGFR, ErbB2. p56. pkA, pkC); MLN518 (TKI, Flt3, PDGFR, c-KIT (T53518: Millennium Pharmaceuticals); lestaurinib (TKI, FLT3, CEP-701, Cephalon); ZD 1839 (TKI, EGFR: gefitinib, Iressa: AstraZeneca); OSI-774 (TKI, EGFR: Erlotinib: Tarceva: OSI Pharmaceuticals); lapatinib (TKI, ErbB-2, EGFR, and GD-2016: Tykerb: GlaxoSmithKline).

[0046] Axitinib is a potent tyrosine kinase inhibitor of vascular endothelial growth factor receptors VEGFR-1, VEGFR-2, and VEGFR-3. These receptors are implicated in pathologic angiogenesis, tumor growth, and metastatic progression of cancer. Axitinib has been shown to potently and selectively inhibit VEGF-mediated signaling and endothelial cell proliferation and survival at picomolar concentrations. Axitinib also inhibits other RTKs at low nanomolar concentrations, including PDGFR- α , PDGFR- β , and c-Kit. The most common metabolites of axitinib, the N-glucuronide (M7) and the sulfoxide (M12) were ≥ 400 -fold less potent against VEGFR-2.

[0047] “CLS-AX” is used herein to describe a formulation comprising axitinib. Exemplary formulations are provided in Tables 1A and 1B and throughout the disclosure.

[0048] In one embodiment, the tyrosine kinase inhibitor (e.g., axitinib) may be used in combination with one or more agents listed herein or with other agents known in the art, either in a single or multiple formulations. In one embodiment, the agent is a VEGF modulator and is administered intravitreally to the patient in need of treatment. In one embodiment, the VEGF modulator is a VEGF antagonist. In one embodiment, the second drug is a VEGF antagonist including, without limitation, a VEGF-receptor kinase antagonist, an anti-VEGF antibody or fragment thereof, an anti-VEGF receptor antibody, an anti-VEGF aptamer, a small molecule VEGF antagonist, a thiazolidinedione, a quinoline or a designed ankyrin repeat protein (DARPin). In one embodiment, the VEGF antagonist includes, but is not limited to, aflibercept, ziv-aflibercept, bevacizumab, sonpeizumab, VEGF sticky trap, cabozantinib, foretinib, vandetanib, nintedanib, regorafenib, cediranib, ranibizumab, lapatinib, sunitinib, sorafenib, plitidepsin, regorafenib, verteporfin, bucillamine, axitinib, pazopanib, fluocinolone acetonide, nintedanib, AL8326, 2C3 antibody, AT001 antibody, XtendVEGF antibody, HuMax-VEGF antibody, R3 antibody, AT001/r84 antibody, HyBEV, ANG3070, APX003 antibody, APX004 antibody, ponatinib, BDM-E, VGX100 antibody, VGX200, VGX300, COSMIX, DLX903/1008 antibody, ENMD2076, INDUS815C, R84 antibody, KD019, NM3, MGCD265, MG516, MP0260, NT503, anti-DLL4/VEGF bispecific antibody, PAN90806, Palomid 529, BD0801 antibody, XV615, lucitanib, motesanib diphosphate, AAV2-sFLT01, soluble Flt1 receptor, AV-951, Volasertib, CEP11981, KH903, lenvatinib, lenvatinib mesylate, terameprocol, PF00337210, PRS050, SP01, carboxyamidotriazole orotate, hydroxychloroquine, linifanib, ALG1001, AGN150998, MP0112, AMG386, ponatinib, PD173074, AVA101, BMS690514, KH902, golvatinib (E7050), dovitinib,

dovitinib lactate (TKI258, CHIR258), ORA101, ORA102, Axitinib (Inlyta, AG013736), PTC299, pegaptanib sodium, troponin, EG3306, vatalanib, Bmab100, GSK2136773, Anti-VEGFR Alterase, Avila, CEP7055, CLT009, ESBA903, GW654652, HMPL010, GEM220, HYB676, JNJ17029259, TAK593, Nova21012, Nova21013, CP564959, smart Anti-VEGF antibody, AG028262, AG13958, CVX241, SU14813, PRS055, PG501, PG545, PTI101, TG100948, ICS283, XL647, enzastaurin hydrochloride, BC194, COT601M06.1, COT604M06.2, MabionVEGF, Apatinib, RAF265 (CHIR-265), Motesanib Diphosphate (AMG-706), Lenvatinib (E7080), TSU-68 (SU6668, Orantinib), Brivanib (BMS-540215), MGCD-265, AEE788 (NVP-AEE788), ENMD-2076, OSI-930, CYC116, Ki8751, Telatinib, KRN 633, SAR131675, Dovitinib (TKI-258) Dilactic Acid, Apatinib, BMS-794833, Brivanib Alaninate (BMS-582664), Golvatinib (E7050), Semaxanib (SU5416), ZM 323881 HCl, Cabozantinib malate (XL184), ZM 306416, AL3818, AL8326, 2C3 antibody, AT001 antibody, HyBEV, bevacizumab (Avastin®), ANG3070, APX003 antibody, APX004 antibody, ponatinib (AP24534), BDM-E, VGX100 antibody (VGX100 CIRCADIAN), VGX200 (c-fos induced growth factor monoclonal antibody), VGX300, COSMIX, DLX903/1008 antibody, ENMD2076, sunitinib malate (Sutent®), INDUS815C, R84 antibody, KD019, NM3, allogenic mesenchymal precursor cells combined with an anti-VEGF antagonist (e.g., anti-VEGF antibody), MGCD265, MG516, VEGF-Receptor kinase inhibitor, MP0260, NT503, anti-DLL4/VEGF bispecific antibody, PAN90806, Palomid 529, BD0801 antibody, XV615, lucitanib (AL3810, E3810), AMG706 (motesanib diphosphate), AAV2-sFLT01, soluble Flt1 receptor, cediranib (Recentin™), AV-951, tivozanib (KRN-951), regorafenib (Stivarga®), volasertib (BI6727), CEP11981, KH903, lenvatinib (E7080), lenvatinib mesylate, terameprocol (EM1421), ranibizumab (Lucentis®), pazopanib hydrochloride (Votrient™), PF00337210, PRS050, SP01 (curcumin), carboxyamidotriazole orotate, hydroxychloroquine, linifanib (ABT869, RG3635), fluocinolone acetonide (Iluvien®), ALG1001, AGN150998, DARPin MP0112, AMG386, ponatinib (AP24534), AVA101, nintedanib (Vargatef™), BMS690514, KH902, golvatinib (E7050), everolimus (Afinitor®), dovitinib lactate (TKI258, CHIR258), ORA101, ORA102, axitinib (Inlyta®, AG013736), plitidepsin (Aplidin®), PTC299, aflibercept (Zaltrap®, Eylea®), pegaptanib sodium (Macugen™, LI900015), verteporfin (Visudyne®), bucillamine (Rimatil, Lamin, Brimani, Lamit, Boomiq), R3 antibody, AT001/r84 antibody, troponin (BLS0597), EG3306, vatalanib (PTK787), Bmab100, GSK2136773, Anti-VEGFR Alterase, Avila, CEP7055, CLT009, ESBA903, HuMax-VEGF antibody, GW654652,

HMPL010, GEM220, HYB676, JNJ17029259, TAK593, XtendVEGF antibody, Nova21012, Nova21013, CP564959, Smart Anti-VEGF antibody, AG028262, AG13958, CVX241, SU14813, PRS055, PG501, PG545, PTI101, TG100948, ICS283, XL647, enzastaurin hydrochloride (LY317615), BC194, quinolines, COT601M06.1, COT604M06.2, MabionVEGF, SIR-Spheres coupled to anti-VEGF or VEGF-R antibody, Apatinib (YN968D1), or AL3818.

[0049] In embodiments, the compositions and methods provided herein are for use in treating ocular disease and disorders. Exemplary ocular diseases and disorders include, without limitation, wet AMD, nonexudative AMD, CNV, RVO (including central RVO, hemi-RVO, branch RVO), retinopathy, diabetic retinopathy, and diabetic macular edema (DME). In embodiments, the disease or disorder is macular edema (ME). ME may occur in association with and/or due to central RVO, hemiretinal RVO, branch RVO, inflammation, uveitis, or CNV. In embodiments, the disease or disorder is a geographic atrophy, for example, from AMD, degenerative retinal disorders, or hereditary retinal disorders. In embodiments, the disease or disorder is retinal neovascularization. For example, in embodiments, retinal neovascularization can result from ischemic causes such as diabetic retinopathy, central RVO, hemiretinal RVO, branch RVO, central retinal artery occlusion, branch retinal artery occlusion, sickle cell retinopathy, or retinopathy of prematurity. In embodiments, retinal neovascularization can result from inflammatory and uveitic disorders.

[0050] Particular conditions in which choroidal neovascularization may occur include wet AMD, angiod streaks, anterior ischemic optic neuropathy, bacterial endocarditis, Best disease, birdshot retinochoroidopathy, choroidal hemangioma, choroidal nevi, choroidal nonperfusion, choroidal osteomas, choroidal rupture, choroideremia, chronic retinal detachment, coloboma of the retina, diabetes mellitus, drusen, endogenous *candida* endophthalmitis, extrapapillary hematomas of the retinal pigment epithelium, fundus flavimaculatus, an idiopathic condition, macular hole, malignant melanoma, membranoproliferative glomerulonephritis (type II), metallic intraocular foreign body, morning-glory disc syndrome, retinitis pigmentosa, retinochoroidal coloboma, Rubella, sarcoidosis, serpiginous or geographic choroiditis, subretinal fluid drainage, tilted disc syndrome, toxoplasma retinochoroiditis, tuberculosis, Vogt-Koyanagi-Harada syndrome, idiopathic polypoidal choroidal vasculopathy, ocular ischemic syndrome, and carotid stenosis.

EXAMPLES

[0051] The present invention is further illustrated by reference to the following Examples. However, it should be noted that these Examples, like the embodiments described above, are illustrative and are not to be construed as restricting the scope of the invention in any way.

Example 1. Axitinib Formulation

[0052] One exemplary Axitinib formulation, denoted CLS-AX, includes the following components.

Table 1A. Exemplary Axitinib Formulation

Ingredient	CLS-AX (%w/v or as noted)
Axitinib	40 mg/mL
Carboxymethylcellulose Sodium (NaCMC)	0.5%
Polysorbate 80 (PS-80)	0.1%
Sodium chloride	0.79%
Sodium phosphate, monobasic, monohydrate	0.059%
Sodium phosphate, dibasic, anhydrous	0.079%
Water for Injection	q.s.
Sodium hydroxide / Hydrochloric acid	Adjust pH to 6.8

[0053] One exemplary axitinib formulation, also denoted in the table below as CLS-AX, is a 1 mg/mL formulation and includes the following components.

Table 1B. Exemplary Axitinib Formulation

Component	Composition (% w/w)	Quantity per Batch (5029g)
Axitinib	0.0994	5.00 g + 0.05 g
Super Refined Tween 80-LQ-(MH), Polysorbate 80	0.050	2.51 g + 0.05 g
7 MF PH Sodium Carboxymethylcellulose	0.497	30.00 g + 0.30 g

Sodium Chloride, Powder	0.794	47.92 g + 0.48 g
Sodium Phosphate, Monobasic, Monohydrate	0.060	3.62 g + 0.04 g
Sodium Phosphate, Dibasic, Anhydrous, Extra Pure	0.080	4.83 g + 0.05 g
Water for Injection (WFI)	98.4194	q.s. to 5,029.0 g + 1%

[0054] In exemplary axitinib formulations, the axitinib is present at a concentration of 1 mg/mL or 10 mg/mL. In addition, in some exemplary axitinib formulations, the polysorbate 80 is present in the formulation at about 0.04% w/v to about 0.05% w/v. Further, in some exemplary embodiments, the sodium chloride is present at a concentration of 0.8%, and/or the sodium phosphate (monobasic, monohydrate) is present at 0.05% w/v; and/or the sodium phosphate (dibasic, anhydrous) is present at 0.085% w/v. Accordingly, the polysorbate 80 concentration may range from about 0.04% w/v to about 0.1% w/v, the sodium chloride concentration may range from about 0.7% w/v to about 0.9% w/v, the sodium phosphate (monobasic, monohydrate) concentration may range from about 0.05% to about 0.06% w/v, and the sodium phosphate (dibasic, anhydrous) may range from about 0.075% w/v to about 0.085% w/v.

[0055] In some embodiments, the NaCMC or a similar compound is included in the formulation as a viscosity modifier. In some embodiments, the polysorbate 80 (PS-80) or a similar agent is included in the formulation as a surfactant wetting agent for the active pharmaceutical ingredient, axitinib. In some embodiments, the sodium chloride is included in the formulation as a tonicity adjuster. In some embodiments, the sodium phosphate, monobasic, monohydrate; and/or the sodium phosphate, dibasic, anhydrous are included as pH buffers. In some embodiments, the sodium hydroxide/hydrochloric acid is included in the formulation as a pH adjuster. In some embodiments, the water for injection is the solvent of the formulation.

[0056] In some embodiments, no preservative is present. In some embodiments, the formulation is terminally sterilized via autoclave.

[0057] Further information providing the solubility, particle size, viscosity, and other product profile parameters of exemplary CLS-AX compositions (active pharmaceutical ingredient (API); and drug product) are provided in Table 1C.

Table 1C. Exemplary Product Profile

API	Chemical formula	C ₂₂ H ₁₈ N ₄ O ₅	
	Molecular weight (g/mol)	386.47	
	Melting point (°C)	213-215	
	Boiling Point (°C)	668.9±55.0	
	Density (g/mL)	1.4	
	Solubility	DMSO 0.2µg/mL in water	
	Particle size (±2)	D50 - 2µm D90 - 3µm	
Drug Product	Particle size (±2)	Preclinical D50 - 1µm D90 - 3µm	Clinical D10 - 2µm D50 - 5µm D90 - 10µm
	Viscosity (cP)	6	

Example 2. Ocular Distribution of CLS-AX Following Suprachoroidal Administration to Pigmented Rabbits

[0058] The purpose of the following studies was to assess the pharmacokinetics and ocular tissue distribution following a single bilateral suprachoroidal microneedle injection of CLS-AX to male pigmented Dutch Belted rabbits. In one study, CLS-AX was administered at a dose of 4 mg/eye (100 µL/injection). In a separate study, CLS-AX was administered at a dose of 0.1 mg/eye or 0.03 mg/eye. The animals' eyes were examined by a board certified veterinary ophthalmologist using a slitlamp biomicroscope and an indirect ophthalmoscope. The exams occurred predose and on the indicated study days prior to sacrifice, as applicable. On specified days, at least two animals/time point were euthanized for the collection of blood (for plasma) and ocular tissues (aqueous humor, vitreous humor, retina, and sclera/choroid-RPE). Plasma and ocular tissues were analyzed for concentrations of CLS-AX using liquid chromatography/mass spectrometry.

[0059] For the initial study, the animals were acclimated to study conditions for 16 days prior to dose administration. At dosing, the animals weighed 1721 to 1941 g and were 5 months of age. All animals were housed in individual, suspended, stainless steel cages during acclimation and the test period. Certified Hi-Fiber Rabbit Diet #5325 (PMI) was provided. Water was provided fresh daily, ad libitum. All animals were housed in individual, suspended, stainless steel cages during acclimation and the test period. Environmental controls for the animal room were set to maintain a temperature of 16 to 22°C (Deviation), a relative humidity of 50 ± 20% (Deviation), and a 12-hour light/12-hour dark cycle. As necessary, the 12-hour dark cycle was interrupted to accommodate study procedures. Each animal was assigned a temporary identification number. At selection, permanent animal numbers were assigned (Deviation). Each animal was uniquely identified with an individually numbered cage card prior to animal selection and an implantable microchip identification device upon assignment to the study. Immediately prior to dosing, animals were anesthetized with an intramuscular (IM) injection of ketamine, dexmedetomidine, and glycopyrrolate. Following application of topical anesthetic, eyes were rinsed with an iodine solution followed by a saline rinse. Animals were not fasted prior to dose administration.

[0060] The dosing formulation was drawn up into a 1-mL luer-lock syringe using a standard 21-gauge, 1-inch needle; any bubbles were expressed, and the standard needle was replaced by a 30-gauge microneedle 700 µm in length. A single suprachoroidal injection of 100 µL was given over approximately 5-10 seconds to each eye (3-4 mm from the limbus, in the superior temporal quadrant) by an OSOD representative according to a study-specific procedure. Following the injection, the needle was kept in the eye for approximately 20 seconds before being withdrawn. Upon withdrawal of the microneedle, a cotton-tipped applicator (CTA, dose wipe) was placed over the injection site for approximately 10 seconds; the dose wipe was discarded. The eye was inspected to confirm accuracy of injection by an OSOD representative. The right eye was dosed first; all postdose times were based on the time of dosing of the second (left) eye. Any dosing observations were recorded.

[0061] Animals were given an intramuscular (IM) injection of flunixin (2 mg/kg) prior to sedation and approximately 24 hours (0.08 mL per animal) post the first flunixin administration, as applicable. Buprenorphine sustained release (SR, 0.2 mg/kg) was administered subcutaneously (SQ) and bland ophthalmic ointment was applied to each eye upon recovery. Animals were also

given neo-poly-bac ointment and atropine ointment topical ocular to both eyes once following dosing on Study Day 1 and twice daily on Study Days 2 and 3.

[0062] Twice daily (a.m. and p.m.), animals were observed for mortality and signs of pain and distress. Cageside observations for general health and appearance, with particular attention paid to the eyes, were done once daily.

[0063] Body weights were taken on the day of arrival, at the time of animal selection, on the day of dose administration, and weekly throughout the remainder of the study, as applicable.

[0064] A board-certified veterinary ophthalmologist conducted ophthalmic examinations predose and on Study Days 4, 15, 28, and 91. At each time point, using a slitlamp biomicroscope to examine the adnexa and anterior portion of each eye, an external examination was conducted. In addition, the eyes were dilated with a mydriatic agent, and the ocular fundus of each eye was examined using an indirect ophthalmoscope.

[0065] The following samples were collected for analysis.

[0066] *Blood and Plasma:* Two animals/time point were euthanized with an overdose of sodium pentobarbital and blood (approximately 5 mL) was collected via cardiac puncture into tubes containing K₂EDTA at 24, 72, and 168 hours postdose and on Study Days 15, 29, 61, and 91. Samples were maintained on wet ice until centrifuged to obtain plasma. All plasma samples were placed on dry ice prior to storage at approximately -70°C until analyzed. The cellular fraction was discarded. Additional blood was collected and discarded to facilitate collection of ocular tissues.

[0067] *Ocular Tissues:* At the time of sacrifice, both eyes were immediately enucleated. The aqueous humor was collected and each eye was flash frozen in liquid nitrogen for 15 to 20 seconds, and subsequently placed on dry ice for at least 2 hours (Deviation). Within approximately one day, the aqueous humor, retina, sclera/choroid (including retinal pigmented epithelium), and vitreous humor tissues were collected. The ocular tissues were rinsed with saline and blotted dry, as appropriate, weighed, and placed on dry ice until stored at approximately -70 °C until analyzed. Residual carcasses and remaining ocular tissues were discarded

[0068] Plasma and ocular tissues were analyzed for concentrations of CLS-AX using liquid chromatography/mass spectrometry.

[0069] Values from instruments such as balances are reported as generated by (or recorded from) each instrument. Unless otherwise noted, calculated values for mean and standard deviation are reported to three significant figures. Statistical analyses were limited to descriptive statistics such as mean and standard deviation. Because the data were computer-generated and rounded appropriately for inclusion in the report, the use of reported values to calculate subsequent parameters will, in some instances, yield minor variations from those listed in the tables. Dose tables were compiled with values calculated using Excel, Version 14.0 (Microsoft Corporation). As applicable, for individual animals, the maximum concentration ($C_{\max}$) in plasma, aqueous humor, retina, sclera/choroid-RPE (SCR), and vitreous humor and the time to reach maximum concentration ($T_{\max}$) were obtained by visual inspection of the raw data. Pharmacokinetic parameters calculated included half-life ($t_{1/2}$), area under the concentration-time curve from time 0 to the last measurable time point (AUC_{0-t}), and area under the concentration-time curve from 0 to infinity ($AUC_{0-\infty}$). Pharmacokinetic parameters were calculated by using Phoenix Winnonlin, version 6.2.1 (Pharsight Corporation).

[0070] All animals appeared clinically healthy throughout acclimation and were released from acclimation and approved for use on the study. The suprachoroidal administration of CLS-AX did not have a deleterious effect on body weight over the duration of the study.

[0071] Sporadic instances of low food consumption were noted throughout the study. Infrequent occurrences of these observations are considered normal for the species in a laboratory environment.

[0072] **Results.** All animals were free of ophthalmologic findings prior to test article administration. Suprachoroidal administration of CLS-AX at 4 mg/eye (100 μ L/injection) was well tolerated through Study Day 91. Subconjunctival white plaques, likely representing test article were commonly observed at later intervals once the initial conjunctival response to the injection had resolved. The observed white plaques were consistent with the dosing observations above, indicating the plaques may be from the minor reflux into the subconjunctival space of CLS-

AX following dosing. Clear subconjunctival channels filled with a clear fluid were also observed in some eyes near the injection site on Study Day 15 and thereafter. Although the exact nature of these channels is unclear, they likely represent congested aqueous veins or lymphatic-like vessels. RPE pigment mottling was observed in one eye on Day 91 of the dosing phase, suggesting that this tissue was disturbed by the injection or test article.

[0073] White deposits were observed during tissue collections. Up to 61 days postdose, white deposits were observed on the exterior of the eye and could be removed with the bulbar conjunctiva. However, on Study Day 91, attempts were made to dislodge the white deposits from adhering to the sclera once the conjunctiva was removed. These attempts were unsuccessful, suggesting that the deposit was sub-scleral in location. It is possible the appearance of the deposits were more pronounced because the white test article is located between the translucent sclera and the deeply pigmented choroid. These white deposits were not seen upon examination of the fundus, indicating the deposit was most likely CLS-AX located in the suprachoroidal space, which could be observed during external examination of the eyes.

[0074] Following many of the injections, small amount of test material may have been trapped under the conjunctiva or within the sclera upon needle withdrawal. The refluxed material may have appeared as subconjunctival white plaques to the examiner. In agreement with the postdose observations and exam findings, white deposits were observed during tissue collections. Up to 61 days postdose, white deposits were observed on the exterior of the eye and could be removed with the bulbar conjunctiva until Study Day 91, suggesting that the deposit was in part suprachoroidal in location.

[0075] The results of the 4mg/eye study are provided graphically in FIG. 1A. Pharmacokinetic analysis results are presented in Table 2. The results of the 0.1 mg/eye or 0.03 mg/eye study are provided graphically in FIG. 1B.

[0076] Following a single bilateral suprachoroidal administration of CLS-AX (4 mg/eye), the analyte was not observed at quantifiable levels in either plasma or aqueous humor samples. CLS-AX was quantifiable at all time points in vitreous humor, retina, and sclera/choroid-RPE (SCR) after administration of 4 mg/eye. A concentration gradient of CLS-AX in tissues was present, with the dose depot (SCR) the highest, followed by the retina, and finally the vitreous humor with the

lowest concentrations (FIG. 1A). The dose depot (SCR) extrapolated initial concentration (C_0) was 17.2 mg/g. The mean concentrations of CLS-AX in the SCR rose slightly on Study Day 4, most likely due to interanimal variability. The dose depot (SCR) concentrations of CLS-AX were the highest early in the study, and then started to decline beyond Study Day 8 through Study Day 91 (FIG. 1A, top panel). The high levels of CLS-AX remaining coincided with observation of sub-scleral white plaques, suggesting that the plaques were remaining dose depot. The elimination half-life ($t_{1/2}$) was calculated to be 102 days; more than 60% of CLS-AX remained in the SCR at 3 months post injection (FIG. 1A, top panel). The level of CLS-AX in the retina was more than 100,000 fold above the IC50 of axitinib for its receptors (VEGFR and PDGFR) (FIG. 1A, bottom panel). The observed exposure (AUC_{0-91}) value of the dose depot was 1260 $\mu\text{g}\cdot\text{day/g}$.

[0077] Although CLS-AX is dosed as a suspension, with limited solubility in aqueous solvent, the immediate presence of CLS-AX 24 hours postdose in retina and vitreous humor indicated a burst release of the test article into tissue following dosing. CLS-AX levels in retina and vitreous humor increased over time, to a maximal mean concentration (C_{max}) of 325 $\mu\text{g/g}$ and 0.857 $\mu\text{g/mL}$, respectively. The C_{max} levels were reached in the vitreous humor on Study Day 4 (T_{max}), and then declined thereafter albeit with some variability. The mean concentrations in retina were similar from Study Day 2 up to Study Day 15, and then increased approximately 3-fold on Study Day 29. The concentrations of CLS-AX in retina were similar to the C_{max} through Study Day 91. The observed exposure to CLS-AX (AUC_{0-91}) in retina and vitreous humor was consistent with the concentration gradient between the two tissues. The retina concentrations of CLS-AX remained above 44 $\mu\text{g/g}$ throughout the duration of the study.

[0078] Individual concentrations of CLS-AX in plasma, aqueous humor, retina, sclera/choroid-RPE (SCR), and vitreous humor are presented in Table 2. The concentrations of CLS-AX were determined using liquid chromatography with tandem mass spectrometric (LC-MS/MS) methods. Sample analysis was performed using a verified method. Analyst[®] software (Version 1.6.2) was used to capture the LC-MS/MS data and integrate the peak areas. Watson LIMS software (Version 7.4.1) was used for data storage, management and reporting.

[0079] As noted above, concentration of CLS-AX was below the limit of quantitation (BLQ) in all plasma samples and aqueous humor samples. In retina samples, concentrations of CLS-AX

ranged from 2750 ng/mL to 60,100 ng/mL of homogenate and 44,500 ng/g to 1,220,000 ng/g tissue. In SCR, concentrations of CLS-AX ranged from 1,870,000 ng/mL to 3,360,000 ng/mL homogenate and to 9,790,000 ng/g to 24,400,000 ng/g tissue. In the vitreous humor, concentrations of CLS-AX ranged from 60 ng/mL to 6,030 ng/mL.

[0080] In summary, following suprachoroidal administration of CLS-AX (4 mg/eye), the analyte was not observed at quantifiable levels (lower limit of quantitation = 1 ng/mL) in either plasma or aqueous humor samples throughout the duration of the study. However, CLS-AX was quantifiable at all time points in vitreous humor, retina, and sclera/choroid-RPE (SCR).

[0081] In the second study, CLS-AX was detectable in the retina and choroid-RPE/sclera well above the IC₅₀ for the full length of the study (67 days) for both doses (FIG. 1B). The concentration detected in the vitreous was significantly lower compared to that in the posterior tissues, and by day 14 CLS-AX was no longer detectable in the vitreous (FIG. 1B).

[0082] Additional data from the second study are provided below and in Table 1C. Mean axitinib concentrations were maximal on day 1 in SCR, retina, and vitreous humor for both doses. Elimination $t_{1/2}$ values of 257 and 379 hours were calculated for 0.03 and 0.1 mg/eye SCR drug depot, respectively. The 0.1 mg/eye mean $C_{\max}$ and $AUC_{0-\infty}$ values were approximately 6- and 7-fold higher than 0.03 mg/eye parameters, respectively.

[0083] Following bilateral suprachoroidal administration, axitinib mean retina concentrations reached $C_{\max}$ values of 4480 and 6260 ng/g on study day 2 ($t_{\max}$ = 24 hours postdose) for 0.03 mg/eye and 0.1 mg/eye, respectively. Beyond Study day 2, the lower dose group mean retina concentrations dropped sharply and were essentially below the limit of quantitation (16.7 ng/g) by day 15, with only 1 of 4 eyes on days 31 and 61 having quantifiable levels of axitinib. The higher dose mean retina concentrations also dropped sharply beyond 24 hours postdose, and were quantifiable in only 1 of 4 eyes on Days 45 through 66. The higher dose mean $C_{\max}$ and AUC_{0-t} values were 1.4- and 1.6-fold higher than the lower dose parameters, respectively. These concentration and exposure ratios are less than dose proportional.

[0084] Beyond day 2, mean vitreous humor concentrations were quantifiable in only a few eyes in each group on Day 8. The mean $C_{\max}$ values for vitreous humor for the two dose groups were

very similar, suggesting that CLS-AX does not readily partition into vitreous humor, independent of dose level.

Table 1D. Pharmacokinetic parameters of Axitinib in rabbits dosed via SCS injection with CLS-AX doses of 0.03 mg/eye or 0.1 mg/eye

Dose Administered (mg/eye)	C ₀ (µg/g or mL) ^b	C _{max} (µg/g or mL) ^b	t _{max} (hour)	AUC _{0-t} (µg·day/g or mL)	AUC _{0-∞} (µg·day/g or mL)	t _{1/2} (hour)
SCR						
0.03	48.40	37.20	24.0	8,120.00	8,160.00	257
0.1	269.00	232.00	24.0	55,600.00	57,300.00	379 ^a
Retina						
0.03	NC	4.48	24.0	414.00	NC	NC
0.1	NC	6.26	24.0	674.00	NC	NC
Vitreous Humor						
0.03	NC	0.004	24.0	NC	NC	NC
0.1	NC	0.0046	24.0	NC	NC	NC

[0085] Taken together, the results of the studies showed that a single bilateral administration of CLS-AX suspension to the suprachoroidal space was well tolerated at all doses tested (4 mg/eye, 0.1 mg/eye, 0.03 mg/eye) and exhibited favorable ocular distribution and pharmacokinetics for durability of treatment of a posterior ocular disorder. Minimal or no systemic exposure, minimal anterior segment exposure, high absorption into the posterior segment, and a much longer half life than expected was achieved. The longer half-life reduces or eliminates the need for repeated injections of axitinib. The problem of effective drug administration to the posterior segment of the eye without detrimental side effects of repeated injections or side effects related to systemic exposure has been a difficult problem for those in the field to solve. With the SCS delivery of an axitinib drug formulation for treatment of a posterior ocular disorder disclosed in the present application and stated in the claims, an unexpected improvement in the method for treatment of a posterior ocular disorder is provided.

Table 2: Mean pharmacokinetic parameters in retina, sclera/choroid-RPE, and vitreous humor of male Dutch Belted rabbits dosed via suprachoroidal injection with Axitinib (4 mg/eye)

Matrix	C ₀ (µg/g)	C _{max} (µg/g or µg/mL)	T _{max} (days)	t _{1/2} (days)	AUC _{0-t} (µg*day/g or µg*day/mL)
Sclera/Choroid-RPE	17200	22000	NC	102 ^a	1260
Retina	NC	325	61	NC	23.9
Vitreous Humor	NC	0.857	4	NC	0.0314

NC Not calculated.

RPE Retinal pigmented epithelium.

a The calculated half-life value should be interpreted with caution as the value was calculated over less than two half-lives.

Example 3. Rat Laser-Induced Choroidal Neovascularization Model

[0086] The purpose of this study was to determine the potential efficacy of suprachoroidal injection of CLS-AX in a laser-induced choroidal neovascularization model with Brown Norway rats using a modified microneedle/SCS microinjector.

[0087] On Day 1, prior to the choroidal neovascularization procedure, mydriatic drops were applied to both eyes, and the animals were anesthetized prior to initiation of the following procedures. A 4-spot pattern was made between the major retinal vessels around the optic disc of each eye. Laser parameters were adjusted as required to ensure rupture of Bruch's membrane. Thereafter, groups of male rats (10/group) were administered the control item (sodium chloride for injection, Unites States Pharmacopeia [USP]) or CLS-AX (batch No. 50311-1, 40 mg/mL) under anesthesia into both eyes by freehand bilateral suprachoroidal injection using a 100 µL Hamilton syringe equipped with a modified Clearside microneedle (33 gauge, < 400 µm). Dosing was performed on Days 1 and 8 at a dose of 0 or 0.4 mg/eye/dose (0.2 mg/eye per each dose administration), respectively. The target dose volume for each animal was 5 µL/eye/injection.

[0088] Parameters evaluated during the study included mortality, clinical signs, body weight, food consumption, ophthalmology (indirect ophthalmoscopy and slit-lamp biomicroscopy) once prestudy and on Day 1 post-dose; and fluorescein angiography (FA) once prestudy and on Days 7 (predose), 14, and 21. Fluorescein angiography is a technique used to assess vascular leakage from

ocular neovascularization lesions. The individual laser spots on the still images were evaluated for leakage semiquantitatively on a scale of Grade 0 to Grade IV by 2 independent readers, who subsequently determined a consensus score. The scale is shown at FIG. 2A. The animals were sacrificed on Day 22, and the eyes and optic nerves were collected and preserved in Davidson's fixative.

[0089] The study design is presented in Table 3.

Table 3: Rat Laser-Induced Choroidal Neovascularization Study Design

Group Number	Test Material	Dose Level (mg/eye/dose)	Dose Volume (μL)	Dose Concentration (mg/mL)	Number of Animals (Male)
1 ^a	Saline for Injection USP	0	5	0	10
2 ^b	CLS-AX	0.4	5	40	10

Abbreviations: USP=United States Pharmacopeia; ^aGroup 1 was dosed on Days 1 and 8. ^bGroup 2 was dosed on Days 1 and 8 (0.2 mg/eye on each occasion).

[0090] There were no deaths, and no treatment-related clinical signs or effects on body weights, body weight gains, or food consumption. Minor retinal iatrogenic trauma resulted from the force applied during the injection procedure, but otherwise no clinically important ocular complications were noted immediately following the first injection.

[0091] Administration of CLS-AX using the microneedle resulted in successful suprachoroidal and/or subretinal injections of CLS-AX in most animals, although assessment of the post-injection results in the control group was complicated by the transparency of the control item. CLS-AX appeared white upon ophthalmic examination, and in Group 2, a total of 11/20 eyes had white test item in the SCS as noted from indirect ophthalmoscopy, and 9/20 had subretinal test item. In 4 eyes, the CLS-AX was observed in both subretinal and suprachoroidal spaces. In 5/20 eyes, the test item could be observed near the needle tip and appeared to have been injected subsclerally or suprachoroidally, but in these eyes, possible visualization of the test item upon funduscopy was negative. Exact localization of CLS-AX in these eyes remained uncertain but was unlikely to be subconjunctival as the conjunctiva did not elevate during the injection.

[0092] Reflux was minimal in most eyes, and similar in the reference group and CLS-AX group. Six eyes in the reference group and 10 eyes in the CLS-AX group were observed with noted reflux. The higher incidence in the CLS-AX group may have been due to the fact that visualization of the CLS-AX was much easier due to its white color. Reflux observed in this study is likely a result of the difficulty of freehand injection in rats, and is not expected to occur when administering CLS-AX to humans using the microinjector.

[0093] Control animals generally showed no decrease in leakage from Days 7 to 21, with the exception of 2 of 20 eyes, from 2 different animals. This rate is typical of the laser-induced choroidal neovascularization model in rats and indicates the model was performing as expected. Decreases in the incidence of clinically important lesions as assessed using FA (scores of 3 or 4) were noted in rats administered CLS-AX, in which 8 of 20 eyes showed improvement (scores of 0 to 2). Comparing the mean scores for control versus treated eyes demonstrated that CLS-AX-treated animals had statistically significant lower scores by Day 21, supporting the activity of CLS-AX in the model. Moreover, the percent of Grade IV lesions at day 21 was significantly less in the CLS-AX group compared to the control group (63.8% and 88.8%, respectively; FIG. 2B). Drug reflux did not appear to correlate with a decreased benefit from CLS-AX treatment.

[0094] The study demonstrated that administration of CLS-AX at 0.4 mg/eye/dose once weekly for 2 weeks by suprachoroidal injection was well tolerated in rats and resulted in significant reduction in clinically important lesions by Day 21 compared to control rats treated with saline.

Example 4. Pig Laser-Induced Choroidal Neovascularization Model

[0095] The purpose of this study was to evaluate the effect of a single SC injection of CLS-AX on the development of choroidal neovascularization in a laser-induced porcine model of choroidal neovascularization. This is a model of human ocular posterior segment neovascular diseases occurring in the choroid, such as nAMD.

[0096] The potential efficacy of a single SC injection of CLS-AX was assessed in 8 male weanling pigs. On Day 1, mydriatic drops were applied to both eyes and the animals were anesthetized prior to the choroidal neovascularization procedure. The diode laser energy was delivered via a laser

indirect headset to create 6 uniform white laser lesions surrounding the optic nerve in each eye. Immediately after laser treatment pigs were administered the control item (balanced salt solution [BSS]) in the left eye and CLS-AX (concentration 40 mg/mL) at a dose of 4 mg in the right eye by freehand SC injection using a syringe equipped with the microneedle (30 gauge, < 400 µm) at a dose volume of 100 µL/eye.

[0097] Parameters evaluated during the in-life portion of the study included ocular fundus photography and FA immediately following treatment as well as 1 and 2 weeks post treatment. The pigs were euthanized 2 weeks following treatment and the eyes were enucleated and processed for retinal flat mounts. Isolectin IB₄ staining, a technique used to evaluate neovascularization, was performed to quantify neo-formed vessels in retinal flat mounts.

[0098] The experimental design is provided in Table 4.

Table 4: Pig Laser-Induced Choroidal Neovascularization Study Design

Group	N (Animals)	Laser (OU)	Drug	Injection Volume	Sample Collection Times N=8 animals per time point
1	8	6 spots around optic nerve	OS: BSS OD: CLS-AX 4 mg	100 µL	Ocular fundus photography (0, 1, and 2 weeks after laser) Fluorescein angiography (0, 1, and 2 weeks after laser) Retinal flat mounts (2 weeks after laser [n=16 eyes])

Abbreviations: BSS=balanced salt solution; OD=right eye; OS=left eye; OU=both eyes

[0099] The laser-induced retinal lesions were readily observed by fundus photography and FA. All lesions became less white and defined over time, but there was no visible difference between eyes dosed with BSS (OS [left eye]) or CLS-AX (OD [right eye]) as evaluated immediately after laser treatment and at 1 and 2 weeks after laser treatment. On FA, at 1 and 2 weeks after laser treatment, the eyes treated with CLS-AX had a 10.5% (P=0.009) and 16.0% (P=0.0015) mean reduction in the area of fluorescence, respectively, compared to the control eyes given BSS (FIG. 3A), indicating treatment with CLS-AX significantly reduced vascular leakage and reduced growth of new blood vessels at the site of the retinal laser lesion. Representative images of a BSS treated eye and a CLS-AX treated eye are provided in FIG. 3B.

[00100] Quantification of neoformed vessels performed on retinal flat mount tissue (measured by isolectin IB₄ signal – a marker of neoformed vasculature) revealed that eyes treated with CLS-AX had reduced isolectin IB₄ signal area by 18% relative to BSS-treated eyes (Representative sample; FIG. 4A; Quantification of mean IB₄ signal, FIG. 4B). These data indicated that CLS-AX treatment significantly suppressed neovascularization relative to the control eyes administered BSS (P=0.0297).

[00101] Thus, a single SCS injection of CLS-AX at a dose of 4 mg/eye significantly reduced vascular leakage (as evidenced by reduced fluorescein signal) and growth of new blood vessels (as assessed by reduced isolectin IB₄ signal) at the site of the retinal laser lesion in pigs as compared with the control treatment of BSS.

[00102] Taken together, the pharmacokinetic and pharmacodynamic data provided herein shows that CLS-AX is generally well tolerated in rats, rabbits, and pigs upon SCS administration with no overt signs of toxicity. Sustained and high exposure of axitinib was observed in ocular tissues throughout the PK study with highest levels in the sclera/choroid/RPE, followed by the retina, followed by the vitreous. No quantifiable axitinib was detected in either plasma or aqueous humor. In rats, CLS-AX decreased the incidence of clinically important neovascular lesions (scores of 3 or 4) where 8/20 eyes (40%) showed a general improvement with reduction in clinically important lesions by Day 21 compared to control group. In pigs, CLS-AX significantly reduced fluorescein leakage (105% and 16% at week 1) and growth of new blood vessels (18%) at the site of the retinal laser lesion as compared to saline treatment. Accordingly, is a potent anti-angiogenic/ anti--neovascularization treatment for nAMD, and his highly effective when non-surgically administered to the SCS of the eye. CLS-AX has potential as a therapy, e.g., a bi-annual therapy, for nAMD, given its pan-VEGF inhibition through receptor blockade as well as the pharmacodynamics effect, prolonged duration, ability to directly target affected tissue layers, and high potency as uncovered in the studies provided herein.

Example 5. Clinical Study

[00103] A Phase 1/2a clinical study will be performed to assess the safety and efficacy of non-surgical administration of axitinib to the suprachoroidal space of the eye of nAMD patients.

In part, the doses to be tested are in line with the data obtained in the pharmacodynamics studies disclosed herein.

[00104] In developed countries, neovascular age-related macular degeneration (nAMD) is the leading cause of irreversible central blindness (Santarelli, 2015). Although AMD pathogenesis is complex and still not fully understood, many of the mechanisms involved are already partially known and, specifically for the 10-15% of AMD classified as the wet type (nAMD), include the vascular endothelial growth factor (VEGF) signaling pathway. In nAMD, abnormal blood vessel growth, choroidal neovascularization (CNV) in the choriocapillaris, immediately below Bruch's membrane, under the retina and macula leads to leakage of blood, lipids, and serum into the retinal layers and causes the macula to bulge or lift up from its normal position, distorting or destroying central vision. In nAMD, VEGF-A, which acts at VEGF-receptors 1 and 2 (VEGFR-1, VEGFR-2) has been shown to promote abnormal blood vessel growth and is therefore an optimal target for treatment. Currently, anti-VEGF drugs are the standard of care for this condition; however significant unmet need remains for significantly improving and maintaining visual acuity in most patients (Martin, 2012). Furthermore, the current treatment paradigm of frequent intravitreal injections is burdensome; for example, a recent "realworld" retrospective study of nearly 50,000 eyes with wet AMD demonstrated that patients are undertreated with only 7.3 injections on average, yielding only a 1-letter gain at 1 year (Ciulla, 2019).

[00105] Primary Endpoint: To assess safety and tolerability of CLS-AX in subjects with neovascular age-related macular degeneration who show stable visual acuity (no loss) following ≥ 3 injections with an intravitreal anti-VEGF therapy in the preceding 5 months.

[00106] Secondary Endpoint: To evaluate and compare the effect of 3 cohort regimens of CLS-AX over 3 months on visual function and anatomy and the need for additional treatment with intravitreal aflibercept.

[00107] Study Design: This is a Phase 1/2a open-label, dose-escalation study to assess the safety and tolerability of single doses of CLS-AX administered suprachoroidally following at least 3 prior treatments (the last of which will be administered at the Screening visit) with an intravitreal (IVT) anti-VEGF agent in nAMD subjects. The study design includes 3 dose cohorts of 5 subjects each. Subject eligibility will be established at Visit 1, Screening (Day -28 ± 3 days). Eligible

subjects will receive an IVT injection of aflibercept, 2 mg (0.05 mL), at Visit 1, Screening (Day - 28 ± 3 days), followed by a suprachoroidal injection of CLS-AX at Visit 2, Baseline (Day 0). Subjects return for safety and tolerability assessments, visual function and ocular anatomy assessments, and the need for additional treatment at Visits 3, 4, and 5 (Weeks 4, 8 and 12), (Follow-up Period). Further, additional treatments will be administered at Visits 3 and 4 (Weeks 4 and 8) based on PRN criteria and will consist of aflibercept (2 mg (0.05 mL) administered by IVT injection unless other therapy is medically necessary. All subjects will be followed until Visit 5 (Week 12, Study Exit) regardless of whether additional therapy is given or not. The 3 dose cohorts will include the following:

- Cohort 1 (lowest dose arm): 0.03 mg suprachoroidal injection of CLS-AX
- Cohort 2 (mid dose arm): 0.06 mg suprachoroidal injection of CLS-AX
- Cohort 3 (high dose arm): 0.10 mg suprachoroidal injection of CLS-AX

[00108] All cohorts will be assessed for safety and tolerability and effects on visual function and anatomy as outlined in the time and events schedule.

[00109] Number of subjects (planned): Approximately 15.

[00110] Inclusion criteria: Subjects are eligible for participation in this study if s/he meets all of the following criteria at the Screening visit (Visit 1) and Baseline visit (Visit 2):

1. Diagnosis of neovascular age-related macular degeneration in the study eye
2. Active subfoveal choroidal neovascularization (CNV) secondary to AMD of any lesion type in the study eye with photos and/or fluorescein angiography (FA) and/or spectral-domain optical coherence tomography (SD-OCT) showing: a. the total lesion area (including blood, neovascularization and scar/atrophy) $\leq 30 \text{ mm}^2$, b. CNV component area of $\geq 50\%$ of total lesion area, c. CNV must not be associated with subfoveal hemorrhage, subfoveal fibrosis or subfoveal atrophy.
3. At Screening, two or more anti-VEGF treatments (aflibercept, ranibizumab, bevacizumab, brolucizumab) in the 4 months preceding Screening (Visit 1), with the last treatment administered

at least 4 weeks prior to Screening (Visit 1), for neovascular AMD in the study eye with a meaningful response, for example, an improvement in vision and/or exudation, based on the Investigator's opinion..

4. Early Treatment Diabetic Retinopathy Study (ETDRS) best-corrected visual activity (BCVA) score of ≥ 20 letters read (20/400 Snellen equivalent) and ≤ 75 letters read (20/32 Snellen equivalent) in the study eye with less than 5 letters change in BCVA between Visit 1 and Visit 2 (Screening and Baseline)

5. Understands the language of the informed consent; willing and able to provide written informed consent prior to any study procedures; willing to comply with the instructions and attend all scheduled study visits

6. At least 50 years of age

[00111] Ophthalmic Exclusion criteria: Subjects are ineligible for participation in this study if s/he meets any of the following criteria:

1. Any atrophy or fibrosis in the fovea of the study eye as assessed by spectral-domain optical coherence tomography

2. Has significant media opacity in the study eye precluding evaluation of the retina and vitreous

3. Has macular edema or CNV in the study eye with etiology other than AMD; has any active ocular disease or infection in the study eye other than AMD; has CNV in the study eye with any of the following on photos and/or FA and/or SD-OCT: a. Total lesion area (including CNV, hemorrhage, fibrosis, atrophy) $>30 \text{ mm}^2$, b. CNV component area of $< 50\%$ of total lesion area, c. Subfoveal hemorrhage, subfoveal fibrosis or subfoveal atrophy, d. Retinal pigment epithelial tear, Retinal angiomatous proliferation (RAP)

4. Other than IVT injected aflibercept, ranibizumab, bevacizumab, or brolucizumab, prior treatment for CNV in the study eye.

5. Intraocular pressure ≥ 25 mmHg or uncontrolled glaucoma (open angle or angle closure) in the study eye at Visit 1; subjects are not excluded if intraocular pressure (IOP) is < 25 mmHg in the study eye with no more than 1 IOP lowering medications; or cup-to-disc ratio > 0.8
6. History of any vitreoretinal surgery (examples include but are not limited to scleral buckle, pars plana vitrectomy, retrieval of a dropped nucleus or intraocular lens) or history of panretinal or macular laser photocoagulation in the study eye; intravitreal injections are acceptable; prior cataract extraction, Yttrium-Aluminum-Garnet laser capsulotomy is allowed, but must not have been within 3 months of Screening (Visit 1)
7. Has had cyclodestructive procedures or filtration surgeries in the study eye in the 3 months prior to Visit 1
8. Has high myopia in the study eye defined as a spherical equivalent > -6 diopters or an axial length ≥ 26 mm. Has a prior history of high myopia, if pseudophakic
9. Has had photocoagulation or cryotherapy in the study eye within the 6 months prior to Visit 1
10. In the study eye, any topical ocular corticosteroid in the 10 days prior to treatment at Visit 2 (Day 0); any intraocular or periocular corticosteroid injection
11. Concomitant therapy with any drug that may be toxic to the lens, retina, or optic nerve
12. At the time of screening, is receiving anti-VEGF therapy in the fellow eye, or is expected to receive anti-VEGF therapy in the fellow eye during the study.

[00112] General Exclusion Criteria: Subjects are ineligible for participation in this study if s/he meets any of the following criteria:

12. Female subjects who are pregnant, lactating or planning a pregnancy. Females of childbearing potential must agree to submit to a pregnancy test at screening and agree to use an acceptable method of contraception throughout participation in the study. Acceptable methods of contraception include double barrier methods (condom with spermicide or diaphragm with spermicide), hormonal methods (oral contraceptives, implantable, transdermal, or injectable contraceptives), or an intrauterine contraceptive device with a documented failure rate of less than

1% per year. Abstinence may be considered an acceptable method of contraception at the discretion of the Investigator, but the subject must agree to use one of the acceptable birth control methods if she becomes sexually active.

13. Any uncontrolled systemic disease that, in the opinion of the Investigator, would preclude participation in the study (e.g., unstable medical status including uncontrolled elevated blood pressure, cardiovascular disease, and glycemic control) or put the subject at risk due to study treatment or procedures

14. Has taken systemic corticosteroids at doses greater than 20 mg per day for oral prednisone (or equivalent for other corticosteroids) in the 2 weeks prior to Visit 2; subjects on 20 mg or less per day can be enrolled if no increase in dosing is anticipated for the duration of the study

15. Likely need for hospitalization or surgery within the study period, including planned elective surgery or hospitalization that cannot be deferred

16. Hypersensitivity to any component of the CLS-AX, fluorescein, or to topical anesthetics

17. Currently enrolled in an investigational drug or device study or has used an investigational drug or device within 30 days of Visit 1

[00113] Investigational product, dosage and mode of administration: CLS-AX, axitinib injectable suspension, doses at 0.03 mg, 0.06 mg and 0.10 mg in 100 µL into the suprachoroidal space, administered with the SCS Microinjector

[00114] Criteria for evaluation include incidence of treatment-emergent adverse events (TEAEs) and serious adverse events, grouped by organ system, relatedness to study treatment, and severity (primary); and incidence/descriptive statistics of changes in ocular safety parameters, number of additional aflibercept treatments following CLS-AX administration, mean change from baseline in central subfield thickness, mean change from baseline in ETDRS BCVA letter score, and the systemic and ocular outcomes (secondary).

[00115] All subjects will receive IVT injected aflibercept, 2 mg (0.05 mL), at Screening (Visit 1) followed by a single dose of CLS-AX administered suprachoroidally at Baseline (Visit

2). Safety assessments from the visits at Week 4 (Visit 3), 8 (Visit 4) and 12 (Visit 5) following CLS-AX SC injection will be reviewed. Subjects will be assessed for additional therapy at Visits 3, 4, and 5 (Weeks 4, 8, and 12). Additional therapy will be administered at Visits 3 and 4 (Weeks 4 and 8), consisting of IVT injection of aflibercept 2 mg (0.05 mL) (unless other therapy is medically necessary), based on the “Additional Therapy Criteria” provided below.

[00116] The following procedures must be performed before the SC CLS-AX injection (the same day as the injection): assess adverse events, review changes to concomitant medications, perform resting heart rate and blood pressure measurements, collect blood for PK analysis, collect urine for pregnancy test in females of child-bearing potential, and perform ophthalmic assessments on the study eye. Ophthalmic assessments include ETDRS BCVA, slit-lamp biomicroscopy including dilated lens grading, intraocular pressure (IOP) measurement in both eyes, dilated indirect ophthalmoscopy, SD-OCT, and OCT-A. The following photographic evaluations will be performed: fluorescein angiograph and fundus photography.

[00117] Eligible subjects will receive suprachoroidal injection of 100 µL of CLS-AX. The following assessments will occur after the suprachoroidal injection: assess adverse events, review changes to concomitant medications, perform resting heart rate (resting 5 minutes) and blood pressure measurements at least 30 minutes after injection, perform 12-lead electrocardiogram at least 30 minutes after injection, collect blood approximately 60 minutes after injection for pharmacokinetic analysis, and perform ophthalmic assessments. Ophthalmic assessments include: indirect ophthalmoscopy immediately after the injection, slit-lamp biomicroscopy, and evaluate IOP 10 to 30 minutes after injection. If IOP remains elevated, the subject must remain on site until the IOP is under control according to the Investigator’s best medical judgment. If IOP is <30 mmHg, then the subject may leave the clinic. At non-dosing visit days, the Investigator will contact the subject and assess adverse events and review changes to concomitant medications.

[00118] At Visit 3 (Week 4) and Visit 4 (Week 8) and Visit 5 (Week 12), the following procedures are performed: assess adverse events, review changes to concomitant medications, perform resting heart rate and blood pressure measurements, perform 12-lead electrocardiogram, collect blood for PK analysis, collect blood and urine samples for central laboratory tests before fluorescein angiogram, and perform ophthalmic assessments on the study eye. Ophthalmic

assessments include ETDRS BCVA, slit-lamp biomicroscopy including dilated lens grading, intraocular pressure (IOP) measurement in both eyes, dilated indirect ophthalmoscopy, SD-OCT, and OCT-A. The following photographic evaluations will be performed: fluorescein angiograph and fundus photography. Subjects will be evaluated for the need of additional therapy with aflibercept.

[00119] If the predefined criteria described below (“Additional Therapy Criteria”) are met, then IVT aflibercept will be administered. Following administration, the following assessments will be performed: assess adverse events, review changes to concomitant medications, perform ophthalmic assessments on the study eye. Ophthalmic assessments will include: assess the study eye by indirect ophthalmoscopy immediately after the injection, perform slit-lamp biomicroscopy, and evaluate IOP 10 to 30 minutes after injection. If IOP remains elevated, the subject must remain on site until IOP is under control according to the Investigator’s best medical judgment. If IOP is <30 mmHg, then the subject may leave the clinic.

[00120] Description of Study Drug: Treatment in each cohort will consist of a single unilateral injection of IVT aflibercept in the study eye at Visit 1 (Screening; Day -14 to -1) and a single unilateral suprachoroidal injection of CLS-AX in 100 µL administered (dosing levels of 0.03 mg, 0.06 mg, or 0.10 mg) on Visit 2 (Day 0). Subjects will be assessed for additional treatment at Visits 3, 4, and 5 (Weeks 4, 8, and 12). Additional treatments will be administered based on PRN criteria below (“Additional Therapy Criteria”) and will consist of IVT aflibercept 2 mg unless other therapy is medically necessary.

[00121] Enrollment into the next higher dose cohort will be based on the recommendation of the SMC following review of the safety data. Five subjects will be enrolled into each cohort.

[00122] Additional Therapy Criteria: At Visits 3, 4, and 5 (Weeks 4, 8, and 12), subjects will be evaluated for the need for additional therapy for neovascular AMD based on the following criteria. If any of the following criteria are met in the study eye at Visit 3 (Week 4) and Visit 4 (Week 8), then IVT aflibercept (2 mg (0.05mL) will be administered:

- Loss of 10 or more letters in BCVA compared to the best prior study-assessed BCVA in the study eye that is attributed to intra- or sub-retinal fluid observed by the Investigator, or

- Increase in central subfield retinal thickness greater than 75 microns from Baseline (Visit 2) in the study eye, or
- Presence of vision-threatening hemorrhage due to AMD in the study eye.

[00123] Additional treatment will be IVT aflibercept 2 mg unless other therapy is medically necessary; even if additional therapy is given the subjects will still be followed until study exit.

[00124] Axitinib systemic blood concentrations will be collected from those subjects who consent, by venipuncture by qualified study personnel, and will be used to estimate standard population PK parameters. A single blood sample will be obtained from each subject at the following time points: Visit 2 (Day 0) pre-dose, Visit 2 (Day 0) approximately 60 minutes post-dose, and Visits 3 and 5 (Weeks 4 and 12).

[00125] Best corrected visual acuity (BCVA) will be evaluated by ETDRS using standardized lighting and standardized lanes. The results shall be reported as the number of letters read. Visual acuity testing should precede any examination requiring contact with the eye. In order to provide standardization and well-controlled assessments of BCVA during the study, all BCVA assessments must be performed by trained staff who are certified on the study procedure using certified VA equipment/lanes.

[00126] Retinal thickness and disease characterization will be assessed via Spectral Domain Optical Coherence Tomography (SD-OCT). The SD-OCT instrument and technician must be certified before screening any subjects. The technician is encouraged to use the same certified equipment throughout the subject's study participation. All images should be taken by the same technician, whenever possible, on each subject per research site. Images will be sent to the Central Reading Center for analysis and interpretation.

[00127] Choroidal neovascular membranes will be classified and follow-up structural changes after the suprachoroidal injection will be assessed via Optical Coherence Tomography Angiography (OCT-A). The OCT-A instrument and technician must be certified before screening any subjects. The technician is encouraged to use the same certified equipment throughout the

subject's study participation. All images should be taken by the same technician, whenever possible, on each subject per research site.

[00128] Intraocular pressure (IOP) will be measured by applanation tonometry and results will be recorded in mmHg. Where available, Goldmann applanation tonometry should be used at all visits. Tonopens may be used for post-injection pressure checks and in cases where no Goldmann is available. The technician is encouraged to use the same tonometry method throughout the subject's study participation. At baseline, where suprachoroidal CLS-AX is to be administered, IOP will be measured 2 times: before suprachoroidal CLS-AX injection, and after suprachoroidal CLS-AX injection. Tonometers must be calibrated for accuracy before the first subject screening at that site and according to the manufacturer specifications during the study, until the last subject has exited the study at that site.

[00129] Slit-lamp biomicroscopy, including magnification, will be performed consistent with standard clinical practice. This procedure should be conducted in the same manner for all subjects and will include an assessment of each of the following as normal or abnormal: eyelids, sclera and conjunctiva, cornea, anterior chamber, iris, and lens. All abnormal findings will be described. Slit lamp examination of the iris is to rule out neovascularization of the iris (NVI).

[00130] Cataract Lens Grading: if an abnormal finding of cataract is noted during the slit-lamp examination, the cataract should be graded for nuclear opalescence, cortical opacity, and posterior subcapsular opacity. Graders must verify training on the grading procedures. Cataract classification will be based on the Lens Opacities Classification System III (LOCS III) grading scale (Chylack, 1993).

[00131] Indirect ophthalmoscopy should be performed according to the Investigator's standard procedure. This procedure should be the same for all subjects observed at the Investigator's site. The fundus will be examined thoroughly, and the following variables will be assessed as normal or abnormal (including but not limited to): vitreous, retina, choroid, and optic nerve/disc, appearance of vessels, and absence of neovascularization.

[00132] Fluorescein angiography will be performed for anatomic assessments and will include the area of fluorescein leakage, area of capillary nonperfusion, the presence of retinal

vascular and optic nerve head staining, and retinal pigment epithelium abnormalities. Digital equipment will be registered, and photographers certified for the imaging procedures. De-identified images will be uploaded to the Central Reading Center.

[00133] Color fundus photographs will be obtained. It is recommended that the fundus photographs should be taken prior to the fluorescein angiography. All photographs should be taken by the same photographer, whenever possible, on all subjects per research site. Digital equipment will be registered, and photographers certified for the imaging procedures. De-identified images will be uploaded to the Central Reading Center.

[00134] Statistical methods: This is an open-label Phase 1 study. The observations and change from baseline will be summarized descriptively for each cohort. Categorical variables will be summarized by counts and percentages and continuous variables by descriptive statistics (n, mean, standard deviation, standard error, median, minimum and maximum). Baseline is defined as the last assessment prior to administration of CLS-AX.

[00135] Dose-escalation criteria: All subjects will receive IVT aflibercept at Screening (Visit 1) followed by CLS-AX administered suprachoroidally at Baseline (Visit 2). Safety data will be assessed 4, 8 and 12 weeks post CLS-AX injection. Dose escalation will not proceed to the next highest dose if:

- A treatment related serious adverse event is reported
- ≥ 2 subjects experience a Grade 3 or higher non-serious treatment related adverse event, based on the CTCAE severity grading scale
- ≥ 3 subjects within the same dose cohort experience a Grade 2 or higher non-serious treatment related adverse event in the same MedDRA system organ class
- ≥ 4 subjects within the same dose cohort experience a Grade 2 or higher non-serious treatment related adverse event in any MedDRA system organ class
- 30 letter decrease in BCVA compared with pre-injection visual activity

- Sustained (> 15 minutes) loss of light perception due to elevated IOP
- 20 mmHg increase or decrease in IOP that does not return to pre-injection levels within 7 days of the onset of the event

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[00136] Publications, patents and patent applications cited herein are specifically incorporated by reference in their entireties. While the described invention has been described with reference to the specific embodiments thereof it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adopt a particular situation, material, composition of matter, process, process step or steps, to the objective spirit and scope of the described invention. All such modifications are intended to be within the scope of the claims appended hereto.

CLAIMS

1. A method of treating neovascular age-related macular degeneration (nAMD), choroidal neovascularization (CNV), or nAMD associated with CNV in a human subject in need thereof, the method comprising,
non-surgically administering an effective amount of a formulation comprising axitinib to the suprachoroidal space (SCS) of the eye of the human subject in need of treatment, wherein the effective amount comprises between about 0.01 and about 0.3 mg of axitinib.
2. The method of claim 1, wherein the effective amount comprises between about 0.03 mg and about 0.1 mg axitinib.
3. The method of claim 1, wherein the effective amount comprises about 0.03 mg axitinib.
4. The method of claim 1, wherein the effective amount comprises about 0.06 mg axitinib.
5. The method of claim 1, wherein the effective amount comprises about 0.1 mg axitinib.
6. The method of claim 1, wherein the effective amount comprises about 0.3 mg axitinib.
7. The method of any one of claims 1-6, wherein the method comprises administering the axitinib to the subject in a first dose and a second dose, wherein the second dose is administered at least 2, at least 3, at least 4 at least 5, or at least 6 months following the first dose.
8. The method of any one of claims 1-7, wherein the method further comprises administering a VEGF antagonist to the subject.
9. The method of claim 8, wherein the VEGF antagonist is administered via intravitreal injection.

10. The method of claim 8 wherein the VEGF antagonist is selected from the group consisting of aflibercept, bevacizumab, ranibizumab, and pegaptinib sodium.
11. The method of any one of claims 1-10, wherein method results in a decrease in retinal thickness in the treated eye, as measured by Spectral Domain Optical Coherence Tomography (SD-OCT) compared to the patient's retinal thickness in the eye in need of treatment prior to the administration of the formulation.
12. The method of claim 11, wherein the decrease in retinal thickness is $\geq 25 \mu\text{m}$, $\geq 50 \mu\text{m}$, $\geq 75 \mu\text{m}$ or ≥ 100 .
13. The method of claim 12, wherein the decrease in retinal thickness is $\geq 5\%$, $\geq 10\%$ or $\geq 25\%$.
14. The method of any one of claims 1-10, wherein the method results in a decrease in retinal thickness in the eye, as measured by SD-OCT, compared to the retinal thickness in the eye of a subject who was not administered a formulation comprising axitinib.
15. The method of any one of claims 1-10, wherein the patient does not exhibit an increase in retinal thickness in the eye within 12 weeks after the administration of the formulation comprising axitinib.
16. The method of claim 15, wherein the patient does not exhibit an increase in retinal thickness in the eye within 2, 3, 4, 5, or 6 months after the administration of the formulation comprising axitinib.
17. The method of any one of claims 1-10, wherein the patient experiences minimal loss in vision as measured by best-corrected visual acuity (BCVA) measurement, compared to the patient's BCVA measurement prior to administration of the formulation, wherein minimal loss of vision is losing no more than 2 letters.

18. The method of any one of claims 1-10, wherein the patient experiences minimal loss in vision as measured by best-corrected visual acuity (BCVA) measurement, compared to the patient's BCVA measurement prior to administration of the formulation, wherein minimal loss of vision is losing no more than 5 letters.

19. The method of any one of claims 1-10, wherein the patient experiences no loss in vision as measured by BCVA, compared to the patient's BCVA measurement prior to administration of the formulation.

20. The method of any one of claims 1-10, wherein the patient experiences an improvement in vision, as measured by gaining ≥ 5 letters, ≥ 10 letters or ≥ 15 letters in BCVA measurement, compared to the patient's BCVA prior to administration of the formulation.

21. The method of claim 20, wherein the patient experiences the improvement in BCVA for at least 2, 3, 4, 5, or 6 months after administration of the formulation comprising axitinib.

22. The method of any one of claims 1-10, wherein the patient experiences minimal loss in vision for 2, 3, 4, 5, or 6 months after administration of the formulation comprising axitinib, wherein the minimal loss of vision is losing no more than 2 letters as measured by BCVA.

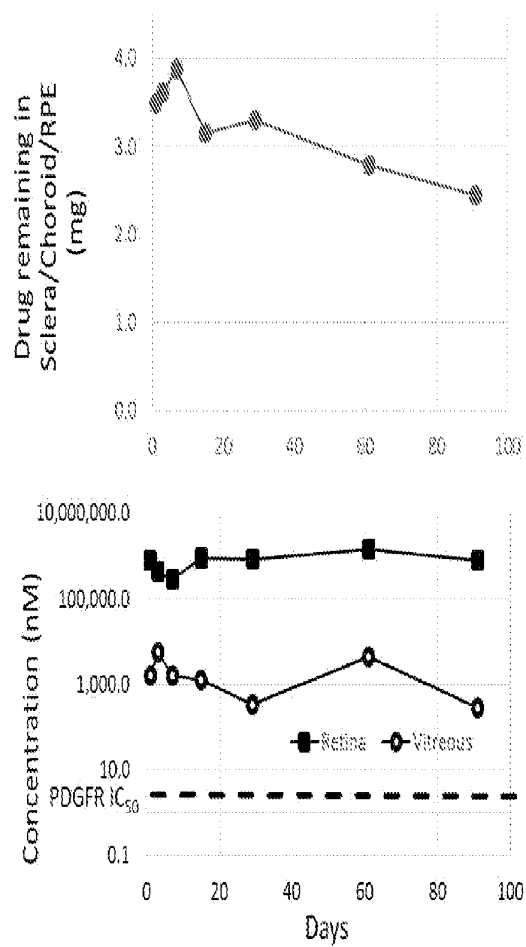
23. The method of any one of claims 1-10, wherein the patient experiences minimal loss in vision for 2, 3, 4, 5, or 6 months after administration of the formulation comprising axitinib, wherein the minimal loss of vision is losing no more than 5 letters as measured by BCVA.

24. The method of any one of claims 1-10, wherein the patient does not experience a loss of vision within 2, 3, 4, 5, or 6 months after the administration of the formulation comprising axitinib, wherein the vision is measured by BCVA.

25. The method of any one of claims 1-24, wherein the axitinib formulation comprises about 1 mg/mL axitinib.

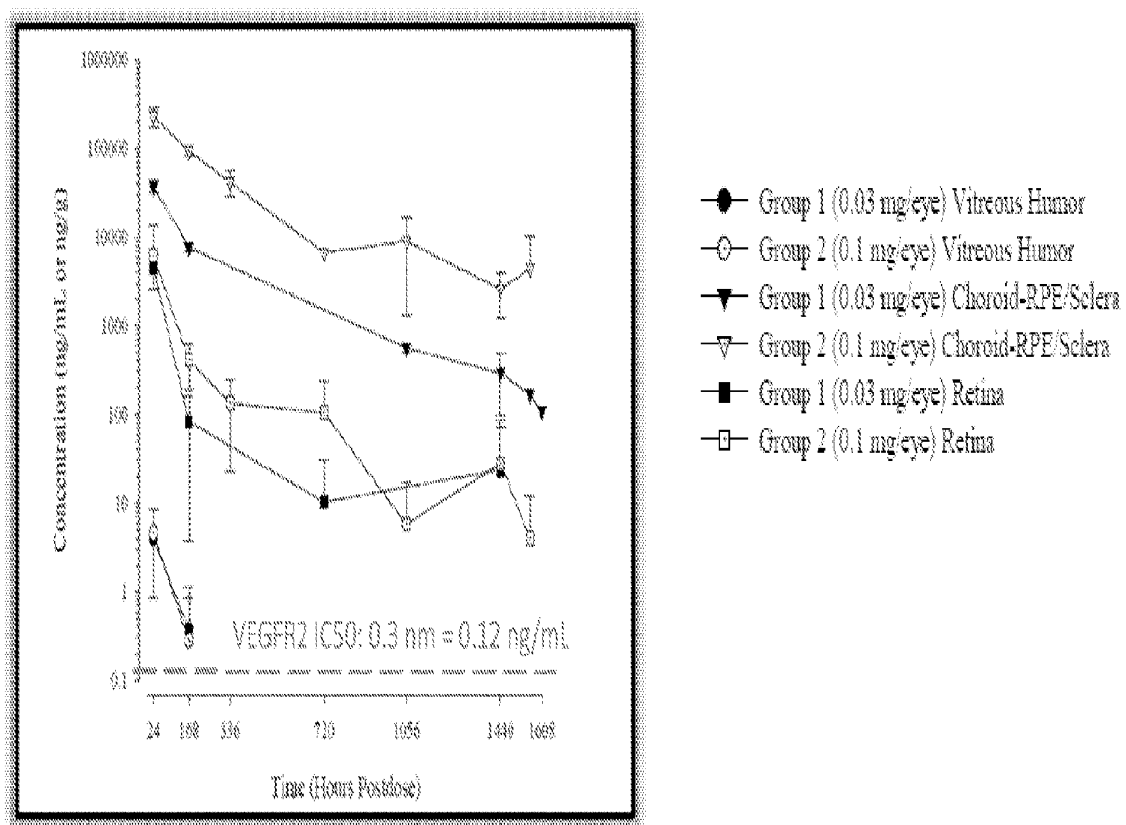
26. The method of any one of claims 1-25, wherein the axitinib formulation comprise about 0.05% polysorbate 80, about 0.5% sodium carboxymethylcellulose, about 0.79% sodium chloride, about 0.06% sodium phosphate monobasic, and about 0.08% sodium phosphate dibasic.

FIG. 1A



SUBSTITUTE SHEET (RULE 26)

FIG. 1B



SUBSTITUTE SHEET (RULE 26)

FIG. 2A

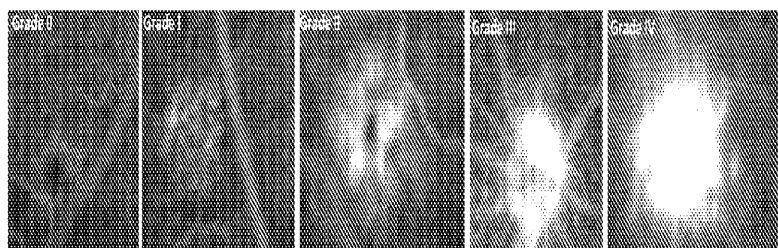


FIG. 2B

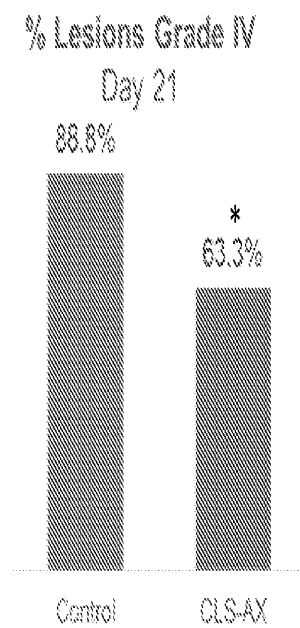


FIG. 3A

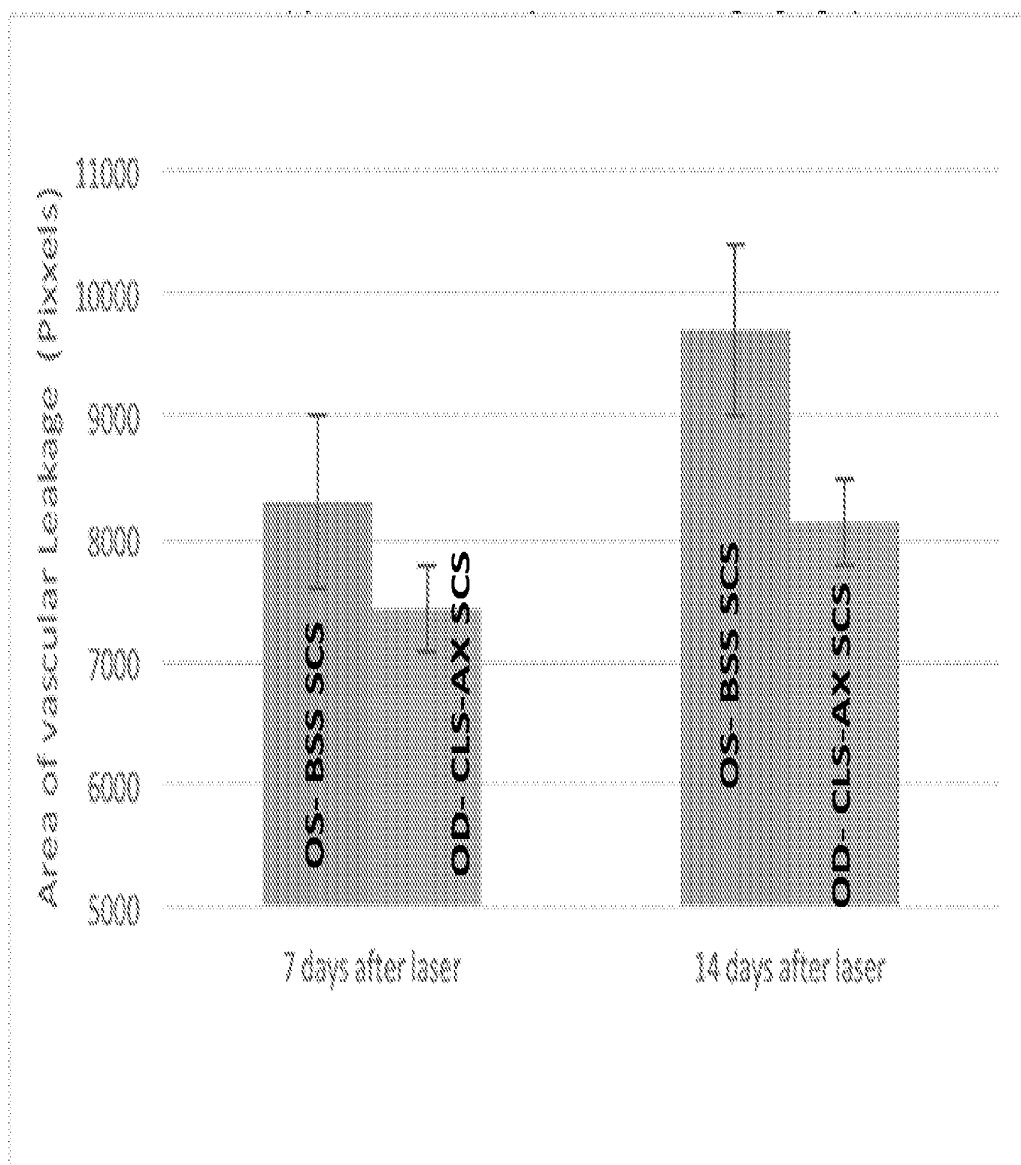


FIG. 3B

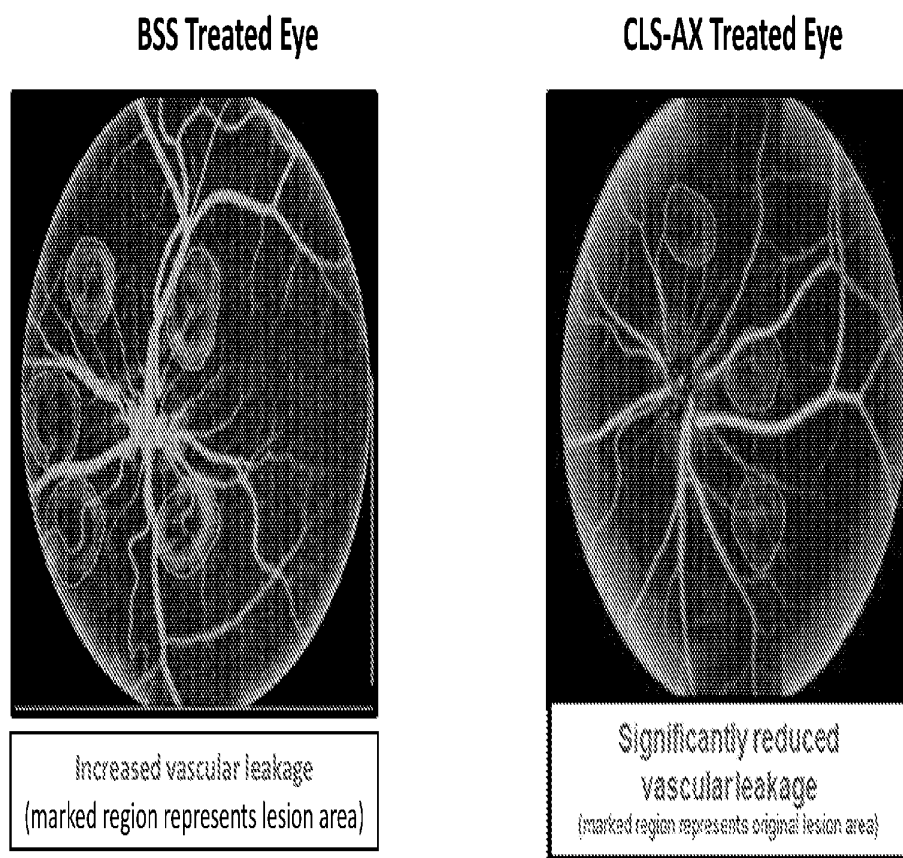
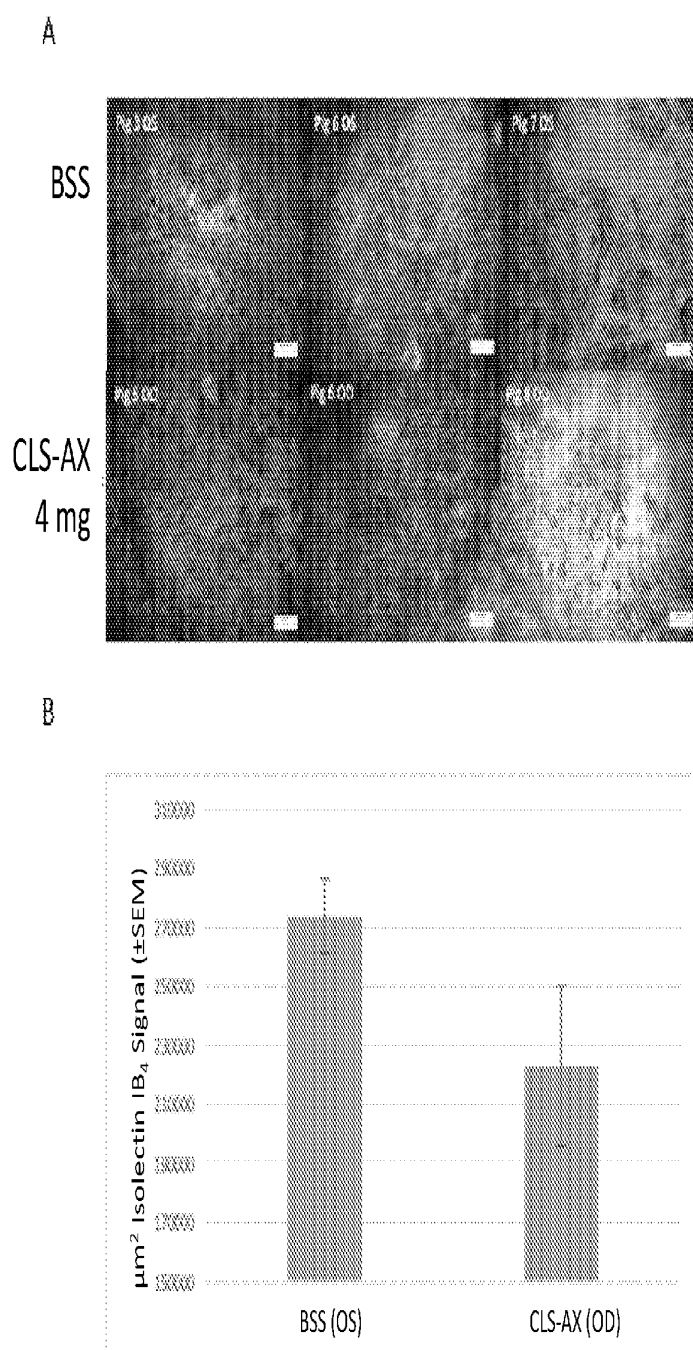


FIG. 4A and 4B



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/18737

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 8-26
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/18737

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 31/4439; A61K 31/496; A61K 9/00 (2021.01)

CPC - A61K 31/44; A61K 31/4439; A61K 31/496

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2017/120600 A1 (Clearside Biomedical, Inc.) 13 July 2017 (13.07.2017), entire document, especially paragraphs [0018] and [0019], example 1, and claim 1.	1-7
A	US 2016/0206628 A1 (Clearside Biomedical, Inc.) 21 July 2016 (21.07.2016), entire document.	1-7
A	WO 2016/183296 A1 (INCEPT, LLC) 17 November 2016 (17.11.2016), entire document.	1-7
A	Giddabasappa et al., "Axitinib inhibits retinal and choroidal neovascularization in in vitro and in vivo models", Experimental Eye Research, Volume 145, 2016, Pages 373-379, ISSN 0014-4835, [Retrieved on 2021-04-12] Retrieved from Internet: <URL: https://doi.org/10.1016/j.exer.2016.02.010 >	1-7

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

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"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

13 April 2021

Date of mailing of the international search report

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1. WO2021203769 - USE OF ANTI-PD-1 ANTIBODY IN PREPARATION OF DRUGS FOR TREATING ACRAL LENTIGINOUS MELANOMA



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A61P 35/00 2006.1

CPC

A61K 31/444

A61K 39/395

A61P 35/00

C07K 16/28

Title

[EN] USE OF ANTI-PD-1 ANTIBODY IN PREPARATION OF DRUGS FOR TREATING ACRAL LENTIGINOUS MELANOMA

[FR] UTILISATION D'ANTICORPS ANTI-PD-1 DANS LA PRÉPARATION DE MÉDICAMENTS POUR LE TRAITEMENT DU MÉLANOME MALIN DES EXTRÉMITÉS

[ZH] 一种抗PD-1抗体在制备治疗肢端黑色素瘤的药物中的用途

Abstract

[EN] Provided is a use of an anti-PD-1 antibody and apatinib in preparation of drugs for treating acral lentiginous melanoma. The solution has a disease control rate of 72.2% against acral lentiginous melanoma, and has an objective response rate of 22.2%. Also provided is a use of an anti-PD-1 antibody, apatinib, and temozolomide in preparation of drugs for treating melanoma.

[FR] L'invention concerne l'utilisation d'un anticorps anti-PD-1 et d'apatinib dans la préparation de médicaments pour le traitement du mélanome malin des extrémités. La solution a un taux de contrôle de maladie de 72,2 % contre le mélanome malin des extrémités, et a un taux de réponse objective de 22,2 %. L'invention concerne également l'utilisation d'un anticorps anti-PD-1, d'apatinib et de témozolomide dans la préparation de médicaments pour le traitement d'un mélanome.

[ZH] 提供一种抗PD-1抗体与阿帕替尼在制备治疗肢端黑色素瘤的药物中的用途, 该方案对肢端黑色素瘤疾病控制率达72.2%, 对疾病客观缓解率为22.2%。还提供了一种抗PD-1抗体、阿帕替尼和替莫唑胺在制备治疗黑色素瘤的药物中的用途。

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Designated States

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1. WO2021203769 - USE OF ANTI-PD-1 ANTIBODY IN PREPARATION OF DRUGS FOR TREATING ACRAL LENTIGINOUS MELANOMA



PCT Biblio. Data Full Text ISR/WOSA/A17[2][a] National Phase Notices Documents

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DESCRIPTION OF THE SPECIFICATION

BRIEF DESCRIPTION OF THE INVENTION 0021 0022 0023 0024 0025 0026 0027 0028 0029 0030 0031 0032 0033 0034 0035 0036
0037 0038 0039 0040 0041 0042 0043 0044 0045 0046 0047 0048 0049 0050 0051 0052 0053 0054 0055 0056 0057 0058 0059
0060 0061 0062 0063 0064 0065 0066 0067 0068 0069 0070 0071 0072 0073 0074 0075 0076 0077 0078 0079 0080 0081 0082
0083 0084 0085 0086 0087 0088 0089 0090 0091 0092 0093 0094 0095 0096 0097 0098 0099 0100 0101 0102 0103 0104 0105
0106 0107 0108 0109 0110 0111 0112 0113 0114 0115 0116 0117 0118

Claims

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

DESCRIPTION OF THE SPECIFICATION

The invention discloses application of an anti-PD -1 antibody in preparation of a drug for treating limb-end melanoma

Technical field

[0001] The invention relates to an application of an anti-PD -1 antibody or an antigen-binding fragment thereof and a VEGFR inhibitor in preparation of a drug for treating limb-end melanoma

BACKGROUND OF THE INVENTION

[0002] Early clinical trials showed specific binding to an anti-programmed death receptor (anti-PD -1), such as Nivolumab and pembrolizumab, so that the prognosis of advanced melanoma patients can be significantly improved (N Engl J Med 2015; 372: 320-30 and N Engl J Med 2015; 372: 2521-32). However, most such research is mainly based on the drug effect data of the anti-PD -1 drug in the high-level cable population, the difference of the melanoma clinical subtype in the population is large, and the response of the anti-PD -1 drug to different subtype responses of the disease is also different.

[0003] A N Shotari et al. (Cancer. 2016; 122 (21): 3354 DOI: 10.1002/CNCR. 30259) Studied the curative effect of the anti-PD -1 antibody pembrolizumab on the melanoma and mucosal melanoma of the limb end, wherein the ORR of the limb-end melanoma group is found to be 32%, the ORR of the mucosal melanoma group is 23%, and the result is equivalent to that of early clinical research data.

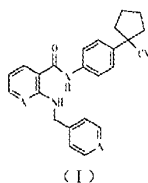
[0004] T Maeda et al. (Br J Dermatol. 2018 Nov;1517 DOI: 10.1111/BJD. 17434) Reports the clinical research data of Natto monoclonal antibody against Asian patients with different clinical subtypes (eg, limb end melanoma, mucosal melanoma and skin melanoma) Asian patients In 58 patients, ORR is 29.4%, wherein 2 patients are partial responses, and 11 cases are diseases stable; 37 patients are disease progression; in three clinical subtypes, the ORR of the skin melanoma group is 42.9%, the ORR of the mucosal melanoma group is 20.7%, and the ORR of the limb end melanoma group is 18.8%, the transfer property and the non-metastatic property are further subdivided, the ORR of the skin melanoma group is 41.7%, the ORR of the mucosal melanoma group is 17.6%, and the ORR of the limb end melanoma group is only 7.7%. Regardless of whether the metastatic, limb-end melanoma groups exhibit poor ORR (7.7% Vs. 17.6% Vs. 41.7%). Therefore, the PD -1 antibody single-drug is used for treatment of limb-end melanoma, and multiple uncertainty is still present.

[0005] The combined use of one or more anti-tumor drugs with different target points has the advantages that the advantages of each component are fully exerted, the anti-tumor activity of the single medicine can be improved, the drug toxicity can be reduced, and the anti-tumor medicine is a commonly accepted anti-tumor therapy

[0006] Apatinib is an oral antiangiogenic drug for the first advanced gastric cancer, and has high selectivity to VEGFR -2 and strong-effect anti-angiogenesis. In a multi-center random double-blind placebo control III-stage test for treating metastatic stomach/stomach esophageal junction cancer patients after an Apatinib II line, the result shows that the Apatinib single medicine can prolong the total survival time of the middle position by 1.8 months compared with the placebo, the middle-position no-progress lifetime is prolonged by 0.8 months, and the adverse event is controllable. The structural formula of apatinib is as shown in formula (I).



[0007]



[0008]

At present, a plurality of PD-1 antibodies and VEGFR inhibitors (such as sunitinib, sorafenib and the like) are currently in the clinical II/III period, and the indications are malignant liver cancer (sorafenib and PD-1 antibody combination) and metastatic renal cell carcinoma (sunitinib and PD-1 antibody combination). The preliminary results show that the two drug combination effects are superior to that of a single drug, but the PD-1 antibody and the VEGF inhibitor are combined to have many uncertainty and are worthy of deep research, and the report of the VEGFR inhibitor and the PD-1 antibody in combination with the treatment limb end melanoma has not been seen. On the other hand, the PD-1 antibody, the VEGF inhibitor and the alkylating agent, such as temozolomide, are used in combination with the drug effect of whether the drug is more excellent in combination, but also cannot be determined, and is worthy of deep research.

[0009]

SUMMARY OF THE INVENTION

[0010]

The disclosure provides an application of an anti-PD-1 antibody and a VEGFR inhibitor in preparation of a drug for treating limb end melanoma (melanoma).

[0011]

The PD-1 antibody is known, and in some alternative embodiments, the anti-PD-1 antibody or antigen-binding fragment thereof is selected from: AMP-224, GLS-010, IBI-308, REGN-2810, PDR-001, BGE-A 317, Pidilumab, PF-06801593, Genolimuzumab, CA-170, MEI1-0680, JS-001, TSR-042, cetuximab, cancomab, pembrolizumab, toritalumab, AK-103, and Nivolumab.

[0012]

In some embodiments, the light chain variable region of the PD-1 antibody comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6, respectively.

[0013]

The heavy chain variable region of the PD-1 antibody comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3, respectively.

[0014]

In some embodiments, the CDR sequence of the PD-1 antibody or antigen-binding fragment light/heavy chain is shown in the following table:

[0015]

[表0001]

Name	Sequence	Numbering
HCDR1-SYMMS		SEQ ID NO: 1
HCDR2	QAGGGGANTYYPDSVKQ	SEQ ID NO: 2
HCDR3	QLYYFDY	SEQ ID NO: 3
LCDR1	LSQHTGTLT	SEQ ID NO: 4
LCDR2	Tattoo D	SEQ ID NO: 5
LCDR3	QQVYSIPWT	SEQ ID NO: 6

[0016]

In an alternative embodiment, the PD-1 antibody is a humanized antibody.

[0017]

In some alternative embodiments, the anti-PD-1 antibody or antigen-binding fragment thereof is selected from the group consisting of Fab, Fab', SH, Fv, Scrv, and (Fab')₂ fragments.

[0018]

Immunoglobulins may be derived from any commonly known isoform, including, but not limited to, IgA, secretory IgA, IgG, and IgM. IgG subclasses are also well known to those skilled in the art, including but not limited to IgG1, IgG2, IgG3 and IgG4 "isotype" refer to class AB or subclasses (eg, IgM or IgG1) encoded by heavy chain constant region genes. In some alternative embodiments, the anti-PD-1 antibody or the antigen-binding fragment thereof in the present application comprises a heavy chain constant region of the same type of human IgG1, IgG2, IgG3 or IgG4, preferably comprising a heavy chain constant region of the isotype of IgG1 or IgG4.

[0019]

In other alternative embodiments, the anti-PD-1 antibody or antigen-binding fragment thereof comprises a light chain constant region of kappa or lambda.

[0020]

In some embodiments, the PD-1 antibody or antigen-binding fragment thereof comprises a light chain variable region or a variant thereof as shown in SEQ ID NO: 10, and the variant preferably has an amino acid change of 0-10 on a light chain variable region sequence shown in SEQ ID NO: 10; more preferably an amino acid change of A43 s; and a heavy chain variable region or a variant thereof as shown in SEQ ID NO: 9, the variant preferably has an amino acid change of 0-10 on a heavy chain variable region sequence shown in SEQ ID NO: 9, more preferably an amino acid change of G44 r.

[0021]

The PD-1 antibody or the antigen-binding fragment thereof is heavy, and the variable region sequence of the light chain is as follows:

[0022]

Heavy chain variable region

[0023]

EVQLVESGGGLVQPGSLRLSCAASGFTFSYMMMSWVRQAPGKGLEWVATISG
GGANTYYPDSVKGRFTISRDNAKNSLYLQMNSLR AEDTAVYYCARQLYYFDY
WGQGTTTVTSS

SEQID NO: 9

[0024]

Light chain variable region

[0025]

DIQMTQSPSSLSASVGDRVTITCLASQTIGTWLTWYQQKPGKAPKLLIYTATSLA
DGVPSPRSGSGSGTDFLTITSLQPEDFATYYCQQVYSIPWTFGGGTKVEIK

SEQID NO: 10

[0026]

In an alternative embodiment, the humanized antibody light chain sequence is a sequence as shown in SEQ ID NO: 8 or a variant thereof, the variant preferably has an amino acid change of 0-10 in the light chain variable region; and more preferably, the amino acid change of A43 s. The humanized antibody heavy chain sequence is a sequence shown as SEQ ID NO: 7 or a variant thereof, the variant preferably has an amino acid change of 0-10 in the heavy chain variable region, and more preferably, the amino acid change of G44 R.

[0027]

In some embodiments, the light chain sequence of the humanized antibody is a sequence as shown in SEQ ID NO: 8, and the heavy chain sequence is a sequence as shown in SEQ ID NO: 7.

[0028]

The humanized anti-weight and light chain sequences are as follows:

{0029} Heavy chain

{0030} EVQLVESGGGLVQPGGSLRLSCAASGFTFSYMMSSWVRQAPGKGLEWVATISG
GGANTYYPDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARQLYYFDY
WGQGTITVTVSSASTKGPVFLAPCSRSTSESTAALGCLVKDYFPEPTVSWNS

{0031} GALTSGVHTFPAVLQSSGLYSLSVVTVPSSSLGKTKYTCNVDHKPSNTKVDKR
VESKYGPPCPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPE
VQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVHLQDVLNGKEYKCKV
SNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEAL
HNHYTQKSLSLSLGK

SEQID NO: 7

{0032} Light chain

{0033} DIQMTQSPSSLSASVGDRTTCLASQTIGTWLTWYQQKPGKAPKLLIYTATSLA
DGVPSTRFSGSGGTDFTLTISSLQPEDFATYYCQVYSIPWTFGGGTKVEIKRTVA
APSVFIPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTE
QDSKSTYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQID NO: 8

{0034} On the other hand, PD-1 (Programmed Death 1), ie, programmed death receptor 1, is an important immunosuppressive molecule. When the switch of PD-1 is opened, a series of reactions can be started to inhibit T cell activity. Tumor cells are combined with PD-1 of T cells by means of PD-L1. "spoofing" T cells, the recognition of T cells is evaded, and the tumor cells continue to run in the body. In addition, the PD-L1/PD-1 antibody can help T cells to uncover the anti-counterfeit face yams of tumor cells and restore the identification and killing of tumor cells. In some alternative embodiments, the anti-PD-1 antibody or the antigen-binding fragment thereof is a Cessium mAb. In some alternative embodiments, the anti-PD-1 antibody or antigen-binding fragment thereof is torellimab. In some alternative embodiments, the anti-PD-1 antibody or antigen-binding fragment thereof is simlimab. In some alternative embodiments, the anti-PD-1 antibody or the antigen-binding fragment thereof is Tislelizumab.

{0035} {表0002}

Antibody universal name	CAS	Reference
CAMARA MAUMAB	1798286-48-2	WO 2017084646
INVOLUMP MAB	946414-94-4	WO 2006121168
gmBrolimumab	1574853-91-4	WO 2008156712
CELIFLICMAB	1801342-60-8	WO 20152011890
toRellimab	1924598-82-2	WO 2014206107
Simlimab	2072873-06-2	WO 2017024465
TISLEELIUMAB	1858168-59-8	WO 2015035606

{0036} In another aspect, in some embodiments, the VEGFR inhibitor is a VEGFR-2 inhibitor.

{0037} In some embodiments, wherein the VEGFR inhibitor is selected from the group consisting of apatinib, amatinib, tatinib, farnid, sorafenib, or a pharmaceutically acceptable salt thereof, preferably apatinib or a pharmaceutically acceptable salt thereof.

{0038} The pharmaceutically acceptable salt is selected from methanesulfonate, maleate, tartrate, succinate, acetate, difluoroacetate, fumarate, citrate, citrate, benzenesulfonate, benzoate, naphthalene sulfonate, lactate, malate, hydrochloride, hydrobromate, sulfate and phosphate. In some embodiments, the apatinib pharmaceutically acceptable salt is selected from methanesulfonate. In some embodiments, the Amatinib pharmaceutically acceptable salt is selected from the group consisting of hydrochloric acid. The combined anti-PD-1 antibody and the VEGFR inhibitor have a synergistic effect.

{0039} Further, the patient in the present disclosure is selected from the group consisting of recurrent, non-surgical resection, or metastatic.

{0040} In some embodiments, the recurrence is post-operative recurrence.

{0041} In an alternative embodiment, the patient is selected from post-operative recurrence.

{0042} In an alternative embodiment, the patient is selected from the group consisting of non-surgical resection.

{0043} In another aspect, the anti-PD-1 antibody and VEGFR inhibitor scheme further comprise an alkylating agent.

{0044} In some embodiments, the use further comprises temozolomide or a pharmaceutically acceptable salt thereof.

{0045} In some embodiments, the anti-PD-1 antibody or antigen-binding fragment thereof is selected from the group consisting of 0.1 mg/kg, 0.2 mg/kg, 0.3 mg/kg, 0.4 mg/kg, 0.5 mg/kg, 0.6 mg/kg, 0.7 mg/kg, 0.8 mg/kg, 0.9 mg/kg, 1.0 mg/kg, 1.2 mg/kg, 1.4 mg/kg, 1.6 mg/kg, 1.8 mg/kg, 2.0 mg/kg, 2.2 mg/kg, 2.4 mg/kg, 2.6 mg/kg, 2.8 mg/kg, 3.0 mg/kg, 3.2 mg/kg, 3.4 mg/kg, 3.6 mg/kg, 3.8 mg/kg, 4.0 mg/kg, 4.2 mg/kg, 4.4 mg/kg, 4.6 mg/kg, 4.8 mg/kg, 5.0 mg/kg, 5.2 mg/kg, 5.4 mg/kg, 5.6 mg/kg, 5.8 mg/kg, 6.0 mg/kg, 6.2 mg/kg, 6.4 mg/kg, 6.6 mg/kg, 6.8 mg/kg, 7.0 mg/kg, 7.2 mg/kg, 7.4 mg/kg, 7.6 mg/kg, 7.8 mg/kg, 8.0 mg/kg, 8.2 mg/kg, 8.4 mg/kg, 8.6 mg/kg, 8.8 mg/kg, 9.0 mg/kg, 9.2 mg/kg, 9.4 mg/kg, 9.6 mg/kg, 9.8 mg/kg, 10.0 mg/kg or any value between any two values, preferably 3 mg/kg.

{0046} In another embodiment, the administration dose of the PD-1 antibody or antigen-binding fragment thereof in a human subject is 1-600 mg, preferably 1.0 mg, 1.2 mg, 1.4 mg, 1.6 mg, 1.8 mg, 2.0 mg, 2.2 mg, 2.4 mg, 2.6 mg, 2.8 mg, 3.0 mg, 3.2 mg, 3.4 mg, 3.6 mg, 3.8 mg, 4.0 mg, 4.2 mg, 4.4 mg, 4.6 mg, 4.8 mg, 5.0 mg, 5.2 mg, 5.4 mg, 5.6 mg, 5.8 mg, 6.0 mg, 6.2 mg, 6.4 mg, 6.6 mg, 6.8 mg, 7.0 mg, 7.2 mg, 7.4 mg, 7.6 mg, 7.8 mg, 8.0 mg, 8.2 mg, 8.4 mg, 8.6 mg, 8.8 mg, 9.0 mg, 9.2 mg, 9.4 mg, 9.6 mg, 9.8 mg, 10.0 mg, 15 mg, 20 mg, 25 mg, 30 mg, 35 mg, 40 mg, 45 mg, 50 mg, 55 mg, 60 mg, 65 mg, 90 mg, 95 mg, 100 mg, 105 mg, 110 mg, 115 mg, 120 mg, 125 mg, 130 mg, 135 mg, 140 mg, 145 mg, 150 mg, 155 mg, 160 mg, 165 mg, 170 mg, 175 mg, 180 mg, 185 mg, 190 mg, 195 mg, 200 mg, 205 mg, 210 mg, 215 mg, 220 mg, 225 mg, 230 mg, 235 mg, 240 mg, 245 mg, 250 mg, 255 mg, 260 mg, 265 mg, 290 mg, 295 mg, 300 mg, 305 mg, 310 mg, 315 mg, 320 mg, 325 mg, 330 mg, 335 mg, 340 mg, 345 mg, 350 mg, 355 mg, 360 mg, 365 mg, 370 mg, 375 mg, 380 mg, 385 mg, 390 mg, 395 mg, 400 mg, 405 mg, 410 mg, 415 mg, 420 mg, 425 mg, 430 mg, 435 mg, 440 mg, 445 mg, 470 mg, 475 mg, 480 mg, 485 mg, 490 mg, 495 mg, 500 mg, 505 mg, 510 mg, 515 mg, 520 mg, 525 mg, 530 mg, 535 mg, 540 mg, 545 mg, 550 mg, 555 mg, 560 mg, 565 mg, 570 mg, 575 mg, 580 mg, 585 mg, 590 mg, 595 mg, 600 mg, or any value between any two values, preferably 200 mg.

{0047} The administration frequency of the anti-PD-1 antibody or the antigen-binding fragment of the anti-PD-1 antibody or the antigen-binding fragment thereof is once every day, once for two days, once for three days, one time of four days, once for five days, once for six days, one week, two weeks, three weeks, one week or one month.

- [0048] In some embodiments, the application dose of the anti-PD-1 antibody or antigen-binding fragment thereof in a human subject is 50-600 mg/2-3 weeks, more preferably 200 mg/2-3 weeks.
- [0049] In other embodiments, the administration dose of the VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof in a human subject is selected from 0.1-500 mg, may be 0.1 mg, 0.25 mg, 0.5 mg, 0.75 mg, 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 11 mg, 12 mg, 12.5 mg, 15 mg, 17.5 mg, 20 mg, 22.5 mg, 25 mg, 30 mg, 45 mg, 50 mg, 60 mg, 70 mg, 75 mg, 80 mg, 90 mg, 100 mg, 125 mg, 150 mg, 175 mg, 200 mg, 225 mg, 250 mg, 275 mg, 300 mg, 375 mg, 400 mg, 500 mg, more preferably 0.25 mg, 0.5 mg, 1 mg, 2 mg, 3 mg, 4 mg, 10 mg, 15 mg, 20 mg, 30 mg, 45 mg, 50 mg, 60 mg, 75 mg, 100 mg, 125 mg, 150 mg, 175 mg, 225 mg, 250 mg, 275 mg, 300 mg, 325 mg, 350 mg, 375 mg, 400 mg, 425 mg, 450 mg, 475 mg, 500 mg or any two values, preferably 250 mg or 375 mg.
- [0050] According to the application, the administration frequency of the VEGFR inhibitor such as apatinib is one-day, one-time, three-day, four-day, five-day, six-day, one week, three days per week, one day, four days per week, one day, five days per week, and one day.
- [0051] In some embodiments, the administration dose of the PD-1 antibody or antigen-binding fragment thereof in a human subject is selected from 60 to 600 mg, intravenous infusion, once every three weeks; the administration dose of VEGFR inhibitors such as apatinib mesylate in a human subject is selected from 250 mg to 500 mg, orally taken, once every two days.
- [0052] In some embodiments, the administration dose of the PD-1 antibody or antigen-binding fragment thereof in a human subject is selected from 1-5 mg/kg, intravenous infusion, once every three weeks; the administration dose of VEGFR inhibitor such as apatinib mesylate in a human subject is selected from 250 mg to 500 mg, orally taken, once every two days.
- [0053] In some embodiments, the administration dose of the PD-1 antibody or antigen-binding fragment thereof in a human subject is selected from 200 mg, intravenous infusion, once every two weeks; the administration dose of VEGFR inhibitor such as apatinib mesylate in a human subject is selected from 250 mg, oral, once daily.
- [0054] In some embodiments, the administration dose of the PD-1 antibody or antigen-binding fragment thereof in a human subject is selected from 3 mg/kg, intravenous infusion, once every two weeks; the administration dose of VEGFR inhibitor such as apatinib mesylate in a human subject is selected from 250 mg, oral, once daily.
- [0055] In some embodiments, the administration dose of the PD-1 antibody or antigen-binding fragment thereof in a human subject is selected from 200 mg, intravenous infusion, once every two weeks; the administration dose of VEGFR inhibitor such as apatinib mesylate in a human subject is selected from 375 mg, oral, once daily.
- [0056] Further, the VEGFR inhibitor disclosed by the invention is a post-meal medication, such as 30 minutes post-meal administration.
- [0057] In another alternative embodiment, the dosage of the alkylating agent such as temozolomide in a human subject is selected from 50 mg/m²-300 mg/m², including 50 mg, 55 mg, 60 mg, 65 mg, 70 mg, 75 mg, 80 mg, 85 mg, 90 mg, 95 mg, 100 mg, 105 mg, 110 mg, 115 mg, 120 mg, 125 mg, 130 mg, 135 mg, 140 mg, 145 mg, 150 mg, 155 mg, 160 mg, 165 mg, 170 mg, 175 mg, 180 mg, 185 mg, 190 mg, 195 mg, 200 mg, 205 mg, 210 mg, 215 mg, 220 mg, 225 mg, 230 mg, 235 mg, 240 mg, 245 mg, 250 mg, 255 mg, 260 mg, 265 mg, 270 mg, 275 mg, 280 mg, 285 mg, 290 mg, 295 mg, 300 mg or any two values, preferably 150 mg/m² or 200 mg/m².
- [0058] The alkylating agent, such as temozolomide, is used for one-day, one-time, three-day, four-day, five-day, six-day, one-week, two-week, three-week, one-week, or one-month.
- [0059] In some embodiments, the dosage of the alkylating agent such as temozolomide in a human subject is selected from 150 mg/m² or 200 mg/m², orally, once daily.
- [0060] In some embodiments, the dosage of the alkylating agent such as temozolomide in a human subject is selected from 150 mg/m² or 200 mg/m², oral, once daily, 5 days.
- [0061] In some embodiments, the dosage of the alkylating agent such as temozolomide in a human subject is selected from the group consisting of 150 mg/m² or 200 mg/m², orally taken once daily, co-five days, and then stopped for 23 days.
- [0062] In other embodiments, the administration dose of the PD-1 antibody or antigen-binding fragment thereof in a human subject is selected from 60 to 600 mg, intravenous infusion, one to three weeks. The administration dose of VEGFR inhibitor such as apatinib mesylate in a human subject is selected from 250 mg to 500 mg, orally taken, and once every two days; the administration dose of the alkylating agent such as temozolomide in a human subject is selected from 150 mg/m² or 200 mg/m², and is orally taken once every day.
- [0063] In other embodiments, the administration dose of the PD-1 antibody or antigen-binding fragment thereof in a human subject is selected from 200 mg, intravenous infusion, once every two weeks. The administration dose of the VEGFR inhibitor such as apatinib mesylate in a human subject is selected from 250 mg, oral administration and once daily; and the administration dose of the alkylating agent such as temozolomide in a human subject is selected from 200 mg/m² and orally taken once every day, and the administration dose of the alkylating agent such as temozolomide in the human subject is 5 days.
- [0064] The invention further provides an application of the anti-PD-1 antibody, the VEGFR inhibitor combined temozolomide or a pharmaceutically acceptable salt thereof in preparation of a medicament for treating melanoma.
- [0065] In some embodiments, the administration dose of the PD-1 antibody or antigen-binding fragment thereof in a human subject is selected from 60 to 600 mg; the administration dose of VEGFR inhibitor such as apatinib mesylate in a human subject is selected from 0.1 mg to 500 mg; the administration dose of the alkylating agent such as temozolomide in a human subject is selected from 50 mg/m² to 200 mg/m².
- [0066] In other embodiments, the administration dose of the PD-1 antibody or antigen-binding fragment thereof in a human subject is selected from 60 to 600 mg, intravenous infusion, one to three weeks. The administration dose of VEGFR inhibitor such as apatinib mesylate in a human subject is selected from 0.1 to 500 mg, oral administration, once every two days; the administration dose of the alkylating agent such as temozolomide in a human subject is selected from 50 mg/m² to 200 mg/m², orally taken once daily.
- [0067] In other embodiments, the administration dose of the PD-1 antibody or antigen-binding fragment thereof in a human subject is selected from 200 mg, intravenous infusion, once every two weeks. The administration dose of the VEGFR inhibitor such as apatinib mesylate in a human subject is selected from 250 mg, oral administration and once daily; and the administration dose of the alkylating agent such as temozolomide in a human subject is selected from 200 mg/m² and orally taken once every day, and the administration dose of the alkylating agent such as temozolomide in the human subject is 5 days.
- [0068] The present disclosure also provides the use of an anti-PD-1 antibody and a VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof in the preparation of a medicament for treating mucosal melanoma (mucosal melanoma), further comprising an alkylating agent, preferably temozolomide or a pharmaceutically acceptable salt thereof.
- [0069] The present disclosure provides the anti-PD-1 antibody combined VEGFR inhibitor, such as apatinib or a pharmaceutically acceptable salt thereof, as a medicament for treating limb-end melanoma, and further comprises an alkylating agent, wherein the alkylating agent is preferably temozolomide or a pharmaceutically acceptable salt thereof.
- [0070] The present disclosure also provides an anti-PD-1 antibody for treating melanoma (eg, limb end melanoma), wherein the anti-PD-1 antibody is administered in combination with a VEGFR inhibitor. In some embodiments, further comprising an alkylating agent, the alkylating agent is preferably temozolomide or a pharmaceutically acceptable salt thereof.
- [0071] In addition, the present disclosure also provides a VEGFR inhibitor for treating melanoma (eg, limb end melanoma), wherein the VEGFR inhibitor is administered in combination with an anti-PD-1 antibody. In some embodiments, further comprising an alkylating agent, the alkylating agent is preferably temozolomide or a pharmaceutically acceptable salt thereof.

- [0072] The present disclosure also provides an alkylating inhibitor, such as temozolomide or a pharmaceutically acceptable salt thereof, for treating melanoma (eg, limb end melanoma), wherein the alkylating inhibitor is administered in combination with an anti-PD -1 antibody and a VEGFR inhibitor. The present disclosure provides the anti-PD -1 antibody combined VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof as a drug for reducing drug adverse reactions. In some embodiments, the adverse drug reaction is selected from the group consisting of anti-PD -1 antibodies or caused by VEGFR inhibitors such as apatinib or pharmaceutically acceptable salts thereof.
- [0073] In another aspect, the present disclosure provides the anti-PD -1 antibody described above, a VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof, or a pharmaceutically acceptable salt thereof as a drug for reducing adverse drug reactions. In some embodiments, the adverse drug reaction is selected from the group consisting of anti-PD -1 antibodies or caused by VEGFR inhibitors such as apatinib or a pharmaceutically acceptable salt thereof, or from temozolomide or a pharmaceutically acceptable salt thereof.
- [0074] The present disclosure provides the anti-PD -1 antibody combined VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof as a drug for reducing the individual application dose of the anti-PD -1 antibody and/or the dosage of the VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof.
- [0075] The present disclosure provides a method of treating limb-end melanoma, comprising administering to a patient an effective amount of said anti-PD -1 antibody and VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof, and further administering to the patient an effective dose of an alkylating agent such as temozolomide or a pharmaceutically acceptable salt thereof.
- [0076] The present disclosure also provides a combination for treating a treatment limb end melanoma. In some embodiments, the combination contains an anti-PD -1 antibody and a VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof. In other embodiments, the aforementioned combination further comprises an alkylating agent such as temozolomide or a pharmaceutically acceptable salt thereof.
- [0077] In another aspect, the present disclosure also provides a combination of an anti-PD -1 antibody, a VEGFR inhibitor, such as apatinib or a pharmaceutically acceptable salt thereof, and an alkylating agent such as temozolomide or a pharmaceutically acceptable salt thereof, for treating melanoma. The combination contains an effective dose of an anti-PD -1 antibody, a VEGFR inhibitor or an alkylating agent. In some embodiments, melanoma includes mucosal melanoma or limb melanoma.
- [0078] The present disclosure also provides a method for reducing the individual application dose of an anti-PD -1 antibody and/or a separately administered dose of a VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof, comprising administering to a patient an anti-PD -1 antibody combined VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof, and further administering an effective dose of an alkylating agent to the patient, said alkylating agent being preferably temozolomide or a pharmaceutically acceptable salt thereof.
- [0079] In some embodiments, when used in conjunction with PD -1, the administration dose of the VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof is 10% to 100%, preferably 10% to 75%, more preferably 75%, 50%, 25%, 12.5% of its individual application dose. In some embodiments, when used in conjunction with PD -1, the dosage of the alkylating agent such as temozolomide or a pharmaceutically acceptable salt thereof is from 10% to 100%, preferably from 10% to 75%, more preferably 75%, 50%, 25%, 12.5%.
- [0080] In some embodiments, the anti-PD -1 antibody dose is 10%-100%, preferably 10%-50%, of the anti-PD -1 antibody alone application dose when used in combination with a VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof. In some embodiments, when used in combination with a VEGFR inhibitor, the dosage of the alkylating agent such as temozolomide or a pharmaceutically acceptable salt thereof is from 10% to 100%, preferably from 10% to 75%, more preferably 75%, 50%, 25%, 12.5%.
- [0081] In embodiments of the present disclosure, when the PD -1 antibody is used in combination with a VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt or/and an alkylating agent such as temozolomide or a pharmaceutically acceptable salt thereof, adverse reactions caused by anti-PD -1 antibodies and/or immune-mediated drugs can be reduced. And preferably, the adverse reaction is selected from the group consisting of angiogenesis-related adverse reactions, adenopathy dysfunction, skin adverse reactions, respiratory system adverse reactions, liver-related adverse reactions, endocrine-related adverse reactions, digestive system adverse reactions, kidney-related adverse reactions, fatigue, and fever, the preferred vascular-related adverse reactions are selected from the group consisting of angiotonia, vasculitis and lymphadenoma, and the decrease in function of the glands is selected from hypothyroidism, hyperparathyroid dysfunction, reduced pancreatic function and reduced prostate function. The skin adverse reaction is selected from pruritus, urticaria, rash and toxic epidermal damage; the respiratory system adverse reaction is selected from pneumonia, bronchitis, chronic obstructive pulmonary disease and pulmonary fibrosis. The liver-related adverse reaction is selected from the group consisting of hepatitis I diabetes, type II diabetes and hypoglycemia; the kidney-related adverse reaction is selected from the group consisting of nephritis and renal failure; the adverse reaction of the digestive system is selected from diarrhea, nausea, vomiting, enteritis and constipation; more preferably, the adverse drug reaction is selected from the group consisting of angiotonia, hypothyroidism, hyperparathyroid dysfunction, pruritus, pneumonia, hepatitis, abnormal liver function, type I diabetes, nephritis and renal failure.
- [0082] The present disclosure also provides a pharmaceutical combination comprising a VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof, and an anti-PD -1 antibody or an antigen-binding fragment thereof, further comprising an alkylating agent selected from the group consisting of temozolomide or a pharmaceutically acceptable salt thereof.
- [0083] In the embodiments of the present disclosure, the PD -1 antibody is administered in an injection manner, such as subcutaneous or intravenous injection, and the PD -1 antibody needs to be formulated into an injectable form before injection. An injectable form of a particularly preferred PD -1 antibody is an injection or freeze-dried powder needle comprising a PD -1 antibody, a buffer, a stabilizer, optionally also a surfactant. The buffering agent may be selected from one or more of acetate, citrate, succinate, and phosphate. The stabilizer may be selected from the group consisting of sugars or amino acids, preferably disaccharides, such as sucrose, lactose, trehalose, maltose. The surfactant is selected from polyoxyethylene hydrogenated castor oil, glycerol fatty acid ester, polyoxyethylene sorbitan fatty acid ester, preferably the polyoxyethylene sorbitan fatty acid ester is polysorbate 20, 40, 60 or 80, and the injectable form of the most preferred PD -1 antibody of polysorbate 20 comprises PD -1 antibody, acetate buffer, trehalose and polysorbate 20.
- [0084] The combination scheme optionally further comprises other components, and the other components include, but are not limited to, other anti-tumor drugs and the like.
- [0085] As is not explained in the contrary, the terms in the present disclosure have the following meanings:
- [0086] The present disclosure is directed to a manner in which at least one dose of VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof and at least one dose of PD -1 antibody or antigen-binding fragment thereof is administered over a period of time, wherein both substances exhibit pharmacological effects. The time period may be within a dosing period, preferably within 4 weeks, within 3 weeks, within 2 weeks, within 1 week, or within 24 hours, more preferably within 12 hours. A VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof and a PD -1 antibody or an antigen-binding fragment thereof May be administered simultaneously or sequentially. Such a period comprises a treatment in which a VEGFR inhibitor such as apatinib or a pharmaceutically acceptable salt thereof and a PD -1 antibody or an antigen-binding fragment thereof is administered by the same administration route or a different administration route. The combined administration mode of the present application is selected from simultaneous administration, independently formulated and co-administered or independently formulated and administered sequentially. In other embodiments, the present disclosure "combined" refers to administration of three different types of drugs such as VEGFR inhibitors, PD -1 antibodies and alkylating agents such as temozolomide within a certain period of time, as described previously.
- [0087] The "effective amount" or "effective dose" in the present disclosure includes an amount sufficient to improve or prevent symptoms or conditions of a medical condition. An effective amount also means an amount sufficient to allow or facilitate diagnosis. The effective amount for a particular patient or veterinary subject may vary according to factors such as the condition to be treated, the overall health of the patient, the route and dose of the administration, and the severity of the side effects. The effective amount can be a maximum dose or dosing regimen that avoids significant side effects or toxic effects.

- [0088] The term "humanized antibody", also referred to as CDR-grafted antibody, refers to an antibody variable region framework for transplanting a CDR sequence of a mouse to a human antibody variable region framework, ie, a different type of human germline antibody framework sequence. The chimeric antibody can be overcome to carry a large number of mouse protein components, so that a strong antibody variable antibody reaction is induced. Such framework sequences can be obtained from a common DNA database or disclosed references that include germline antibody gene sequences. A germline DNA sequence, such as a human heavy chain and a light chain variable region gene, may be in the "Abase" human germline sequence database (at the Internet WWW). MRCCPE. COMM AC. UK (Ybase can be obtained) and found in Kabat, E A et al. 1991 Sequences of Proteins of Immunological Interest, 5th ed. In A preferred embodiment of the present application, the CDR sequence of the PD-1 humanized antibody is selected from SEQ ID NO: 1, 2, 3, 4, 5, and 6.
- [0089] The term "antigen-binding fragment" refers to a Fab fragment having antigen binding activity, a Fab fragment, an F(ab')₂ fragment, and an Fv fragment. The Fv fragment bound to human PD-1; the antibody comprising one or more CDR regions of the antibody of the present application selected from SEQ ID NO: 1 to SEQ ID NO: 6. The Fv fragment contains an antibody heavy chain variable region and a light chain variable region, but does not have a constant region and has the minimum antibody fragment of all antigen binding sites. Generally, the Fv antibody further comprises a polypeptide linker between the VH and VL domains and is capable of forming the structure required for antigen binding. The two antibody variable regions can also be connected to a polypeptide chain, called a single chain antibody or a single chain Fv (Schriv), with different linkers. The term "combined with PD-1" in the present application refers to the interaction with human PD-1. The term "antigen binding site" in the present application refers to a three-dimensional space site recognized by an antibody or antigen-binding fragment of the present application.
- [0090] Progress-free survival (PFS): time to record tumor objective progress date from random to first recording tumor or to any reason leading to death in order to first occur.
- [0091] The total lifetime (OS) refers to the period of death from a random period to any reason. In the last follow-up time, a living subject is still stored, and the OS is deleted from data at the last follow-up time. The visited subject, its OS, confirms the survival time as data deletion at the last last confirmed survival time. The OS of the data deletion is defined as a time from random grouping to deletion.
- [0092] Objective response rate (ORR) refers to the proportion of patients with tumor reduction reaching a certain time and maintaining a certain time, and the disease examples of CR and PR are contained. Tumors are evaluated objectively by adopting a solid tumor mitigation evaluation standard (Recipe 1.1 standard). The subject has to be accompanied by a measurable tumor lesion at baseline, and the curative effect assessment standard is divided into complete mitigation (CR), partial mitigation (PR), stability (SD), progress (PD) according to the Recipe 1.1 standard.
- [0093] The Disease Control Rate (DCR) refers to the percentage of the number of cases in which the confirmed complete alleviation, partial alleviation and Disease stability (≥ 8 weeks) in the patient can be evaluated.
- [0094] Complete mitigation (CR): All target lesions disappear, and the short diameter of all pathological lymph nodes (including target nodules and non-target nodules) must be reduced to < 10 mm.
- [0095] Partial mitigation (PR): the sum of the target lesion diameter is reduced by at least 30% greater than the baseline level.
- [0096] Disease progression (PD): the minimum value of the sum of all measured target lesion diameters in the course of the whole experiment is referenced, the diameter and the relative increase by at least 20% (with reference to the baseline value if the baseline measurement value is minimal); in addition, it is necessary to increase the absolute value of the diameter and the absolute value of at least 5 mm (the occurrence of one or more new lesions is also regarded as disease progression).
- [0097] Disease stability (SD): the degree to which the target lesion is reduced does not reach Pr, the increase degree does not reach the Pd level, and the minimum value of the diameter and the minimum value can be used as a reference during research.

DETAILED DESCRIPTION OF THE EMBODIMENTS

- [0098] The following embodiments are used to further describe the present disclosure, but these embodiments do not limit the scope of the present disclosure.
- [0099] Example 1: Study of Anti-PD-1 Antibody Combined Mesylate Atnib Therapy Limb Melanoma
- [0100] Compound A: PD-1 antibody is prepared according to the method in patent application WO 2017054646 A, and the heavy and light chain sequences are as shown in SEQ ID NO: 7 and SEQ ID NO: 8. 200 mg per bottle in the present disclosure, and are prepared into 20 mg/ml of standby.
- [0101] Compound B: apatinib mesylate can be prepared according to the method in patent application WO 2010031266 A1.
- [0102] Group criteria: (1) pathological histological verification, recurrence after surgery, no surgical resection or metastatic limb end melanoma patients; and (2) 3 not receiving any system anti-tumor drug treatment.
- [0103] Administration method:
- [0104] The PD-1 antibody comprises a fixed dose of 200 mg, intravenous infusion (no need to prevent medication), or a subject having a body weight of less than 50 kg for a baseline period, using 3 mg/kg, once every 2 weeks, and 4 weeks;
- [0105] Apatinib: 250 mg of tablets, orally taken, once daily, once and after meals.
- [0106] Conclusion:
- [0107] The sum of the target lesion diameters of the 4 patients is reduced by at least 30% compared with the baseline level in the 18 example subjects, the 9 example subjects appear as disease stability (SD), 5 patients appear as disease progression, the overall disease control rate is 72.2%, the disease mitigation rate is 22.2%, the single-drug PD-1 disease mitigation rate is 15%, and the medication safety and effectiveness of the patients are obviously improved.
- [0108] Example 2: Study of Anti-PD-1 Antibody Combined Mesylate Atnib Therapy Limb Melanoma
- [0109] Compound A: PD-1 antibody is prepared according to the method in patent application WO 2017054646 A, and the heavy and light chain sequences are as shown in SEQ ID NO: 7 and SEQ ID NO: 8. 200 mg per bottle in the present disclosure, and are prepared into 20 mg/ml of standby.
- [0110] Compound B: apatinib mesylate can be prepared according to the method in patent application WO 2010031266 A1.
- [0111] Compound C: temozolomide, temozolomide for injection (powder needle, Shanghai constant-Rayleigh) or temozolomide capsule (Mersatan).
- [0112] Group criteria: (1) pathological histological verification, recurrence after surgery, surgical resection or metastatic melanoma patients (including limb ends); and (2) 3 not receiving any system anti-tumor drug treatment.
- [0113] Administration method:



- [0114] The PD -1 antibody comprises a fixed dose of 200 mg, intravenous infusion (no need to prevent medication), or a subject having a body weight of less than 50 kg for a baseline period, using 3 mg/kg, once every 2 weeks, and 4 weeks;
- [0115] Apatinib: 250 mg of tablets, orally taken, once every day, once and after meals, and every 28 days is a period;
- [0116] Temozolomide: 200 mg/m², oral or intravenous injection, once daily, continuously administered for 5 days, and every 28 days
- [0117] Conclusion;
- [0118] It can be evaluated that in 23 subjects, all target lesions of the patient disappear, no new lesion appears, and the sum of the tumor marker normal (CR) and 11 patients target lesion diameter is reduced by at least 30% (PR) than the baseline level, and 10 patients appear as disease stability (SD); 1 case patient outlier group, disease control rate 96.63% (22), and objective mitigation rate 52.17% (12). Compared with other treatment methods, such as two drugs (ORR 22.2%, DCR 72.2%) or single-drug PD -1 (15%) has obvious advantages.

Claims

- [Claim 1] Use of anti-PD -1 antibody and VEGFR inhibitor in preparation of medicine for treating limb end melanoma
- [Claim 2] The method of claim 1, wherein the VEGFR inhibitor is selected from the group consisting of Apatinib, Amatinib, Tazatinib, Farnetinib, Sorafenib, or a pharmaceutically acceptable salt thereof, preferably Apatinib or a pharmaceutically acceptable salt thereof.
- [Claim 3] The use of claim 1 or 2, wherein the PD -1 antibody or antigen-binding fragment thereof is selected from the group consisting of AMP -224, GLS -0010, B1-308, REGN -2810, PDR-001, BGB-A 317, Pidizumab, PF -06801591, Genolizumab, CA -170, MEDI -0680, JS -001, TSR -042, celipitumab, cancorumab, pembrolizumab, tofitatimab, AK -103, and Nivolumab.
- [Claim 4] The method of claim 1, wherein the light chain variable region of the PD -1 antibody or antigen-binding fragment thereof comprises SEQ ID NO: 5 and SEQ ID NO: 6, the heavy chain variable region of the PD -1 antibody comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3, respectively.
- [Claim 5] The method of claim 4, wherein the PD -1 antibody is selected from a humanized antibody or an antigen-binding fragment thereof.
- [Claim 6] Use according to claim 4 or 5, wherein the anti-PD -1 antibody or antigen-binding fragment thereof comprises a heavy chain constant region of the same type of human IgG1, IgG2, IgG3 or IgG4, preferably comprising a heavy chain constant region of the isotype of IgG1 or IgG4.
- [Claim 7] Use according to any one of claims 4-6, wherein the anti-PD -1 antibody or antigen-binding fragment thereof comprises a light chain constant region of kappa or lambda.
- [Claim 8] The use according to any one of claims 4 to 6, wherein the light chain variable region sequence of the humanized antibody is a sequence as shown in SEQ ID NO: 8 or a variant thereof, and the variant preferably has an amino acid change of 0-10 in the light chain variable region, more preferably an amino acid change of A43 s, the heavy chain variable region sequence is a sequence represented by SEQ ID NO: 7 or a variant thereof, and the variant preferably has an amino acid change of 0-10 in the heavy chain variable region, more preferably an amino acid change of G44 i.
- [Claim 9] The use according to any one of claims 4 to 6, wherein the PD -1 antibody or the antigen-binding fragment thereof comprises the light chain as shown in SEQ ID NO: 8 and the heavy chain as shown in SEQ ID NO: 7.
- [Claim 10] Use according to any one of claims 1 to 9, wherein the patient is selected from the group consisting of relapsed, non-operative resection or metastatic, a preferred post-operative recurrence of the recurrence.
- [Claim 11] The use according to any one of claims 1 to 10, wherein the PD -1 antibody or the antigen-binding fragment dose thereof is selected from 1-10 mg/kg, such as 3 mg/kg.
- [Claim 12] The use according to any one of claims 1 to 10, wherein the PD -1 antibody or the antigen-binding fragment dose thereof is selected from 50 to 600 mg, such as 200 mg.
- [Claim 13] Use according to any one of claims 1 to 12, wherein the VEGFR inhibitor dose is selected from 0.1 to 500 mg, such as 250 mg or 375 mg.
- [Claim 14] Use according to any one of claims 1 to 13, further comprising an alkylating agent, preferably temozolomide or a pharmaceutically acceptable salt thereof.
- [Claim 15] The method of claim 14, wherein the temozolomide dose is selected from 50 mg/m² to 300 mg/m², such as 150 mg/m² or 200 mg/m².
- [Claim 16] The use of any one of claims 2-15, wherein the apatinib pharmaceutically acceptable salt is selected from the group consisting of methanesulfonate, maleate, tartrate, succinate, acetate, difluoroacetate, fumarate, citrate, benzenesulfonate, benzoate, naphthalene sulfonate, lactate, malate, hydrochloride, hydrobromate, sulfate and phosphate, preferably methanesulfonate.
- [Claim 17] Use of anti-PD -1 antibody, VEGFR inhibitor combined temozolomide or pharmaceutically acceptable salt thereof in preparation of drugs for treating melanoma
- [Claim 18] An anti-PD -1 antibody for treating melanoma, wherein the anti-PD -1 antibody is administered in combination with a VEGFR inhibitor, further comprising an alkylating agent, preferably temozolomide or a pharmaceutically acceptable salt thereof.
- [Claim 19] A VEGFR inhibitor for treating melanoma, wherein the VEGFR inhibitor is administered in combination with an anti-PD -1 antibody, further comprising an alkylating agent, preferably temozolomide or a pharmaceutically acceptable salt thereof.
- [Claim 20] A pharmaceutical composition comprising an effective therapeutic amount of a PD -1 antibody or an antigen-binding fragment thereof and a VEGFR inhibitor, further comprising an alkylating agent, preferably temozolomide or a pharmaceutically acceptable salt thereof.



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本国际公布:

- 包括国际检索报告 (条约第 21 条 (3))。
- 包括说明书序列表部分 (细则 5.2(a))。

(54) Title: USE OF ANTI-PD-1 ANTIBODY IN PREPARATION OF DRUGS FOR TREATING ACRAL LENTIGINOUS MELANOMA

(54) 发明名称: 一种抗 PD-1 抗体在制备治疗肢端黑色素瘤的药物中的用途

(57) Abstract: Provided is a use of an anti-PD-1 antibody and apatinib in preparation of drugs for treating acral lentiginous melanoma. The solution has a disease control rate of 72.2% against acral lentiginous melanoma, and has an objective response rate of 22.2%. Also provided is a use of an anti-PD-1 antibody, apatinib, and temozolomide in preparation of drugs for treating melanoma.

(57) 摘要: 提供一种抗 PD-1 抗体与阿帕替尼在制备治疗肢端黑色素瘤的药物中的用途, 该方案对肢端黑色素瘤疾病控制率达 72.2%, 对疾病客观缓解率为 22.2%。还提供了一种抗 PD-1 抗体、阿帕替尼和替莫唑胺在制备治疗黑色素瘤的药物中的用途。



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一种抗 PD-1 抗体在制备治疗肢端黑色素瘤的药物中的用途

技术领域

本公开涉及一种抗 PD-1 抗体或其抗原结合片段和 VEGFR 抑制剂联合在制备
5 治疗肢端黑色素瘤的药物中的用途。

背景技术

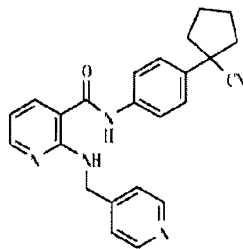
早期临床试验显示特异性结合抗程序性死亡受体（抗 PD-1），如 nivolumab 和 pembrolizumab，可使晚期黑色素瘤患者预后得到显著改善（*N Engl J Med* 2015; 372: 320-30 和 *N Engl J Med* 2015; 372: 2521-32）。然而大多数此类研究主要基于抗 PD-1
10 药物在高加索人群中的药效数据进行分析，黑色素瘤临床亚型在人群中差异较大，且抗 PD-1 药物对疾病的不同亚型响应也有所不同。

A. N. Shoushtari 等人（*Cancer*. 2016, 122(21): 3354. doi:10.1002/cncr.30259）研究了抗 PD-1 抗体 pembrolizumab 对肢端黑色素瘤和粘膜黑色素瘤的疗效，他们发
15 现肢端黑色素瘤组的 ORR 为 32%，粘膜黑色素瘤组的 ORR 为 23%，其结果与早期临床研究数据相当。

T. Maeda 等人（*Br J Dermatol*. 2018 Nov 17. doi: 10.1111/bjd.17434）报道纳武单抗在不同临床亚型的黑色素瘤（如肢端黑色素瘤、粘膜黑色素瘤和皮肤黑色素瘤）亚洲患者的临床研究数据，在 68 例患者中，ORR 为 29.4%，其中 2 例患者为
20 完成反应，18 例患者为部分反应，11 例为疾病稳定，37 例患者为疾病进展，在细分三种临床亚型中，皮肤黑色素瘤组的 ORR 为 42.9%，粘膜黑色素瘤组的 ORR 为 20.7%，肢端黑色素瘤组的 ORR 为 18.8%，进一步细分转移性与非转移性的，皮肤黑色素瘤组的 ORR 为 41.7%，粘膜黑色素瘤组的 ORR 为 17.6%，肢端黑色素瘤组的 ORR 只有 7.7%。不论是否细分转移性的，肢端黑色素瘤组均展现差的 ORR
25 （7.7% Vs 17.6% Vs 41.7%）。故 PD-1 抗体单药用于肢端黑色素瘤的治疗仍存在诸多不确定性。

联合使用一种以上靶点各异又相互关联的抗肿瘤药物，充分发挥各组分优势，既能提高单药的抗肿瘤活性又可降低药物毒性，是一种被普遍接受的抗肿瘤疗法。

阿帕替尼是全球首个晚期胃癌的口服抗血管生成药物，对 VEGFR-2 具有高度
30 选择性，强效抗血管生成。在一项关于阿帕替尼二线以后治疗转移性胃/胃食管结合部癌患者的多中心随机双盲安慰剂对照 III 期试验中，结果显示与安慰剂相比，阿帕替尼单药能将中位总生存期延长 1.8 个月，中位无进展生存期延长 0.8 个月，且不良事件可控。阿帕替尼的结构式如式(I)所示。



(I)

目前已有多个 PD-1 抗体与 VEGFR 抑制剂（如舒尼替尼，索拉菲尼等）正处于临床 II/III 期，适应症分别为恶性肝癌（索拉菲尼与 PD-1 抗体联用）和转移性
 5 肾细胞癌（舒尼替尼与 PD-1 抗体联用），初步结果显示两种药物联用效果均优于单药，但 PD-1 抗体与 VEGF 抑制剂联用具有很多不确定性，值得深入研究，同时尚未见 VEGFR 抑制剂与 PD-1 抗体联用于治疗肢端黑色素瘤的报告。另一方面，PD-1 抗体、VEGF 抑制剂与烷基化剂如替莫唑胺三药联用是否取得更为优异联合用药的
 10 药效也是不能确定性，值得深入研究。

发明内容

本公开 (the disclosure) 提供了一种抗 PD-1 抗体和 VEGFR 抑制剂联合在制备治疗肢端黑色素瘤（黑色素瘤）的药物中的用途。

PD-1 抗体是已知的，在一些可选实施方案中，所述抗 PD-1 抗体或其抗原结合片段选自：AMP-224、GLS-010、IBI-308、REGN-2810、PDR-001、BGB-A317、
 15 Pidilizumab、PF-06801591、Genolimzumab、CA-170、MEDI-0680、JS-001、TSR-042、Cemiplimab、Camrelizumab、Pembrolizumab、Toripalimab、Sintilimab、Tislelizumab、LZM-009、AK-103 和 Nivolumab。

在一些实施方案中，所述的 PD-1 抗体的轻链可变区包含分别如 SEQ ID NO:4、
 20 SEQ ID NO:5 和 SEQ ID NO: 6 所示的 LCDR1、LCDR2 和 LCDR3。

所述的 PD-1 抗体的重链可变区包含分别如 SEQ ID NO:1、SEQ ID NO:2 和 SEQ ID NO:3 所示的 HCDR1、HCDR2 和 HCDR3。

在一些实施方案中，所述 PD-1 抗体或抗原结合片段轻/重链中 CDR 序列如下表所示：

名称	序列	编号
HCDR1	SYMMS	SEQID NO: 1
HCDR2	TISGGGANTYYPDSVKG	SEQID NO: 2
HCDR3	QLYYFDY	SEQID NO: 3
LCDR1	LASQTIGTWLT	SEQID NO: 4
LCDR2	TATSLAD	SEQID NO: 5
LCDR3	QQVYSIPWT	SEQID NO: 6

在可选实施方案中，所述的 PD-1 抗体为人源化抗体。

在一些可选实施方案中，所述抗 PD-1 抗体或其抗原结合片段选自 Fab，Fab'-SH，Fv，scFv，和(Fab')₂ 片段组成的组的抗体片段。

免疫球蛋白可以来源于任何通常已知的同种型，包括但不限于 IgA、分泌型 IgA、IgG 和 IgM。IgG 亚类也是本领域技术人员众所周知的，包括但不限于 IgG1、IgG2、IgG3 和 IgG4。“同种型”是指由重链恒定区基因编码的 Ab 种类或亚类(例如，IgM 或 IgG1)。在一些可选实施方案中，本申请中所述抗 PD-1 抗体或其抗原结合片段包含人源 IgG1、IgG2、IgG3 或 IgG4 同种型的重链恒定区，优选包含 IgG1 或 IgG4 同种型的重链恒定区。

10 在另一些可选实施方案中，所述抗 PD-1 抗体或其抗原结合片段包含 κ 或 λ 的轻链恒定区。

在一些实施方案中，所述 PD-1 抗体或其抗原结合片段包含如 SEQ ID NO:10 所示的轻链可变区或其变体，所述变体优选在 SEQ ID NO:10 所示的轻链可变区序列上有 0-10 的氨基酸变化，更优选 A43S 的氨基酸变化；和如 SEQ ID NO:9 所示的重链可变区或其变体，所述变体优选在 SEQ ID NO:9 所示的重链可变区序列上有 0-10 的氨基酸变化，更优选 G44R 的氨基酸变化。

前述 PD-1 抗体或其抗原结合片段重、轻链的可变区序列如下所示：

重链可变区

EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYMMSWVRQAPGKGLEWVATISG
20 GGANTYYPD SVKGRFTISRDN AKNSLYLQMNSLRAEDTAVYYCARQLYYFDY
WGQGT TTVTVSS

SEQID NO: 9

轻链可变区

DIQMTQSPSSLSASVGDRVTITCLASQTIGTWLTWYQQKPGKAPKLLIYTATSLA
25 DGVPSRFSGSGSGTDFLTITSLQPEDFATYYCQQVYSIPWTFGGGTKVEIK

SEQID NO: 10

在可选实施方案中，所述的人源化抗体轻链序列为如 SEQ ID NO:8 所示的序列或其变体；所述的变体优选在轻链可变区有 0-10 的氨基酸变化；更优选为 A43S 的氨基酸变化。所述人源化抗体重链序列为如 SEQ ID NO:7 所示的序列或其变体；所述变体优选在重链可变区有 0-10 的氨基酸变化；更优选为 G44R 的氨基酸变化。

30 在一些实施方案中，所述人源化抗体的轻链序列为如 SEQ ID NO:8 所示的序列，重链序列为如 SEQ ID NO:7 所示的序列。

所述人源化抗体重、轻链的序列如下所示：

重链

35 EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYMMSWVRQAPGKGLEWVATISG
GGANTYYPD SVKGRFTISRDN AKNSLYLQMNSLRAEDTAVYYCARQLYYFDY
WGQGT TTVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNS

GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKR
 VESKYGPPCPPCAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPE
 VQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKV
 SNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAV
 5 EWESNGQPENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEAL
 HNHYTQKSLSLSLGK

SEQID NO: 7

轻链

DIQMTQSPSSLSASVGDRVTITCLASQTIGTWLTWYQQKPGKAPKLLIYTATSLA
 10 DGVPSRFGSGSGTDFTLTISLQPEDFATYYCQQVYSIPWTFGGGKVEIKRTVA
 APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTE
 QDSKSTYSLSSLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQID NO: 8

另一方面, PD-1(programmed death 1), 即程序性死亡受体 1, 是一种重要的免
 疫抑制分子。当 PD-1 的开关打开, 就会启动一系列反应, 抑制 T 细胞活性。肿瘤
 15 细胞借助 PD-L1 与 T 细胞的 PD-1 结合, “欺骗” T 细胞, 逃避 T 细胞的识别, 继
 续在体内横行霸道。而 PD-L1/PD-1 抗体则可以帮助 T 细胞揭开肿瘤细胞伪善的面
 纱, 恢复其对肿瘤细胞识别和杀伤。在一些可选联用实施方案中, 所述抗 PD-1 抗
 体或其抗原结合片段为 Cemiplimab。在一些可选联用实施方案中, 所述抗 PD-1 抗
 20 体或其抗原结合片段为 Toripalimab。在一些可选联用实施方案中, 所述抗 PD-1 抗
 体或其抗原结合片段为 Sintilimab。在一些可选联用实施方案中, 所述抗 PD-1 抗
 体或其抗原结合片段为 Tislelizumab。

抗体通用名	CAS 号	参考文献
Camrelizumab	1798286-48-2	WO2017054646
Nivolumab	946414-94-4	WO2006121168
Pembrolizumab	1374853-91-4	WO2008156712
Cemiplimab	1801342-60-8	WO2015112800
Toripalimab	1924598-82-2	WO2014206107
Sintilimab	2072873-06-2	WO2017024465
Tislelizumab	1858168-59-8	WO2015035606

另一方面, 在一些实施方案中, 其中所述 VEGFR 抑制剂是 VEGFR-2 抑制剂。

在一些实施方案中, 其中所述的 VEGFR 抑制剂选自阿帕替尼、安罗替尼、他
 25 菲替尼、法米替尼、索拉非尼或其可药用盐, 优选阿帕替尼或其可药用盐。

本公开所述可药用盐选自甲磺酸盐、马来酸盐、酒石酸盐、琥珀酸盐、醋酸
 盐、二氟醋酸盐、富马酸盐、柠檬酸盐、枸橼酸盐、苯磺酸盐、苯甲酸盐、萘磺
 酸盐、乳酸盐、苹果酸盐、盐酸盐、氢溴酸盐、硫酸盐、以及磷酸盐。在一些实
 施方案中, 所述阿帕替尼可药用盐选自甲磺酸盐。在一些实施方案中, 所述安罗
 30 替尼可药用盐选自盐酸。本公开所述联合抗 PD-1 抗体和 VEGFR 抑制剂具有协同

药效作用。

进一步地,本公开中所述患者选自复发的,不能手术切除的或转移性的。

在一些实施方案中,所述复发的为手术后复发的。

在可选实施方案中,所述患者选自手术后复发的

5 在可选实施方案中,所述患者选自不能手术切除的。

另一方面,前述抗 PD-1 抗体与 VEGFR 抑制剂方案中还进一步包含烷基化剂。

在一些实施方案中,所述用途还进一步包括替莫唑胺或其可药用盐。

在一些实施方案中,所述抗 PD-1 抗体或其抗原结合片段在人类受试者中的施用剂量(按患者体重给药)选自 0.1mg/kg、0.2mg/kg、0.3mg/kg、0.4mg/kg、0.5mg/kg、
10 0.6mg/kg、0.7mg/kg、0.8mg/kg、0.9mg/kg、1.0mg/kg、1.2mg/kg、1.4mg/kg、1.6mg/kg、
1.8mg/kg、2.0mg/kg、2.2mg/kg、2.4mg/kg、2.6mg/kg、2.8mg/kg、3.0mg/kg、3.2mg/kg、
3.4mg/kg、3.6mg/kg、3.8mg/kg、4.0mg/kg、4.2mg/kg、4.4mg/kg、4.6mg/kg、4.8mg/kg、
5.0mg/kg、5.2mg/kg、5.4mg/kg、5.6mg/kg、5.8mg/kg、6.0mg/kg、6.2mg/kg、6.4mg/kg、
6.6mg/kg、6.8mg/kg、7.0mg/kg、7.2mg/kg、7.4mg/kg、7.6mg/kg、7.8mg/kg、8.0mg/kg、
15 8.2mg/kg、8.4mg/kg、8.6mg/kg、8.8mg/kg、9.0mg/kg、9.2mg/kg、9.4mg/kg、9.6mg/kg、
9.8mg/kg、10.0mg/kg 或任意两数值间任意值,优选 3mg/kg。

在另一选实施方案中,所述 PD-1 抗体或其抗原结合片段在人类受试者中的施用剂量为 1~600mg,优选 1.0mg、1.2mg、1.4mg、1.6mg、1.8mg、2.0mg、2.2mg、
2.4mg、2.6mg、2.8mg、3.0mg、3.2mg、3.4mg、3.6mg、3.8mg、4.0mg、4.2mg、
20 4.4mg、4.6mg、4.8mg、5.0mg、5.2mg、5.4mg、5.6mg、5.8mg、6.0mg、6.2mg、
6.4mg、6.6mg、6.8mg、7.0mg、7.2mg、7.4mg、7.6mg、7.8mg、8.0mg、8.2mg、
8.4mg、8.6mg、8.8mg、9.0mg、9.2mg、9.4mg、9.6mg、9.8mg、10.0mg、15mg、
20mg、25mg、30mg、35mg、40mg、45mg、50mg、55mg、60mg、65mg、70mg、
75mg、80mg、85mg、90mg、95mg、100mg、105mg、110mg、115mg、120mg、
25 125mg、130mg、135mg、140mg、145mg、150mg、155mg、160mg、165mg、170mg、
175mg、180mg、185mg、190mg、195mg、200mg、205mg、210mg、215mg、220mg、
225mg、230mg、235mg、240mg、245mg、250mg、255mg、260mg、265mg、270mg、
275mg、280mg、285mg、290mg、295mg、300mg、305mg、310mg、315mg、320mg、
325mg、330mg、335mg、340mg、345mg、350mg、355mg、360mg、365mg、370mg、
30 375mg、380mg、385mg、390mg、395mg、400mg、405mg、410mg、415mg、420mg、
425mg、430mg、435mg、440mg、445mg、450mg、455mg、460mg、465mg、470mg、
475mg、480mg、485mg、490mg、495mg、500mg、505mg、510mg、515mg、520mg、
525mg、530mg、535mg、540mg、545mg、550mg、555mg、560mg、565mg、570mg、
575mg、580mg、585mg、590mg、595mg、600mg 或任意两数值间任意值,优选
35 200mg。

本公开所述抗 PD-1 抗体或其抗原结合片段的给药频为一天一次、两天一次、

三天一次、四天一次、五天一次、六天一次、一周一次、二周一次、三周一次、四周一次或一月一次。

在一些实施方案中，本公开所述抗 PD-1 抗体或其抗原结合片段在人类受试者中的施用剂量为 50~600mg/2-3 周一次，更优选为 200mg/2-3 周一次。

5 在另一些实施方案中，所述 VEGFR 抑制剂如阿帕替尼或其可药用盐在人类受试者中的施用剂量选自 0.1-500mg，可以为 0.1mg、0.25mg、0.5mg、0.75mg、1mg、2mg、3mg、4mg、5mg、6mg、7mg、8mg、9mg、10mg、11mg、12mg、12.5mg、15mg、17.5mg、20mg、22.5mg、25mg、30mg、45mg、50mg、60mg、70mg、75mg、80mg、90mg、100mg、125mg、150mg、175mg、200mg、225mg、250mg、275mg、
10 300mg、375mg、400mg、500mg，更优选 0.25mg、0.5mg、1mg、2mg、3mg、4mg、10mg、15mg、20mg、30mg、45mg、50mg、60mg、75mg、100mg、125mg、150mg、175mg、225mg、250mg、275mg、300mg、325mg、350mg、375mg、400mg、425mg、450mg、475mg、500mg 或任意两数值间任意值，优选 250mg 或 375mg。

本公开所述的用途，所述 VEGFR 抑制剂如阿帕替尼的给药频率为一天一次，
15 两天一次，三天一次，四天一次，五天一次，六天一次，一周一次，每周给药三天、一天一次，每周给药四天、一天一次，每周给药五天、一天一次。

在一些实施方案中，所述的 PD-1 抗体或其抗原结合片段在人类受试者中的施用剂量选自 60 至 600mg，静脉输注，每一至三周一次；VEGFR 抑制剂如甲磺酸阿帕替尼在人类受试者中的施用剂量选自 250mg 至 500mg，口服，每一到两日一次。
20

在一些实施方案中，所述的 PD-1 抗体或其抗原结合片段在人类受试者中的施用剂量选自 1-5mg/kg，静脉输注，每一至三周一次；VEGFR 抑制剂如甲磺酸阿帕替尼在人类受试者中的施用剂量选自 250mg 至 500mg，口服，每一到两日一次。

在一些实施方案中，所述的 PD-1 抗体或其抗原结合片段在人类受试者中的施用剂量选自 200mg，静脉输注，每两周一次；VEGFR 抑制剂如甲磺酸阿帕替尼在人类受试者中的施用剂量选自 250mg，口服，每日一次。
25

在一些实施方案中，所述的 PD-1 抗体或其抗原结合片段在人类受试者中的施用剂量选自 3mg/kg，静脉输注，每两周一次；VEGFR 抑制剂如甲磺酸阿帕替尼在人类受试者中的施用剂量选自 250mg，口服，每日一次。

30 在一些实施方案中，所述的 PD-1 抗体或其抗原结合片段在人类受试者中的施用剂量选自 200mg，静脉输注，每两周一次；VEGFR 抑制剂如甲磺酸阿帕替尼在人类受试者中的施用剂量选自 375mg，口服，每日一次。

进一步地，本公开所述 VEGFR 抑制剂为餐后用药，譬如餐后 30 分钟内服药。

另一方面，在另一选实施方案中，所述烷基化剂如替莫唑胺在人类受试者中的施用剂量（按体表面积给药）选自 50mg/m²~300mg/m²，包括 50mg、55mg、60mg、
35 65mg、70mg、75mg、80mg、85mg、90mg、95mg、100mg、105mg、110mg、115mg、

120mg、125mg、130mg、135mg、140mg、145mg、150mg、155mg、160mg、165mg、170mg、175mg、180mg、185mg、190mg、195mg、200mg、205mg、210mg、215mg、220mg、225mg、230mg、235mg、240mg、245mg、250mg、255mg、260mg、265mg、270mg、275mg、280mg、285mg、290mg、295mg、300mg 或任意两数值间任意值，

5 优选 150mg/m² 或 200mg/m²。

本公开所述烷基化剂如替莫唑胺给药频为一天一次、两天一次、三天一次、四天一次、五天一次、六天一次、一周一次、二周一次、三周一次、四周一次或一月一次。

10 在一些实施方案中，所述烷基化剂如替莫唑胺在人类受试者中的施用剂量选自 150mg/m² 或 200mg/m²，口服，每日一次。

在一些实施方案中，所述烷基化剂如替莫唑胺在人类受试者中的施用剂量选自 150mg/m² 或 200mg/m²，口服，每日一次，共 5 天。

在一些实施方案中，所述烷基化剂如替莫唑胺在人类受试者中的施用剂量选自 150mg/m² 或 200mg/m²，口服，每日一次，共 5 天，然后停药 23 天。

15 在另一些实施方案中，其中所述的 PD-1 抗体或其抗原结合片段在人类受试者中的施用剂量选自 60 至 600mg，静脉输注，每一至三周一次；VEGFR 抑制剂如甲磺酸阿帕替尼在人类受试者中的施用剂量选自 250mg 至 500mg，口服，每一到两日一次；所述烷基化剂如替莫唑胺在人类受试者中的施用剂量选自 150mg/m² 或 200mg/m²，口服，每日一次。

20 在另一些实施方案中，其中所述的 PD-1 抗体或其抗原结合片段在人类受试者中的施用剂量选自 200mg，静脉输注，每两周一次；VEGFR 抑制剂如甲磺酸阿帕替尼在人类受试者中的施用剂量选自 250mg，口服，每日一次；所述烷基化剂如替莫唑胺在人类受试者中的施用剂量选自 200mg/m²，口服，每日一次，共 5 天。

25 本公开另一方面还提供一种抗 PD-1 抗体、VEGFR 抑制剂联合替莫唑胺或其可药用盐在制备治疗黑素瘤的药物中的用途。

在一些实施方案中，其中所述的 PD-1 抗体或其抗原结合片段在人类受试者中的施用剂量选自 60 至 600mg；VEGFR 抑制剂如甲磺酸阿帕替尼在人类受试者中的施用剂量选自 0.1mg 至 500mg；所述烷基化剂如替莫唑胺在人类受试者中的施用剂量选自 50mg/m² 至 200mg/m²。

30 在另一些实施方案中，其中所述的 PD-1 抗体或其抗原结合片段在人类受试者中的施用剂量选自 60 至 600mg，静脉输注，每一至三周一次；VEGFR 抑制剂如甲磺酸阿帕替尼在人类受试者中的施用剂量选自 0.1 至 500mg，口服，每一到两日一次；所述烷基化剂如替莫唑胺在人类受试者中的施用剂量选自 50mg/m² 至 200mg/m²，口服，每日一次。

35 在另一些实施方案中，其中所述的 PD-1 抗体或其抗原结合片段在人类受试者中的施用剂量选自 200mg，静脉输注，每两周一次；VEGFR 抑制剂如甲磺酸阿帕

替尼在人类受试者中的施用剂量选自 250mg，口服，每日一次；所述烷基化剂如替莫唑胺在人类受试者中的施用剂量选自 200mg/m²，口服，每日一次，共 5 天。

本公开还提供了一种抗 PD-1 抗体和 VEGFR 抑制剂如阿帕替尼或其可药用盐联合在制备治疗粘膜黑色素瘤（粘膜型黑色素瘤）的药物中的用途，还进一步包含烷基化剂，所述烷基化剂优选替莫唑胺或其可药用盐。

本公开提供上述抗 PD-1 抗体联合 VEGFR 抑制剂如阿帕替尼或其可药用盐作为治疗制备治疗肢端黑色素瘤的药物，还进一步包含烷基化剂，所述烷基化剂优选替莫唑胺或其可药用盐。

本公开还提供一种用于治疗黑色素瘤（如肢端黑色素瘤）的抗 PD-1 抗体，其中抗 PD-1 抗体与 VEGFR 抑制剂组合施用。在一些实施方案中，其中还包括烷基化剂，所述烷基化剂优选替莫唑胺或其可药用盐。

另外，本公开还提供一种用于治疗黑色素瘤（如肢端黑色素瘤）的 VEGFR 抑制剂，其中 VEGFR 抑制剂与抗 PD-1 抗体组合施用。在一些实施方案中，其中还包括烷基化剂，所述烷基化剂优选替莫唑胺或其可药用盐。

本公开还提供一种用于治疗黑色素瘤（如肢端黑色素瘤）的烷基化抑制剂如替莫唑胺或其可药用盐，其中所述烷基化抑制剂与抗 PD-1 抗体和 VEGFR 抑制剂组合施用。本公开提供上述抗 PD-1 抗体联合 VEGFR 抑制剂如阿帕替尼或其可药用盐作为减少药物不良反应的药物。在一些实施方案中，所述的药物不良反应选自由抗 PD-1 抗体引起或由 VEGFR 抑制剂如阿帕替尼或其可药用盐引起。

另一方面，本公开提供上述抗 PD-1 抗体、VEGFR 抑制剂如阿帕替尼或其可药用盐联合替莫唑胺或其可药用盐作为减少药物不良反应的药物。在一些实施方案中，所述的药物不良反应选自由抗 PD-1 抗体引起或由 VEGFR 抑制剂如阿帕替尼或其可药用盐，或者由替莫唑胺或其可药用盐引起。

本公开提供上述抗 PD-1 抗体联合 VEGFR 抑制剂如阿帕替尼或其可药用盐作为降低抗 PD-1 抗体单独施用剂量和/或 VEGFR 抑制剂如阿帕替尼或其可药用盐单独施用剂量的药物。

本公开提供了一种治疗肢端黑色素瘤的办法，包括向患者施用有效量的上述抗 PD-1 抗体和 VEGFR 抑制剂如阿帕替尼或其可药用盐，还进一步向患者施用有效剂量的烷基化剂如替莫唑胺或其可药用盐。

本公开还提供一种用于治疗治疗肢端黑色素瘤的组合。在一些实施方案中，该组合中含有抗 PD-1 抗体和 VEGFR 抑制剂如阿帕替尼或其可药用盐的。在另一些实施方案中，前述组合还包含烷基化剂如替莫唑胺或其可药用盐。

另一方面，本公开还提供一种用于治疗黑色素瘤的、含有抗 PD-1 抗体、VEGFR 抑制剂如阿帕替尼或其可药用盐和烷基化剂如替莫唑胺或其可药用盐的组合。该组合中含有有效剂量的抗 PD-1 抗体、VEGFR 抑制剂或烷基化剂。在一些实施方案中，黑色素瘤包括粘膜型黑色素瘤或肢端黑色素瘤。

本公开还提供了一种降低抗 PD-1 抗体单独施用剂量和/或 VEGFR 抑制剂如阿帕替尼或其可药用盐单独施用剂量的方法,包括向患者施用上述抗 PD-1 抗体联合 VEGFR 抑制剂如阿帕替尼或其可药用盐,还进一步向患者施用有效剂量的烷基化剂,所述烷基化剂优选替莫唑胺或其可药用盐。

5 在一些实施方案中,与 PD-1 联合使用时,所述 VEGFR 抑制剂如阿帕替尼或其可药用盐的给药剂量是其单独施用剂量的 10%~100%,优选 10%~75%,更优选 75%、50%、25%、12.5%。在一些实施方案中,与 PD-1 联合使用时,所述烷基化剂如替莫唑胺或其可药用盐的给药剂量是其单独施用剂量的 10%~100%,优选 10%~75%,更优选 75%、50%、25%、12.5%。

10 在一些实施方案中,与 VEGFR 抑制剂如阿帕替尼或其可药用盐联用时,抗 PD-1 抗体剂量是抗 PD-1 抗体单独施用剂量的 10%~100%,优选 10%~50%。在一些实施方案中,与 VEGFR 抑制剂联合使用时,所述烷基化剂如替莫唑胺或其可药用盐的给药剂量是其单独施用剂量的 10%~100%,优选 10%~75%,更优选 75%、50%、25%、12.5%。

15 在本公开实施方案中,当 PD-1 抗体与 VEGFR 抑制剂如阿帕替尼或其可药用盐或/和烷基化剂如替莫唑胺或其可药用盐联合使用时,可减少由抗 PD-1 抗体和/或免疫介导的药物不良反应;优选地,所述的不良反应选自血管相关不良反应、腺体机能减退、皮肤不良反应、呼吸系统不良反应、肝脏相关不良反应、内分泌相关不良反应、消化系统不良反应、肾脏相关不良反应、疲劳、发热;所述优选
20 血管相关不良反应选自血管瘤、血管炎、淋巴管瘤、所述腺体功能减退选自甲状腺机能减退、甲状旁腺功能减退、胰腺功能减退、前列腺功能减退;所述皮肤不良反应选自瘙痒症、荨麻疹、皮疹、毒性表皮坏死症;所述呼吸系统不良反应选自肺炎、支气管炎、慢性阻塞性肺病、肺纤维化;所述肝脏相关不良反应选自肝炎、肝功能异常;所述内分泌相关不良反应选自 I 型糖尿病、II 型糖尿病、低血糖
25 症;肾脏相关不良反应选自肾炎、肾衰竭;所述消化系统不良反应选自腹泻、恶心、呕吐、肠炎、便秘;更优选的,所述药物不良反应选自血管瘤、甲状腺机能减退、甲状旁腺功能减退、瘙痒症、肺炎、肝炎、肝功能异常、I 型糖尿病、肾炎、肾衰竭。

本公开还提供了一种药物组合,其中含有 VEGFR 抑制剂如阿帕替尼或其可药用盐,和抗 PD-1 抗体或其抗原结合片段,进一步包括烷基化剂,所述烷基化剂选自替莫唑胺或其可药用盐。
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在本公开实施方案中,所述的 PD-1 抗体以注射的方式给药,例如皮下或静脉注射,注射前需将 PD-1 抗体配制成药液或冻干粉针的形式。特别优选的 PD-1 抗体的可注射形式是注射液或冻干粉针,其包含 PD-1 抗体、缓冲剂、稳定剂,任选地还含有
35 表面活性剂。缓冲剂可选自醋酸盐、柠檬酸盐、琥珀酸盐、以及磷酸盐中的一种或几种。稳定剂可选自糖或氨基酸,优选二糖,例如蔗糖、乳糖、海藻糖、麦芽

糖。表面活性剂选自聚氧乙烯氢化蓖麻油、甘油脂肪酸酯、聚氧乙烯山梨醇酐脂肪酸酯，优选所述聚氧乙烯山梨醇酐脂肪酸酯为聚山梨酯 20、40、60 或 80，最优选聚山梨酯 20。最为优选的 PD-1 抗体的可注射形式包含 PD-1 抗体、醋酸盐缓冲剂、海藻糖和聚山梨酯 20。

- 5 本公开联合方案任选的还包含其他组分，所述其他组分包括但不限于其他抗肿瘤药等。

如无相反解释，本公开中术语具有如下含义：

- 本公开关于“联合”是一种给药方式，是指在一定时间期限内给予至少一种剂量的 VEGFR 抑制剂如阿帕替尼或其可药用盐和至少一种剂量的 PD-1 抗体或其抗原结合片段，其中两种物质都显示药理学作用。所述的时间期限可以是一个给药周期内，优选 4 周内，3 周内，2 周内，1 周内，或 24 小时以内，更优选 12 小时以内。可以同时或依次给予 VEGFR 抑制剂如阿帕替尼或其可药用盐和 PD-1 抗体或其抗原结合片段。这种期限包括这样的治疗，其中通过相同给药途径或不同给药途径给予 VEGFR 抑制剂如阿帕替尼或其可药用盐和 PD-1 抗体或其抗原结合片段。本申请所述联合的给药方式选自同时给药、独立地配制并共给药或独立地配制并相继给药。在另一些实施方案中，本公开“联合”是指在一定时间期限内给予三种不同种的药物如 VEGFR 抑制剂、PD-1 抗体以及烷基化剂如替莫唑胺，所述的时间期限如前述所描述。
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- 本公开中所述“有效量”或“有效剂量”包含足以改善或预防医学病症的症状或病症的量。有效量还意指足以允许或促进诊断的量。用于特定患者或兽医学受试者的有效量可依据以下因素而变化：如待治疗的病症、患者的总体健康情况、给药的方法途径和剂量以及副作用严重性。有效量可以是避免显著副作用或毒性作用的最大剂量或给药方案。
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- 术语“人源化抗体(humanized antibody)”，也称为 CDR 移植抗体(CDR-grafted antibody)，是指将小鼠的 CDR 序列移植到人的抗体可变区框架，即不同类型的人种系抗体构架序列中产生的抗体。可以克服嵌合抗体由于携带大量小鼠蛋白成分，从而诱导的强烈的抗体可变抗体反应。此类构架序列可以从包括种系抗体基因序列的公共 DNA 数据库或公开的参考文献获得。如人重链和轻链可变区基因的种系 DNA 序列可以在“VBase”人种系序列数据库（在因特网 www.mrcpe.com.ac.uk/vbase 可获得），以及在 Kabat, E.A. 等人，1991 Sequences of Proteins of Immunological Interest, 第 5 版中找到。在本申请一个优选的实施方案中，所述的 PD-1 人源化抗体的 CDR 序列选自 SEQ ID NO:1, 2, 3, 4, 5, 6。
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- 术语“抗原结合片段”，指具有抗原结合活性的 Fab 片段，Fab ‘片段，F(ab’) 2 片段，以及与人 PD-1 结合的 Fv 片段 sFv 片段；包含本申请所述抗体的选自 SEQ ID NO:1 至 SEQ ID NO:6 中的一个或多个 CDR 区。Fv 片段含有抗体重链可变区和轻链可变区，但没有恒定区，并具有全部抗原结合位点的最小抗体片段。一般地，
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Fv 抗体还包含在 VH 和 VL 结构域之间的多肽接头，且能够形成抗原结合所需的结构。也可以用不同的连接物将两个抗体可变区连接成一条多肽链，称为单链抗体（single chain antibody）或单链 Fv（sFv）。本申请的术语“与 PD-1 结合”，指能与 PD-1 相互作用。本申请的术语“抗原结合位点”指抗原上不连续的，由本申请抗体或抗原结合片段识别的三维空间位点。

无进展生存期（PFS）：从随机开始到首次记录肿瘤客观进展日期或到任何原因导致死亡的时间，以先出现者为准。

总生存期（OS）指从随机期至任何原因导致死亡的期。末次随访时仍存活的受试者，其 OS 以末次随访时间计为数据删失。失访的受试者，其 OS 以失访前末次证实存活时间计为数据删失。数据删失的 OS 定义为从随机分组到删失的时间。

客观缓解率（Objective response rate，ORR）指肿瘤缩小达到一定并且保持一定时间的病人的比例，包含了 CR 和 PR 的病例。采用实体瘤缓解评估标准（RECIST 1.1 标准）来评定肿瘤客观缓解。受试者在基线时必须伴有可测量的肿瘤病灶，疗效评定标准根据 RECIST 1.1 标准分为完全缓解（CR）、部分缓解（PR）、稳定（SD）、进展（PD）。

疾病控制率（Disease Control Rate，DCR）指经确认的完全缓解、部分缓解和疾病稳定（≥ 8 周）病例数在可评价疗效患者中的百分比。

完全缓解（CR）：所有靶病灶消失，全部病理淋巴结(包括靶结节和非靶结节)短直径必须减少至 <10 mm。

部分缓解（PR）：靶病灶直径之和比基线水平减少至少 30%。

疾病进展（PD）：以整个实验研究过程中所有测量的靶病灶直径之和的最小值为参照，直径和相对增加至少 20%（如果基线测量值最小就以基线值为参照）；除此之外，必须满足直径和的绝对值增加至少 5 mm（出现一个或多个新病灶也视为疾病进展）。

疾病稳定（SD）：靶病灶减小的程度没达到 PR，增加的程度也没达到 PD 水平，介于两者之间，研究时可以直径之和的最小值作为参考。

具体实施方式

以下结合实施例用于进一步描述本公开，但这些实施例并非限制本公开的范围。

实施例1：抗PD-1抗体联合甲磺酸阿帕替尼治疗肢端黑色素瘤的研究

化合物 A：PD-1 抗体按照专利申请 WO2017054646A 中的方法制备，其重、轻链的序列如本公开中 SEQID NO: 7 和 SEQID NO: 8。200mg/每瓶，配成 20mg/ml 备用。

化合物 B：甲磺酸阿帕替尼，可按照专利申请 WO2010031266A1 中的方法制备。

入组标准：(1) 经病理组织学证实,手术后复发、不能手术切除或转移性肢端黑色素瘤患者；(2) 3. 既往未接受过任何系统抗肿瘤药物治疗

给药方法：

PD-1抗体：固定剂量200mg，静脉输注（无需预防用药），或针对基线期体重
5 <50kg的受试者，采用3mg/kg，每2周一次，4周一个周期；

阿帕替尼：250mg/片，口服，每日一次，一次一片，餐后给药。

结论：

共计入组 20 例受试者，可评价 18 例受试者中，4 例患者靶病灶直径之和比基
线水平减少至少 30%，9 例受试者表现为疾病稳定（SD），5 例患者表现为疾病进展，
10 整体疾病控制率 72.2%，疾病缓解率 22.2%，而单药 PD-1 疾病缓解率为 15%，明显
改善患者用药安全性、有效性。

实施例2：抗PD-1抗体联合甲磺酸阿帕替尼治疗肢端黑色素瘤的研究

化合物 A：PD-1 抗体按照专利申请 WO2017054646A 中的方法制备，其重、
15 轻链的序列如本公开中 SEQID NO: 7 和 SEQID NO: 8。200mg/每瓶，配成 20mg/ml
备用。

化合物 B：甲磺酸阿帕替尼，可按照专利申请 WO2010031266A1 中的方法制
备。

化合物 C：替莫唑胺，注射用替莫唑胺（粉针，上海恒瑞）或替莫唑胺胶囊（默
20 沙东）

入组标准：(1) 经病理组织学证实,手术后复发、不能手术切除或转移性黑色
素瘤患者（包括肢端的）；(2) 3. 既往未接受过任何系统抗肿瘤药物治疗。

给药方法：

PD-1抗体：固定剂量200mg，静脉输注（无需预防用药），或针对基线期体重
25 <50kg的受试者，采用3mg/kg，每2周一次，4周一个周期；

阿帕替尼：250mg/片，口服，每日一次，一次一片，餐后给药，每28天为一个周
期；

替莫唑胺：200mg/m²，口服或静脉注射，每日一次，连续给药5天，每28天一
周期。

30 结论：

可评价 23 例受试者中，1 例患者所有靶病灶消失，无新病灶出现，且肿瘤标志
物正常（CR），11 例患者靶病灶直径之和比基线水平减少至少 30%（PR），10 例患
者表现为疾病稳定（SD），1 例患者出组，疾病控制率 96.65%（22 例），客观缓解
率 52.17%（12 例）。相比于其他治疗方式，如两药（ORR22.2%，DCR72.2%）或单
35 药 PD-1（15%）有明显优势的。

权利要求书

1. 一种抗 PD-1 抗体和 VEGFR 抑制剂联合在制备治疗肢端黑色素瘤的药物中的用途。
- 5 2. 如权利要求 1 所述的用途，其中所述的 VEGFR 抑制剂选自阿帕替尼、安罗替尼、他菲替尼、法米替尼、索拉非尼或其可药用盐，优选阿帕替尼或其可药用盐。
- 10 3. 如权利要求 1 或 2 所述的用途，其中所述的 PD-1 抗体或其抗原结合片段选自：AMP-224、GLS-010、IBI-308、REGN-2810、PDR-001、BGB-A317、Pidilizumab、PF-06801591、Genolimzumab、CA-170、MEDI-0680、JS-001、TSR-042、Cemiplimab、Camrelizumab、Pembrolizumab、Toripalimab、Sintilimab、Tislelizumab、LZM-009、AK-103 和 Nivolumab。
- 15 4. 如权利要求 1 所述的用途，其中所述的 PD-1 抗体或其抗原结合片段的轻链可变区包含分别如 SEQ ID NO:4、SEQ ID NO:5 和 SEQ ID NO:6 所示的 LCDR1、LCDR2 和 LCDR3，所述的 PD-1 抗体的重链可变区包含分别如 SEQ ID NO:1、SEQ ID NO:2 和 SEQ ID NO:3 所示的 HCDR1、HCDR2 和 HCDR3。
- 20 5. 如权利要求 4 所述的用途，其中所述的 PD-1 抗体选自人源化抗体或其抗原结合片段。
6. 如权利要求 4 或 5 所述的用途，其中所述抗 PD-1 抗体或其抗原结合片段包含人源 IgG1、IgG2、IgG3 或 IgG4 同种型的重链恒定区，优选包含 IgG1 或 IgG4 同种型的重链恒定区。
- 25 7. 权利要求 4-6 任一项所述的用途，其中所述抗 PD-1 抗体或其抗原结合片段包含 κ 或 λ 的轻链恒定区。
- 30 8. 如权利要求 4-6 任一项所述的用途，其中所述人源化抗体的轻链可变区序列为如 SEQ ID NO:8 所示的序列或其变体，所述的变体优选在轻链可变区有 0-10 的氨基酸变化，更优选为 A43S 的氨基酸变化；重链可变区序列为如 SEQ ID NO:7 所示的序列或其变体，所述变体优选在重链可变区有 0-10 的氨基酸变化，更优选为 G44R 的氨基酸变化。
- 35 9. 如权利要求 4-6 任一项所述的用途，其中所述 PD-1 抗体或其抗原结合片段

包含 SEQ ID NO:8 所示轻链, 和如 SEQ ID NO:7 所示重链。

10. 如权利要求 1-9 任一项所述的用途, 其中所述患者选自复发的, 不能手术切除的或转移性的, 所述复发的优选手术后复发的。

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11. 如权利要求 1-10 任一项所述的用途, 其中所述的 PD-1 抗体或其抗原结合片段剂量选自 1-10mg/kg, 例如 3mg/kg。

12. 如权利要求 1-10 任一项所述的用途, 其中所述的 PD-1 抗体或其抗原结合片段剂量选自 50-600mg, 例如 200mg。

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13. 如权利要求 1-12 任一项所述的用途, 其中所述的 VEGFR 抑制剂剂量选自 0.1-500mg, 例如 250mg 或 375mg。

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14. 如权利要求 1-13 任一项所述的用途, 其中还进一步包含烷基化剂, 所述烷基化剂优选替莫唑胺或其可药用盐。

15. 如权利要求 14 所述的用途, 其中所述的替莫唑胺剂量选自 50mg/m²~300mg/m², 例如 150mg/m² 或 200mg/m²。

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16. 如权利要求 2-15 任一项所述的用途, 其中所述阿帕替尼可药用盐选自甲磺酸盐、马来酸盐、酒石酸盐、琥珀酸盐、醋酸盐、二氟醋酸盐、富马酸盐、柠檬酸盐、枸橼酸盐、苯磺酸盐、苯甲酸盐、萘磺酸盐、乳酸盐、苹果酸盐、盐酸盐、氢溴酸盐、硫酸盐和磷酸盐, 优选甲磺酸盐。

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17. 一种抗 PD-1 抗体、VEGFR 抑制剂联合替莫唑胺或其可药用盐在制备治疗黑色素瘤的药物中的用途。

18. 一种用于治疗黑色素瘤的抗 PD-1 抗体, 其中抗 PD-1 抗体与 VEGFR 抑制剂组合施用, 进一步还包括烷基化剂, 所述烷基化剂优选替莫唑胺或其可药用盐。

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19. 一种用于治疗黑色素瘤的 VEGFR 抑制剂, 其中所述 VEGFR 抑制剂与抗 PD-1 抗体组合施用, 进一步还包括烷基化剂, 所述烷基化剂优选替莫唑胺或其可药用盐。

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20. 一种药物组合, 其包含有效治疗量的 PD-1 抗体或其抗原结合片段和 VEGFR 抑制剂, 进一步包括烷基化剂, 所述烷基化剂优选替莫唑胺或其可药用盐。

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2021/000069

A. CLASSIFICATION OF SUBJECT MATTER

A61K 39/395(2006.01)i; A61K 31/444(2006.01)i; C07K 16/28(2006.01)i; A61P 35/00(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNABS, CNKI, CNTXT, DWPI, SIPOABS, EPTXT, USTXT, WOTXT, JPTXT, NCBI, 中国专利生物序列检索系统, Genbank, EMBL, STN: PD-1抗体, 阿帕替尼, 替莫唑胺, 烷基化, 黑色素瘤, VEGFR抑制剂, apatinib, anti-PD-1 antibody, melanoma, vascular endothelial growth factor receptors inhibitor, VEGFR inhibitor, cancer, temozolomide, alkylate, SEQ ID Nos: 1-8, A43S, G44R

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CN 108601831 A (SUZHOU SUNCADIA BIOPHARMACEUTICALS CO., LTD.; JIANGSU HENGRUI MEDICINE COMPANY) 28 September 2018 (2018-09-28) claims 1-21, description paragraphs 15-27	1-20
Y	SHOUSHTARI, A. N. et al. "Efficacy of anti-PD-1 agents in acral and mucosal melanoma" <i>Cancer</i> , Vol. 122, No. 21, 15 November 2016 (2016-11-15), ISSN: 0008-543X, see abstract	1-20
Y	CN 106999591 A (JIANGSU HENGRUI MEDICINE CO., LTD.; SHANGHAI HENGRUI PHARMACEUTICAL CO., LTD.) 01 August 2017 (2017-08-01) description paragraphs 8-17, 45-49	4-9
Y	CN 101676267 A (JIANGSU HENGRUI MEDICINE CO., LTD.) 24 March 2010 (2010-03-24) description page 3 paragraph 2	16
Y	CN 105026428 A (SHANGHAI HENGRUI MEDICINE CO., LTD.; JIANGSU HENGRUI MEDICINE CO., LTD.) 04 November 2015 (2015-11-04) description paragraphs 29-41, 135-143	4-9

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

25 June 2021

Date of mailing of the international search report

15 July 2021

Name and mailing address of the ISA/CN

China National Intellectual Property Administration (ISA/
CN)
No. 6, Xitucheng Road, Jimenqiao, Haidian District, Beijing
100088
China

Authorized officer

Facsimile No. (86-10)62019451

Telephone No.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2021/000069**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN 108079292 A (SUZHOU SUNCADIA BIOPHARMACEUTICALS CO., LTD.; JIANGSU HENGRUI MEDICINE COMPANY) 29 May 2018 (2018-05-29) entire document	1-20
A	CN 106963948 A (GU, Yanhong) 21 July 2017 (2017-07-21) entire document	1-20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2021/000069

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☒ forming part of the international application as filed:
☒ in the form of an Annex C/ST.25 text file.
☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2021/000069

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
CN	108601831	A	28 September 2018	EP	3524268	A4	01 July 2020
				RU	2019112029	A3	21 January 2021
				BR	112019005908	A2	11 June 2019
				CA	3038586	A1	19 April 2018
				TW	201813667	A	16 April 2018
				US	2020040078	A1	06 February 2020
				CN	108601831	B	12 November 2019
				AU	2017342880	A9	09 May 2019
				WO	2018068691	A1	19 April 2018
				MX	2019003780	A	01 July 2019
				EP	3524268	A1	14 August 2019
				RU	2019112029	A	13 November 2020
				KR	20190066012	A	12 June 2019
				JP	2019530752	A	24 October 2019
				AU	2017342880	A1	18 April 2019
CN	106999591	A	01 August 2017	RU	2018110333	A3	30 December 2019
				US	10786567	B2	29 September 2020
				TW	201711699	A	01 April 2017
				EP	3357508	A4	24 April 2019
				WO	2017054646	A1	06 April 2017
				JP	2018532730	A	08 November 2018
				AU	2016329960	A1	26 April 2018
				CA	2999079	A1	06 April 2017
				BR	112018005349	A2	09 October 2018
				CN	106999591	B	23 February 2021
				RU	2018110333	A	28 October 2019
				KR	20180054791	A	24 May 2018
				EP	3357508	A1	08 August 2018
				RU	2731418	C2	02 September 2020
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CN	101676267	A	24 March 2010	US	2018339045	A1	29 November 2018
				US	2021000954	A1	07 January 2021
				AU	2009295168	B2	13 February 2014
				HK	1141514	A1	12 November 2010
				JP	5649132	B2	07 January 2015
				CN	101676267	B	26 December 2012
				EP	2327697	A4	06 June 2012
				RU	2011113229	A	27 October 2012
				KR	20110059877	A	07 June 2011
				KR	101674227	B1	08 November 2016
				AU	2009295168	A1	25 March 2010
				EP	2327697	A1	01 June 2011
				WO	2010031266	A1	25 March 2010
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				ZA	201101891	B	30 May 2012
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				JP	2012502885	A	02 February 2012
				EP	2327697	B1	12 March 2014
				BR	PI0919018	A2	08 December 2015

Form PCT/ISA/210 (patent family annex) (January 2015)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2021/000069

Patent document cited in search report				Publication date (day/month/year)		Patent family member(s)		Publication date (day/month/year)	
				RU	2499796	C2	27 November 2013		
				US	8362256	B2	29 January 2013		
				CA	2736664	C	24 May 2016		
CN	105026428	A	04 November 2015	ZA	201604660	B	18 December 2019		
				CR	20160319	A	08 November 2016		
				SG	10201804945 W	A	30 July 2018		
				IL	246137	D0	31 July 2016		
				UA	119659	C2	25 July 2019		
				US	2019309069	A1	10 October 2019		
				HR	P20191678	T1	13 December 2019		
				PT	3081576	T	15 October 2019		
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				EP	3081576	B1	21 August 2019		
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				JP	2017500889	A	12 January 2017		
				WO	2015085847	A1	18 June 2015		
				DO	P2016000133	A	30 November 2016		
				US	10344090	B2	09 July 2019		
				KR	20160113113	A	28 September 2016		
				ES	2746805	T3	06 March 2020		
				RS	59480	B1	31 December 2019		
				TW	201605901	A	16 February 2016		
				TW	I654201	B	21 March 2019		
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				HK	1213910	A1	15 July 2016		
				EP	3081576	A1	19 October 2016		
				AU	2014361473	B2	26 September 2019		
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				CL	2016001460	A1	20 January 2017		
				PH	12016501120	A1	15 August 2016		
				HU	E046249	T2	28 February 2020		
				ME	03527	B	20 April 2020		
				EA	201691225	A1	30 September 2016		
				JP	6502959	B2	17 April 2019		
				EC	SP16060104	A	31 August 2017		
				EP	3081576	A4	05 July 2017		
				AU	2014361473	A1	07 July 2016		
				CN	105026428	B	16 January 2018		
				US	2016376367	A1	29 December 2016		
				SI	3081576	T1	31 December 2019		
				PE	20160953	A1	26 September 2016		
				EA	035037	B1	21 April 2020		
				CN	108079292	A	29 May 2018	None	
				CN	106963948	A	21 July 2017	None	

Form PCT/ISA/210 (patent family annex) (January 2015)

国际检索报告

国际申请号

PCT/CN2021/000069

A. 主题的分类		
A61K 39/395(2006.01)i; A61K 31/444(2006.01)i; C07K 16/28(2006.01)i; A61P 35/00(2006.01)i		
按照国际专利分类(IPC)或者同时按照国家分类和IPC两种分类		
B. 检索领域		
检索的最低限度文献(标明分类系统和分类号)		
A61K C07K A61P		
包含在检索领域中的除最低限度文献以外的检索文献		
在国际检索时查阅的电子数据库(数据库的名称, 和使用的检索词(如使用))		
CNABS, CNKI, CNTXT, DWPI, SIPOABS, EPTXT, USTXT, WOTXT, JPTXT, NCBI, 中国专利生物序列检索系统, Genbank, EMBL, STN和检索项: PD-1抗体、阿帕替尼, 替莫唑胺, 烷基化, 黑色素瘤, VEGFR抑制剂, apatinib, anti-PD-1 antibody, melanoma, vascular endothelial growth factor receptors inhibitor, VEGFR inhibitor, cancer, temozolomide, alkylate, SEQ ID Nos:1-8, A43S, G44R		
C. 相关文件		
类 型*	引用文件, 必要时, 指明相关段落	相关的权利要求
Y	CN 108601831 A (苏州盛迪亚生物医药有限公司 江苏恒瑞医药股份有限公司) 2018年 9月 28日 (2018 - 09 - 28) 权利要求1-21, 说明书第15-27段	1-20
Y	SHOUSHITARI, A.N.等. "Efficacy of anti-PD-1 agents in acral and mucosal melanoma" Cancer, 第122卷, 第21期, 2016年 11月 15日 (2016 - 11 - 15), ISSN: 0008 543X, 参见摘要	1-20
Y	CN 106999591 A (江苏恒瑞医药股份有限公司 上海恒瑞医药有限公司) 2017年 8月 1日 (2017 - 08 - 01) 说明书第8-17、45-49段	4-9
Y	CN 101676267 A (江苏恒瑞医药股份有限公司) 2010年 3月 24日 (2010 - 03 - 24) 说明书第3页第二段	16
Y	CN 105026428 A (上海恒瑞医药有限公司 江苏恒瑞医药股份有限公司) 2015年 11月 4日 (2015 - 11 - 04) 说明书第29-41、135-143段	4-9
☒ 其余文件在C栏的续页中列出。 ☒ 见同族专利附件。		
* 引用文件的具体类型: "A" 认为不特别相关的表示了现有技术一般状态的文件 "E" 在国际申请日的当天或之后公布的在先申请或专利 "L" 可能对优先权要求构成怀疑的文件, 或为确定另一篇引用文件的公布日而引用的或者因其他特殊理由而引用的文件(如具体说明的) "O" 涉及口头公开、使用、展览或其他方式公开的文件 "P" 公布日先于国际申请日但迟于所要求的优先权日的文件 "T" 在申请日或优先权日之后公布, 与申请不相抵触, 但为了理解发明之理论或原理的在后文件 "X" 特别相关的文件, 单独考虑该文件, 认定要求保护的发明不是新颖的或不具有创造性 "Y" 特别相关的文件, 当该文件与另一篇或者多篇该类文件结合并且这种结合对于本领域技术人员为显而易见时, 要求保护的发明不具有创造性 "&" 同族专利的文件		
国际检索实际完成的日期		国际检索报告邮寄日期
2021年 6月 25日		2021年 7月 15日
ISA/CN的名称和邮寄地址		受权官员
中国国家知识产权局(ISA/CN) 中国北京市海淀区蓟门桥西土城路6号 100088		王翔宇
传真号 (86-10)62019451		电话号码 62089318

PCT/ISA/210 表(第2页) (2015年1月)

国际检索报告

国际申请号

PCT/CN2021/000069

C. 相关文件

类 型*	引用文件，必要时，指明相关段落	相关的权利要求
A	CN 108079292 A (苏州盛迪亚生物医药有限公司 江苏恒瑞医药股份有限公司) 2018年 5月 29日 (2018 - 05 - 29) 全文	1-20
A	CN 106963948 A (顾艳宏) 2017年 7月 21日 (2017 - 07 - 21) 全文	1-20

第1栏 核苷酸和/或氨基酸序列(续第1页第1. c项)

1. 关于国际申请中所公开的任何核苷酸和/或氨基酸序列, 国际检索是基于下列序列表进行的:
 - a. ☒ 作为国际申请的一部分提交的:
 - ☒ 附件C/ST. 25文本文件形式
 - ☐ 纸件或图形文件形式
 - b. ☐ 根据细则13之三. 1(a) 仅为国际检索目的以附件C/ST. 25文本文件形式与国际申请同时提交的:
 - c. ☐ 仅为国际检索目的在国际申请日之后提交的:
 - ☐ 附件C/ST. 25文本文件形式(细则13之三. 1(a))
 - ☐ 纸件或图形文件形式(细则13之三. 1(b) 和行政规程第713段)
2. ☐ 另外, 在提交/提供了多个版本或副本的序列表的情况下, 提供了关于随后提交的或附加的副本中的信息与申请时提交的作为申请一部分的序列表的信息相同或未超出申请时提交的申请中的信息范围(如适用)的所需声明。
3. 补充意见:

国际检索报告
关于同族专利的信息

国际申请号

PCT/CN2021/000069

检索报告引用的专利文件			公布日 (年/月/日)	同族专利		公布日 (年/月/日)	
CN	108601831	A	2018年 9月 28日	EP	3524268	A4	2020年 7月 1日
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				BR	112019005908	A2	2019年 6月 11日
				CA	3038586	A1	2018年 4月 19日
				TW	201813667	A	2018年 4月 16日
				US	2020040078	A1	2020年 2月 6日
				CN	108601831	B	2019年 11月 12日
				AU	2017342880	A9	2019年 5月 9日
				WO	2018068691	A1	2018年 4月 19日
				MX	2019003780	A	2019年 7月 1日
				EP	3524268	A1	2019年 8月 14日
				RU	2019112029	A	2020年 11月 13日
				KR	20190066012	A	2019年 6月 12日
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CN	106999591	A	2017年 8月 1日	RU	2018110333	A3	2019年 12月 30日
				US	10786567	B2	2020年 9月 29日
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				EP	3357508	A4	2019年 4月 24日
				WO	2017054646	A1	2017年 4月 6日
				JP	2018532730	A	2018年 11月 8日
				AU	2016329960	A1	2018年 4月 26日
				CA	2999079	A1	2017年 4月 6日
				BR	112018005349	A2	2018年 10月 9日
				CN	106999591	B	2021年 2月 23日
				RU	2018110333	A	2019年 10月 28日
				KR	20180054791	A	2018年 5月 24日
				EP	3357508	A1	2018年 8月 8日
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US	2018339045	A1	2018年 11月 29日				
US	2021000954	A1	2021年 1月 7日				
CN	101676267	A	2010年 3月 24日	AU	2009295168	B2	2014年 2月 13日
				HK	1141514	A1	2010年 11月 12日
				JP	5649132	B2	2015年 1月 7日
				CN	101676267	B	2012年 12月 26日
				EP	2327697	A4	2012年 6月 6日
				RU	2011113229	A	2012年 10月 27日
				KR	20110059877	A	2011年 6月 7日
				KR	101674227	B1	2016年 11月 8日
				AU	2009295168	A1	2010年 3月 25日
				EP	2327697	A1	2011年 6月 1日
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				ZA	201101891	B	2012年 5月 30日
				US	2011184023	A1	2011年 7月 28日
				CA	2736664	A1	2010年 3月 25日
				JP	2012502885	A	2012年 2月 2日
				EP	2327697	B1	2014年 3月 12日
				BR	PI0919018	A2	2015年 12月 8日

PCT/ISA/210 表(同族专利附件) (2015年1月)

国际检索报告
关于同族专利的信息

国际申请号

PCT/CN2021/000069

检索报告引用的专利文件				公布日 (年/月/日)		同族专利			公布日 (年/月/日)		
						RU	2499796	C2	2013年 11月 27日		
						US	8362256	B2	2013年 1月 29日		
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CN	105026428	A	2015年 11月 4日	ZA	201604660	B	2019年 12月 18日				
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				PE	20160953	A1	2016年 9月 26日				
				EA	035037	B1	2020年 4月 21日				
CN	108079292	A	2018年 5月 29日	无							
CN	106963948	A	2017年 7月 21日	无							

PCT/ISA/210 表(同族专利附件) (2015年1月)



(51) International Patent Classification:

A61K 9/00 (2006.01) *C07K 14/28* (2006.01)
A61K 47/10 (2017.01) *A61K 39/395* (2006.01)
A61K 47/12 (2006.01) *A61P 13/00* (2006.01)
A61K 47/26 (2006.01) *A61P 35/00* (2006.01)

(21) International Application Number:

PCT/US2021/028701

(22) International Filing Date:

22 April 2021 (22.04.2021)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/013,834 22 April 2020 (22.04.2020) US
63/033,161 01 June 2020 (01.06.2020) US

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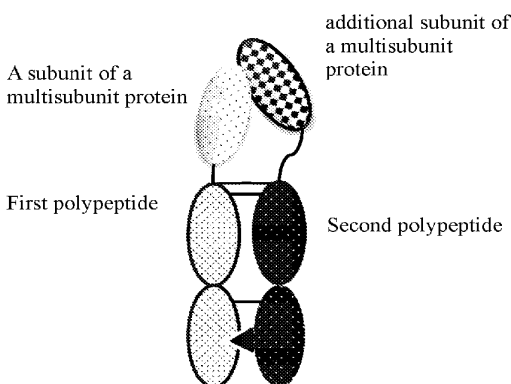
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(54) Title: FORMULATION, DOSAGE REGIMEN, AND MANUFACTURING PROCESS FOR HETERODIMERIC FC-FUSED PROTEINS

FIG. 1A



(57) Abstract: The present invention relates to pharmaceutical formulations for heterodimeric Fc-fused proteins which are advantageous for achieving higher titers of the proteins during production, higher stability during storage, and improved efficacy when used as a therapeutic. Also provided are dosage regimens for such heterodimeric Fc-fused proteins and pharmaceutical formulations for use in treating cancer, such as locally advanced or metastatic solid tumor.

[Continued on next page]

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FORMULATION, DOSAGE REGIMEN, AND MANUFACTURING PROCESS FOR HETERODIMERIC FC-FUSED PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and benefit of U.S. Provisional Application No. 63/013,834, filed on April 22, 2020, and U.S. Provisional Application No. 63/033,161, filed on June 1, 2020, each of which is hereby incorporated by reference herein in its entirety.

SEQUENCE LISTING

[0002] The content of the electronically submitted sequence listing in ASCII text file (Name: 3338_259PC03_Seqlisting_ST25.txt; Size: 946,336 bytes; and Date of Creation: April 21, 2021) filed with the application is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0003] The present disclosure relates to pharmaceutical formulations and dosage regimens for heterodimeric Fc-fused proteins, and methods of using such proteins to treat cancers.

BACKGROUND

[0004] Physiologically active proteins mostly have the disadvantage of having a short *in vivo* half-life. In order to solve this disadvantage, there has been an attempt to conjugate them to PEG (polyethylene glycol) or the like, or to fuse them to an antibody Fc (crystallizable fragment) region. Proteins composed of two or more different subunits, in which the two or more different subunits form a protein complex to exhibit physiological activity, can be fused to wild-type Fc domains to prepare Fc-fused protein forms, forming a homodimer due to the homodimeric nature of Fc. Proteins composed of two or more different subunits, in which the two or more different subunits form a protein complex to exhibit physiological activity, can also be fused to heterodimeric Fc regions derived not only from IgG1, but also from other isotype antibodies such as IgG2, IgG3 and IgG4, to form a heterodimeric Fc-fused protein. Thus, one or more subunit(s) of the protein, which is composed of two or more different subunits and in which two or more subunits exhibit physiological activity by forming a protein complex, can be fused to the terminus of heterodimeric Fc variant regions to form improved Fc-fused protein forms.

[0005] Fc heterodimerization is a technology that induces mutations in two different CH3 domains of Fc by genetic engineering, such that the two Fc fragments form a heterodimer with

minimal sequence variations while they have tertiary structures very similar to those of naturally occurring antibodies (*see, e.g.*, U.S. Patent No. 7,695,936).

[0006] The inventions described in the present disclosure provide designs for improving the Fc-fused protein forms, in which the two subunits of a heterodimeric protein are connected to two Fc domains having different heterodimerization domains, by introducing linkers of varying lengths, or mutations in the CH2 and the CH3 domains of the Fc. Further the inventions described in the present disclosure provides pharmaceutical formulations for such proteins, method of making such proteins and formulations, and methods for treating cancer using such proteins.

SUMMARY

[0007] The invention generally relates to pharmaceutical formulations comprising certain heterodimeric Fc-fused proteins comprising IL12 subunit(s), processes for preparing such proteins and pharmaceutical formulations. Also provided are dosage regimens for using such heterodimeric Fc-fused proteins and pharmaceutical formulations to treat cancer, such as locally advanced or metastatic solid tumors.

[0008] Accordingly, in one aspect, provided herein is a pharmaceutical formulation comprising a heterodimeric Fc-fused protein comprising a first polypeptide comprising a first antibody Fc domain polypeptide and a first subunit of a multisubunit cytokine and a second polypeptide comprising a second antibody Fc domain polypeptide and a second, different subunit of the multisubunit cytokine, citrate, a sugar, a sugar alcohol, and a non-ionic surfactant, at pH 6.0 to 7.0, wherein the first and second antibody Fc domain polypeptides each comprise different mutations promoting heterodimerization, and wherein the first subunit and second, different subunit of the multisubunit cytokine are bound to each other. In some embodiments, the first and/or second antibody Fc domain polypeptides comprise one or more mutation(s) that reduce(s) an effector function of an Fc.

[0009] In some embodiments, the concentration of citrate in the pharmaceutical formulation is about 10 mM to about 30 mM. In certain embodiments, the concentration of citrate in the pharmaceutical formulation is about 20 mM. In some embodiments, the concentration of the sugar in the pharmaceutical formulation is about 3% to about 12% (w/v). In certain embodiments, the concentration of the sugar in the pharmaceutical formulation is about 6% (w/v). In certain embodiments, the sugar is a disaccharide. In certain embodiments, the disaccharide is sucrose. In some embodiments, the concentration of the sugar alcohol in the pharmaceutical formulation is between about 0.5% to about 6% (w/v). In certain embodiments, the concentration of the sugar

alcohol in the pharmaceutical formulation is about 1% (w/v). In certain embodiments, the sugar alcohol is derived from a monosaccharide. In certain embodiments, the sugar alcohol is mannitol.

[0010] In some embodiments, the concentration of the non-ionic surfactant in the pharmaceutical formulation is between about 0.005% to about 0.02% (w/v). In certain embodiments, the concentration of polysorbate 80 in the pharmaceutical formulation is about 0.01% (w/v). In certain embodiments, the non-ionic surfactant is a polysorbate. In certain embodiments, the polysorbate is polysorbate 80.

[0011] In some embodiments, the pH is between about 6.1 and about 6.9. In certain embodiments, the pH is between about 6.2 and about 6.8. In certain embodiments, the pH is between about 6.3 and about 6.7. In some embodiments, the pH is between about 6.4 and about 6.6. In certain embodiments, the pH is about 6.5.

[0012] In some embodiments, the pharmaceutical formulation further comprises water. In certain embodiments, the water is Water for Injection, USP.

[0013] In some embodiments, the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 1 g/L to about 10 g/L. In certain embodiments, the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 2 g/L to about 8 g/L. In certain embodiments, the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 4 g/L to about 6 g/L. In certain embodiments, the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 5 g/L.

[0014] In some embodiments, the pharmaceutical formulation comprises a concentration of the protein for administration of about 0.5 g/L to about 1.5 g/L. In certain embodiments, the pharmaceutical formulation comprises a concentration of the protein for administration of about 0.75 g/L to about 1.25 g/L. In certain embodiments, the pharmaceutical formulation comprises a concentration of the protein for administration of about 1 g/L.

[0015] In some embodiments, the formulation is designed to be stored at a temperature between 2°C and 8°C. In some embodiments, the pharmaceutical formulation is a clear, colorless solution and free of visible particulates.

[0016] In some embodiments, the formulation has a thermal stability profile as defined by a T_{m1} of greater than about 60°C, greater than about 61°C, greater than about 62°C, greater than about 63°C, greater than about 64°C, greater than about 65°C, or greater than about 66°C; and/or a T_{m2} of greater than about 70°C, greater than about 71°C, greater than about 72°C, greater than about 73°C, greater than about 74°C, greater than about 75°C, greater than about 76°C, or greater than

about 77°C, as measured by differential scanning fluorimetry. In certain embodiments, the formulation has a thermal stability profile as defined by a T_{m1} of about 67.0°C and a T_{m2} of about 77.3°C. In certain embodiments, the thermal stability profile of the pharmaceutical formulation, as defined by T_{m1} and/or T_{m2} is changed by less than about 2°C or less than about 1°C when the pharmaceutical formulation is incubated for 1 week at 50°C, as compared to the same pharmaceutical formulation that is incubated for 1 week at 5°C, as measured by differential scanning fluorimetry.

[0017] In some embodiments, the formulation has a thermal stability profile as defined by a T_{agg} of greater than about 60°C, greater than about 61°C, greater than about 62°C, greater than about 63°C, greater than about 64°C, greater than about 65°C, greater than about 66°C, or greater than about 67°C, as measured by differential scanning fluorimetry. In certain embodiments, the thermal stability profile of the pharmaceutical formulation, as defined by T_{agg} is changed by less than about 2°C or less than about 1°C when the pharmaceutical formulation is incubated for 1 week at 50°C, as compared to the same pharmaceutical formulation that is incubated for 1 week at 5°C, as measured by differential scanning fluorimetry.

[0018] In some embodiments, the pH of the pharmaceutical formulation does not change by more than about 0.2 or about 0.1 in pH value after the pharmaceutical formulation is incubated for 1 week at 5°C. In some embodiments, the pH of the pharmaceutical formulation does not change by more than about 0.2 or about 0.1 in pH value after the pharmaceutical formulation is incubated for 1 week at 50°C.

[0019] In some embodiments, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less than about 15 nm, less than about 14 nm, less than about 13 nm, or less than about 12 nm, as measured by dynamic light scattering at 25°C. In certain embodiments, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of about 11.6 nm. In some embodiments, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less than about 20 nm, less than about 19 nm, less than about 18 nm, less than about 17 nm, less than about 16 nm, or less than about 15 nm, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is incubated for 2 weeks at 50°C. In certain embodiments, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of about 14.4 nm. In some embodiments, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less than about 20 nm, less than about 19 nm, less than about 18 nm, less than about 17 nm, or less than

about 16 nm, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is subjected to five freeze thaw cycles. In certain embodiments, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of about 15.3 nm.

[0020] In some embodiments, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is less than about 0.30, less than about 0.29, less than about 0.28, or less than about 0.27, as measured by dynamic light scattering at 25°C. In some embodiments, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is about 0.26. In some embodiments, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is less than about 0.30, less than about 0.29, less than about 0.28, less than about 0.27, or less than about 0.26, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is incubated for 2 weeks at 50°C. In some embodiments, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is about 0.25. In some embodiments, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is less than about 0.40, less than about 0.35, or less than about 0.34, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is subjected to five freeze thaw cycles. In some embodiments, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is about 0.33.

[0021] In some embodiments, the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is greater than about 90%, greater than about 91%, greater than about 92%, greater than about 93%, greater than about 94%, greater than about 95%, greater than about 96%, greater than about 97%, greater than about 98%, or greater than about 99%. In certain embodiments, the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is about 99.0%.

[0022] In some embodiments, the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is greater than about 75%, greater than about 80%, greater than about 81%, greater than about 82%, greater than about 83%, greater than about 84%, or greater than about 85%, after the pharmaceutical formulation is incubated for 2 weeks at 50°C. In certain embodiments, the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is about 85.2%.

[0023] In some embodiments, the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is greater than about 90%, greater than about 91%, greater than about 92%, greater than about 93%, greater than about 94%, greater than about 95%, greater than about 96%, greater than about 97%, or greater than about 98%, after the pharmaceutical formulation is subjected to five freeze thaw cycles. In certain embodiments, the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is about 98.9%.

[0024] In another aspect, the disclosure provides for a method comprising administering to a subject in need thereof, the pharmaceutical formulation as a single-dose therapy.

[0025] In another aspect, the disclosure provides for a method comprising administering to a subject in need thereof, the pharmaceutical formulation in a multiple-dose therapy at an interval of at least three weeks between the doses or at least four weeks between the doses. In some embodiments, the pharmaceutical formulation is administered to the subject once every three weeks. In some embodiments, the pharmaceutical formulation is administered to the subject once every four weeks. In certain embodiments, the pharmaceutical formulation is administered to the subject once every six weeks.

[0026] In some embodiments, the method further comprises stopping the multi-dose therapy if the subject develops progressive disease, unacceptable toxicity, or meets a criterion for withdrawal. In some embodiments, if the subject experiences a complete response (CR) during the multi-dose therapy, then the multi-dose therapy is further administered for at least 12 months after the confirmation of the complete response. In certain embodiments, the total duration of the multi-dose therapy is equal to or less than 24 months. In certain embodiments, the total treatment duration is greater than 24 months.

[0027] In some embodiments, the pharmaceutical formulation is administered by subcutaneous injection.

[0028] In some embodiments, the pharmaceutical formulation is administered to the subject in an amount sufficient to provide the heterodimeric Fc-fused protein at a dosage of between about 0.05 µg/kg to about 1.75 µg/kg, based on the subject's weight. In some embodiments, the pharmaceutical formulation is administered to the subject in an amount sufficient to provide the heterodimeric Fc-fused protein at a dosage of about 0.05 µg/kg, about 0.10 µg/kg, about 0.20 µg/kg, about 0.40 µg/kg, about 0.60 µg/kg, about 0.80 µg/kg, about 1.00 µg/kg, about 1.20 µg/kg, about 1.40 µg/kg, or about 1.75 µg/kg, based on the subject's weight. In certain embodiments, the

pharmaceutical formulation is administered to the subject in an amount sufficient to provide the heterodimeric Fc-fused protein at a dosage of greater than 0.00 µg/kg and less than about 0.05 µg/kg, based on the subject's weight. In certain embodiments, the pharmaceutical formulation is administered to the subject in an amount sufficient to provide the heterodimeric Fc-fused protein at a dosage of greater than about 1.75 µg/kg, based on the subject's weight.

[0029] In some embodiments, the subject has cancer. In certain embodiments, the subject has a locally advanced or metastatic solid tumor. In certain embodiments, the presence of the cancer in the subject is confirmed using the Response Evaluation Criteria for Solid Tumors (RECIST), version 1.1. In certain embodiments, the cancer is selected from the group consisting of: melanoma, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), head and neck squamous cell carcinoma (HNSCC), classical Hodgkin lymphoma, primary mediastinal large B-Cell lymphoma, bladder cancer, urothelial carcinoma, micro-satellite instability high cancer, colorectal cancer, gastric cancer, oesophageal cancer, cervical cancer, hepatocellular carcinoma, Merkel cell carcinoma, renal cell carcinoma (RCC), endometrial carcinoma, cutaneous T cell lymphoma, and triple negative breast cancer. In certain embodiments, the cancer is selected from the group consisting of: melanoma, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), head and neck squamous cell carcinoma (HNSCC), classical Hodgkin lymphoma, primary mediastinal large B-Cell lymphoma, bladder cancer, urothelial carcinoma, micro-satellite instability high cancer, colorectal cancer, gastric cancer, oesophageal cancer, cervical cancer, ovarian cancer, prostate cancer, hepatocellular carcinoma, Merkel cell carcinoma, renal cell carcinoma (RCC), endometrial carcinoma, cutaneous T cell lymphoma, and triple negative breast cancer. In certain embodiments, the subject is anti-PD-1 refractory.

[0030] In some embodiments, the subject has melanoma. In certain embodiments, the subject has previously been treated with an anti-PD-1 antibody for at least 6 weeks. In certain embodiments, the subject has been confirmed of progression of disease at least 4 weeks after the initial diagnosis of progression of disease while receiving an anti-PD-1 antibody. In certain embodiments, progression of disease is confirmed by radiological or clinical observation. In certain embodiments, if the subject has a tumor comprising a BRAF activating mutation, then the subject has previously been treated with a BRAF inhibitor.

[0031] In some embodiments, the subject has RCC. In certain embodiments, the RCC has clear cell histology. In certain embodiments, the patient has previously been treated with an anti-PD-1/PD-L1 antibody and/or an anti-vascular endothelial growth factor therapy. In certain embodiments, the subject has previously received three or fewer lines of therapy.

[0032] In some embodiments, the subject has urothelial carcinoma. In certain embodiments, the subject has locally advanced or metastatic transitional cell carcinoma of the urothelium. In certain embodiments, the subject has previously been treated with a single treatment comprising a platinum-containing regimen and has shown radiographic progression recurrence within 6 months after the last administration of the platinum-containing regimen. In certain embodiments, the subject has previously received two or less lines of therapy. In certain embodiments, the subject has not previously received a checkpoint inhibitor (e.g., anti-PD-1 or anti-PD-L1 antibody) therapy as a monotherapy or in combination with a platinum based chemotherapy.

[0033] In some embodiments, the pharmaceutical formulation is administered to the subject as a monotherapy.

[0034] In some embodiments, the pharmaceutical formulation is administered to the subject as a combination therapy.

[0035] In some embodiments, the method further comprises administering to the subject an anti-PD-1 antibody. In certain embodiments, the anti-PD-1 antibody is pembrolizumab. In certain embodiments, the pembrolizumab is administered intravenously. In certain embodiments, the pembrolizumab is administered at a dose of 200 mg. In certain embodiments, the administration of pembrolizumab precedes each administration of the pharmaceutical formulation. In certain embodiments, the pharmaceutical formulation is administered within 1 hour after completion of administration of pembrolizumab.

[0036] In certain embodiments, the anti-PD-1 antibody is nivolumab. In certain embodiments, the nivolumab is administered intravenously. In certain embodiments, the nivolumab is administered at a dose of about 480 mg. In certain embodiments, the administration of nivolumab precedes each administration of the pharmaceutical formulation. In certain embodiments, the pharmaceutical formulation is administered within 1 hour after completion of administration of nivolumab.

[0037] In some embodiments the combination therapy is for treatment of a cancer selected from the group consisting of: melanoma, NSCLC, SCLC, RCC, classical Hodgkin lymphoma, HNSCC, urothelial carcinoma, colorectal cancer, hepatocellular carcinoma, and oesophageal cancer. In some embodiments the combination therapy is for treatment of a cancer selected from the group consisting of: melanoma, NSCLC, SCLC, RCC, classical Hodgkin lymphoma, HNSCC, urothelial carcinoma, colorectal cancer, hepatocellular carcinoma, oesophageal cancer, gastric cancer, ovarian cancer, and prostate cancer. In some embodiments, the cancer is melanoma. In some embodiments, the melanoma is unresectable. In some embodiments the cancer is colorectal cancer.

In some embodiments, the colorectal cancer is microsatellite instability-high (MSI-H) or mismatch repair deficient metastatic (dMMR) colorectal cancer.

[0038] In some embodiments, the method further comprises performing a surgical intervention to lyse cancer cells, remove a tumor, or debulk a tumor in the subject. In certain embodiments, the surgical intervention comprises cryotherapy. In certain embodiments, the surgical intervention comprises hyperthermic therapy. In certain embodiments, the surgical intervention comprises administering to the subject a radiotherapy. In certain embodiments, the radiotherapy is a stereotactic body radiation therapy (SBRT).

[0039] In some embodiments, the method further comprises administering to the subject an NK cell-targeting therapy. In certain embodiments, the subject is administered a multi-specific binding protein. In some embodiments, the method further comprises administering to the subject a chimeric antigen receptor therapy. In some embodiments, the method further comprises administering to the subject a cytokine therapy. In some embodiments, the method further comprises administering to the subject an innate immune system agonist therapy. In some embodiments, the method further comprises administering to the subject a chemotherapy. In some embodiments, the method further comprises administering to the subject a targeted antigen therapy. In some embodiments, the method further comprises administering to the subject an oncolytic virus therapy.

[0040] In another aspect, the disclosure provides for a method of detecting toxicity in a subject receiving a pharmaceutical formulation comprising measuring the concentration of C-reactive protein (CRP) in the subject's blood, wherein the pharmaceutical formulation comprises a heterodimeric Fc-fused protein and a pharmaceutically acceptable carrier, and wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization. In some embodiments, if the CRP concentration in the subject's blood is higher than a threshold CRP concentration, then the subject is identified as being at risk for developing an adverse drug reaction; and if the CRP concentration in the subject's blood is about the same or lower than the threshold C-reactive protein concentration, the subject is not identified as being at risk for developing an adverse drug reaction. In certain embodiments, if the

CRP concentration in the subject's blood is higher than the threshold CRP concentration, then the administration of the pharmaceutical formulation is paused, the heterodimeric Fc-fused protein is administered at a lower dose, or a remedial action is taken to reduce or alleviate the formulation's toxicity effects in the subject.

[0041] In some embodiments of the pharmaceutical formulation or the method described herein, the first and second antibody Fc domain polypeptides are human IgG1 Fc domain polypeptides. In certain embodiments, the multisubunit cytokine is a human IL12. In certain embodiments, the human IgG1 Fc domain polypeptides comprise one or more mutation(s) that reduce(s) an effector function of an Fc. In certain embodiments, the first and second antibody Fc domain polypeptides comprise mutations selected from L234A, L235A or L235E, G237A, P329A, A330S, and P331S, numbered according to the EU numbering system. In certain embodiments, the first and second antibody Fc domain polypeptides each comprise mutations L234A, L235A, and P329A. In certain embodiments, the first subunit of a multisubunit cytokine is a p40 subunit of IL12 and the second subunit of a multisubunit cytokine is a p35 subunit of IL12. In certain embodiments of the pharmaceutical formulation or the method, the first subunit of a multisubunit cytokine comprises the amino acid sequence of SEQ ID NO: 127 and the second subunit of a multisubunit cytokine comprises the amino acid sequence of SEQ ID NO: 128. In certain embodiments of the pharmaceutical formulation or the method, the second subunit of a multisubunit cytokine is fused to the second antibody Fc domain by a linker comprising the amino acid sequence of SEQ ID NO: 108. In certain embodiments of the pharmaceutical formulation or the method, the first antibody Fc domain comprises mutations L234A, L235A, P329A, Y349C, K360E, and K409W, and the second antibody Fc domain comprises mutations L234A, L235A, P329A, Q347R, S354C, D399V, and F405T. In certain embodiments of the pharmaceutical formulation or the method, the first antibody Fc domain comprises the amino acid sequence of SEQ ID NO:215, and the second antibody Fc domain comprises the amino acid sequence of SEQ ID NO:216. In certain embodiments of the pharmaceutical formulation or the method, the first antibody Fc domain peptide comprises the amino acid sequence of SEQ ID NO:290 and the second antibody Fc domain peptide comprises the amino acid sequence of SEQ ID NO:291.

[0042] In another aspect, provided herein is a kit comprising one or more vessels comprising a pharmaceutical formulation, wherein the pharmaceutical formulation comprises a heterodimeric Fc-fused protein comprising a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the

second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization; and a pharmaceutically acceptable carrier, and the one or more vessels collectively comprise about 0.1 mg – about 2 mg of heterodimeric Fc-fused protein. In certain embodiments, the one or more vessels collectively comprise about 0.5 mg to about 2 mg of heterodimeric Fc-fused protein. In certain embodiments, the one or more vessels collectively comprise about 1 mg of heterodimeric Fc-fused protein. In certain embodiments, the kit comprises one vessel comprising about 1 mg of heterodimeric Fc-fused protein. In some embodiments, the pharmaceutical formulation is a lyophilized formulation or a liquid formulation. In certain embodiments, the pharmaceutical formulation is a liquid formulation supplied in a volume of 1 mL.

[0043] In another aspect, the present disclosure provides for a use of a heterodimeric Fc-fused protein in the manufacture of a medicament for treating a cancer, wherein the medicament is manufactured in a liquid pharmaceutical formulation comprising about 0.5 g/L to about 1.5 g/L of the heterodimeric Fc-fused protein contained in one or more vessels, wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization. In some embodiments, the liquid pharmaceutical formulation comprises about 1.0 g/L of the heterodimeric Fc-fused protein.

[0044] In another aspect, provided herein is a use of a heterodimeric Fc-fused protein in the manufacture of a medicament for treating a cancer, wherein the medicament is manufactured in a liquid pharmaceutical formulation comprising about 0.1 mg – about 2 mg of heterodimeric Fc-fused protein contained in one or more vessels, wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the

second Fc region each comprise one or more mutations promoting heterodimerization. In some embodiments, the liquid pharmaceutical formulation comprises 1 mg of heterodimeric Fc-fused protein. In some embodiments, the medicament is contained in one vessel. In some embodiments, wherein each vessel contains 1 mg of heterodimeric Fc-fused protein. In certain embodiments, the medicament is administered to the subject on day 1, every 3 weeks. In some embodiments, the medicament is administered to the subject on day 1, every 4 weeks. In some embodiments, the medicament is administered subcutaneously. In some embodiments, the medicament is administered in a volume of about 0.1 mL to about 1 mL. In certain embodiments, the medicament is administered in a volume of about 1 mL. In some embodiments, the medicament is administered to a maximum of two injection sites. In certain embodiments, a second injection is completed within 10 minutes after a first injection. In some embodiments, the medicament is administered at a dose of about 0.05 mg/kg to about 1.75 mg/kg. In certain embodiments, the medicament is administered at a dose of about 1 mg/kg. In some embodiments, the medicament is diluted prior to administration in a solution of 0.9% saline (sodium chloride for injection) and 0.01% polysorbate 80.

[0045] In another aspect, provided herein is a method of manufacturing a heterodimeric Fc-fused protein for the preparation of a pharmaceutical formulation thereof, the method comprising adding acetic acid to a solution comprising the heterodimeric Fc-fused protein obtained from a Chinese Hamster Ovary (CHO) cell culture expressing the heterodimeric Fc-fused protein for 30 minutes to 90 minutes, wherein the acetate adjusts and maintains the pH of the solution at pH 3.55 to 3.75, and wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization. In certain embodiments, the acetic acid is added to the solution comprising the heterodimeric Fc-fused protein for about 60 minutes. In certain embodiments, the acetic acid adjusts and maintains the pH of the solution to about 3.65. In certain embodiments, the CHO cell culture expressing the heterodimeric Fc-fused protein is maintained in suspension. In certain embodiments, the CHO cell culture expressing the heterodimeric Fc-fused protein is cultured for 7-21 days in a bioreactor. In certain embodiments, the CHO cell culture expressing the heterodimeric Fc-fused protein is cultured for 14 days in a bioreactor. In some

embodiments, the CHO cell culture expressing the heterodimeric Fc-fused protein is harvested by depth filtration to yield a CHO harvest medium. In certain embodiments, the depth filtration is a two-stage single-use depth filtration consisting of DOHC and XOHC filters. In certain embodiments, the heterodimeric Fc-fused protein is purified from the CHO harvest medium using Protein A capture chromatography, mixed mode chromatography, and cation exchange chromatography to yield the solution comprising the heterodimeric Fc-fused protein.

[0046] In some embodiments, the Protein A capture chromatography comprises equilibrating a Protein A resin with 20 mM Tris, 150 mM NaCl at pH 7.5, loading CHO harvest medium onto the Protein A resin; washing the loaded Protein A resin with 20 mM Tris, 150 mM NaCl at pH 7.5; washing the loaded Protein A resin with 50 mM acetate at pH 5.4; and eluting the heterodimeric Fc-fused protein from the Protein A resin with 50 mM acetate, 100 mM arginine at pH 3.7 and collecting by 280 nm UV starting at 1.25 AU/cm ascending and ending at 1.25 AU/cm descending. In certain embodiments, the acetic acid is added at a concentration of 0.5M to the solution comprising the heterodimeric Fc-fused protein eluted from the Protein A resin, wherein the acetic acid acidifies the pH of the solution to pH 3.65 for 60 minutes, followed by neutralization of the solution to pH 5.2 by adding 2M Tris. In certain embodiments, following acidification and neutralization of the solution, the solution comprising the heterodimeric Fc-fused protein is passed through a 0.2 µm filter. In certain embodiments, the filtered solution comprising the heterodimeric Fc-fused protein eluted from the Protein A resin is passed through X0SP depth filtration.

[0047] In some embodiments, mixed mode chromatography comprises equilibrating a mixed mode chromatography column with 50 mM acetate at pH 5.2; loading the solution passed through X0SP filtration onto the mixed mode chromatography column; washing the loaded mixed mode chromatography column with 50 mM acetate at pH 5.2; and eluting the heterodimeric Fc-fused protein from the mixed mode chromatography column with 50 mM Acetate, 250 mM NaCl at pH 5.2 and collecting by 280 nm UV starting at 0.625 AU/cm ascending and ending at 1.50 AU/cm descending. In certain embodiments, the solution comprising the heterodimeric Fc-fused protein eluted from the mixed mode chromatography column is passed through a 0.2 µm filter.

[0048] In some embodiments, cation exchange chromatography comprises equilibrating a cation exchange chromatography resin with 50 mM Tris at pH 7.4; loading the filtered solution eluted from the mixed mode chromatography column onto the cation exchange chromatography resin; washing the loaded cation exchange chromatography resin with 50 mM Tris at pH 7.4; and eluting the heterodimeric Fc-fused protein from the cationic exchange chromatography resin with a gradient of 50 mM Tris at pH 7.4 and 50 mM Tris, 0.5 M NaCl at pH 7.4, and collecting by 280

nm UV starting at 2.5 AU/cm ascending and ending at 4.5 AU/cm descending. In certain embodiments, the solution comprising the heterodimeric Fc-fused protein eluted from the cation exchange chromatography resin is passed through a 0.2 μ m filter. In certain embodiments, the filtered solution comprising the heterodimeric Fc-fused protein eluted from the cation exchange chromatography resin is nanofiltrated through a prefilter, a 20 nm nominal filter, and a 0.2 μ m membrane.

[0049] In some embodiments, the nanofiltrated solution comprising the heterodimeric Fc-fused protein is ultrafiltrated and diafiltrated, wherein ultrafiltration and diafiltration comprises equilibrating an ultrafiltration system with 50 mM Tris, 265 mM NaCl at pH 7.4; concentrating the nanofiltrated solution comprising the heterodimeric Fc-fused protein to a concentration of about 5.0 g/L; exchanging the buffer using 7 diavolumes of 20 mM citrate at pH 6.5; concentration the diafiltrated solution comprising the heterodimeric Fc-fused protein to a concentration of about 11.0 g/L; diluting the concentration solution comprising the heterodimeric Fc-fused protein to a concentration of about 5 g/L to about 10 g/L with 20 mM citrate at pH 6.5; and adding 20 mM citrate, 18% (w/v) sucrose, 3% (w/v) mannitol, 0.03% (w/v) polysorbate-80 at pH 6.5 to achieve a final concentration of the ultrafiltration/diafiltration retentate solution comprising the heterodimeric Fc-fused protein of 20 mM citrate, 6% (w/v) sucrose, 1% (w/v) mannitol, 0.01% (w/v) polysorbate-80. In certain embodiments, the ultrafiltrated/diafiltrated solution comprising the heterodimeric Fc-fused protein is passed through a 0.2 μ m membrane to yield a bulk drug substance.

[0050] In some embodiments, the bulk drug substance is diluted to an 80% drug product solution in a 0.2 μ m filtered buffer comprising 20 mM citrate, 6% (w/v) sucrose, 1% (w/v) mannitol, and 0.01% (w/v) polysorbate-80 at pH 6.5. In certain embodiments, the bulk drug substance or 80% drug product is diluted to a concentration for administration of 1 mg/mL of the heterodimeric Fc-fused protein in a 0.2 μ m filtered buffer comprising 20 mM citrate, 6% (w/v) sucrose, 1% (w/v) mannitol, and 0.01% (w/v) polysorbate-80 at pH 6.5.

[0051] A method of treating cancer in a subject who has received treatment with a checkpoint inhibitor antibody for at least 6 weeks, the method comprising administering a pharmaceutical formulation comprising a heterodimeric Fc-fused protein and a pharmaceutically acceptable carrier to the subject, wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and

wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization. In certain embodiments, the checkpoint inhibitor antibody is an anti-programmed cell death protein 1 (PD-1) antibody. In certain embodiments, the cancer is melanoma. In certain embodiments, the melanoma is unresectable or metastatic. In certain embodiments, the subject is confirmed to have progressive disease at least 4 weeks after the initial diagnosis of progressive disease while receiving the anti-PD-1 antibody. In certain embodiments, the subject is confirmed to have progressive disease at least 4 weeks after the initial diagnosis of progressive disease while receiving the anti-PD-1 antibody. In certain embodiments, the progressive disease is confirmed by radiological or clinical observation.

[0052] In another aspect, provided herein is a method of treating cancer in a subject who has received treatment with a checkpoint inhibitor antibody or an anti-vascular endothelial growth factor therapy as a monotherapy, or in combination, the method comprising administering a pharmaceutical formulation comprising a heterodimeric Fc-fused protein and a pharmaceutically acceptable carrier to the subject, wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization. In certain embodiments, the checkpoint inhibitor antibody is an anti-PD-1 antibody or an anti-PD-L1 antibody.

[0053] In certain embodiments, the cancer is advanced renal cell carcinoma (RCC). In certain embodiments, the RCC is unresectable or metastatic. In certain embodiments, the RCC has a clear cell component. In certain embodiments, the subject received no more than 3 previous lines of therapy. In certain embodiments, the subject has not received treatment with a checkpoint inhibitor. In certain embodiments, the checkpoint inhibitor comprises an anti-PD-1 antibody or anti-PD-L1 antibody as a monotherapy or in combination with a platinum based chemotherapy.

[0054] In another aspect, provided herein is a method of treating cancer in a subject who has received treatment with only one platinum-containing regimen, the method comprising administering a pharmaceutical formulation comprising a heterodimeric Fc-fused protein and a pharmaceutically acceptable carrier to the subject, wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-

12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization. In certain embodiments, the platinum containing regimen is platinum in combination with an agent selected from gemcitabine, methotrexate, vinblastine, and doxorubicin. In certain embodiments, the cancer is locally advanced or metastatic transitional cell urothelial carcinoma. In certain embodiments, the urothelial carcinoma includes one or more of the group consisting of the renal pelvis, ureters, urinary urothelium, and urethra. In certain embodiments, the urothelial carcinoma is inoperable. In certain embodiments, the urothelial carcinoma is characterized with radiographic progression or with recurrence within 6 months after the last administration of a platinum-containing regimen as an adjuvant.

[0055] In some embodiments, the urothelial carcinoma is considered failure of a first-line, platinum-containing regimen. In certain embodiments, the subject has received no more than 2 lines of therapy (including the platinum-containing regimen) for the treatment of the urothelial carcinoma prior to administration of the pharmaceutical formulation. In certain embodiments, the subject has not received treatment with a checkpoint inhibitor (CPI) as a first-line therapy. In certain embodiments, the checkpoint inhibitor is an anti-PD-1 antibody or anti-PD-L1 antibody. In certain embodiments, the checkpoint inhibitor is a monotherapy or in combination with a platinum based chemotherapy.

[0056] In some embodiments, the pharmaceutical formulation is administered in combination with pembrolizumab. In certain embodiments, pembrolizumab is administered once every 3 weeks. In certain embodiments, pembrolizumab is administered before administration of the pharmaceutical formulation. In certain embodiments, the pharmaceutical formulation is administered within one hour after the completion of administration of pembrolizumab. In some embodiments, pembrolizumab is administered at a dose of 200 mg. In some embodiments, pembrolizumab is administered intravenously. In some embodiments, the combination is for treatment of a cancer selected from the group consisting of: melanoma, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), head and neck squamous cell carcinoma (HNSCC), classical Hodgkin lymphoma, primary mediastinal large B-cell lymphoma, urothelial carcinoma, microsatellite instability-high cancer, gastric cancer, oesophageal cancer, cervical cancer, hepatocellular carcinoma, Merkel cell carcinoma, renal cell carcinoma, and endometrial carcinoma. In some embodiments, the combination is for treatment of a cancer selected from the

group consisting of: melanoma, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), head and neck squamous cell carcinoma (HNSCC), classical Hodgkin lymphoma, primary mediastinal large B-cell lymphoma, urothelial carcinoma, microsatellite instability-high cancer, gastric cancer, oesophageal cancer, cervical cancer, ovarian cancer, prostate cancer, hepatocellular carcinoma, Merkel cell carcinoma, renal cell carcinoma, and endometrial carcinoma.

[0057] In some embodiments, the pharmaceutical formulation is administered in combination with nivolumab. In certain embodiments, nivolumab is administered once every 4 weeks. In some embodiments, the nivolumab is administered before administration of the pharmaceutical formulation. In some embodiments, the pharmaceutical formulation is administered within one hour after the completion of administration of nivolumab. In some embodiments, the nivolumab is administered at a dose of about 480 mg. In some embodiments, the nivolumab is administered intravenously. In some embodiments the combination is for treatment of a cancer selected from the group consisting of: melanoma, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), renal cell carcinoma, classical Hodgkin lymphoma, head and neck squamous cell carcinoma (HNSCC), colorectal cancer, hepatocellular carcinoma, bladder cancer, and oesophageal cancer. In some embodiments the combination is for treatment of a cancer selected from the group consisting of: melanoma, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), renal cell carcinoma, classical Hodgkin lymphoma, head and neck squamous cell carcinoma (HNSCC), colorectal cancer, hepatocellular carcinoma, bladder cancer, oesophageal cancer, gastric cancer, ovarian cancer, and prostate cancer. In some embodiments, the cancer is melanoma. In certain embodiments, the melanoma is unresectable or metastatic. In some embodiments the cancer is colorectal cancer. In certain embodiments, the colorectal cancer is microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer.

[0058] In some embodiments, the pharmaceutical formulation is administered to the subject on day 1, every 3 weeks. In some embodiments, the pharmaceutical formulation is administered to the subject on day 1, every 4 weeks. In some embodiments, the pharmaceutical formulation is administered subcutaneously. In some embodiments, the pharmaceutical formulation is administered in a volume of about 0.1 mL to about 1 mL. In some embodiments, the pharmaceutical formulation is administered in a volume of about 1 mL. In some embodiments, the pharmaceutical formulation is administered to a maximum of two injection sites. In certain embodiments, a second injection is completed within 10 minutes after a first injection.

[0059] In some embodiments, the pharmaceutical formulation is administered at a dose of about 0.05 mg/kg to about 1.75 mg/kg. In certain embodiments, the pharmaceutical formulation is administered at a dose of about 1 mg/kg. In some embodiments, the pharmaceutical formulation is diluted prior to administration in a solution of 0.9% saline (sodium chloride for injection) and 0.01% polysorbate 80.

[0060] In some embodiments, the presence of the cancer is determined using the Response Evaluation Criteria for Solid Tumors (RECIST), version 1.1. In some embodiments, a subject who has a confirmed complete response is treated with the pharmaceutical formulation for at least 12 months after confirmation unless a criterion for discontinuation is met.

[0061] In another aspect, provided herein is a method of treating a subject whose blood concentration of C-reactive protein (CRP) is monitored, the method comprising administering to the subject a pharmaceutical formulation comprising a heterodimeric Fc-fused protein and a pharmaceutically acceptable carrier, wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization. In some embodiments, if the CRP concentration in the subject's blood is higher than a threshold CRP concentration, then the subject is identified as being at risk for developing an adverse drug reaction; and if the CRP concentration in the subject's blood is about the same or lower than the threshold C-reactive protein concentration, the subject is not identified as being at risk for developing an adverse drug reaction. In some embodiments, if the CRP concentration in the subject's blood is higher than the threshold CRP concentration, then (1) the administration of the pharmaceutical formulation is paused; (2) the heterodimeric Fc-fused protein is administered at a lower dose; or (3) a remedial action is taken to reduce or alleviate the formulation's toxicity effects in the subject.

[0062] In another aspect, provided herein is a method of treating cancer in a subject in need thereof, the method comprising subcutaneous administration of a pharmaceutical formulation comprising a heterodimeric Fc-fused protein and pharmaceutically acceptable carrier to the subject, wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the

second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization; and the pharmaceutical formulation comprises citrate; a sugar; a sugar alcohol; and a non-ionic surfactant, and the pH of the formulation is between 5.5 and 7.0.

[0063] In some embodiments of the kit, the use, or the method provided herein, the first Fc region and second Fc region of the heterodimeric Fc-fused protein are human IgG1 Fc regions. In some embodiments, the human IgG1 Fc regions comprise one or more mutation(s) that reduce(s) an effector function of an Fc. In some embodiments of the kit, the use, or the method provided herein, the first Fc region and second Fc region comprise one or more mutation(s) selected from L234A, L235A or L235E, G237A, P329A, A330S, and P331S, numbered according to the EU numbering system. In some embodiments, the first Fc region and second Fc region each comprise mutations L234A, L235A, and P329A.

[0064] In certain embodiments of the kit, the use, or the method provided herein, the p40 subunit of IL12 comprises the amino acid sequence of SEQ ID NO: 127 and the p35 subunit of IL-12 comprises the amino acid sequence of SEQ ID NO: 128. In certain embodiments of the kit, the use, or the method provided herein, the p35 subunit of IL-12 is fused to the second Fc region by a linker comprising the amino acid sequence of SEQ ID NO: 108. In some embodiments, the first Fc region comprises mutations L234A, L235A, P329A, Y349C, K360E, and K409W, and the second Fc region comprises mutations L234A, L235A, P329A, Q347R, S354C, D399V, and F405T. In certain embodiments, the first Fc region comprises the amino acid sequence of SEQ ID NO:215, and the second Fc region comprises the amino acid sequence of SEQ ID NO:216. In some embodiments, the first Fc region linked to the p40 subunit of IL12 comprises the amino acid sequence of SEQ ID NO:290 and the second Fc region linked to the p35 subunit of IL-12 comprises the amino acid sequence of SEQ ID NO:291.

[0065] In some embodiments of the kit, the use, or the method provided herein, the pharmaceutical formulation comprises: (a) citrate; (b) a sugar; (c) a sugar alcohol; and (d) a non-ionic surfactant, further wherein the pH of the formulation is between about 6.0 and about 7.0. In some embodiments of the kit, the use, or the method provided herein, the concentration of citrate in the pharmaceutical formulation is about 10 to about 30 mM. In some embodiments, the concentration of citrate in the pharmaceutical formulation is about 20 mM. In some embodiments, the concentration of the sugar in the pharmaceutical formulation is about 3% to about 12% (w/v). In some embodiments, the concentration of the sugar in the pharmaceutical formulation is about

6% (w/v). In some embodiments, the sugar is a disaccharide. In some embodiments, the disaccharide is sucrose. In some embodiments, the concentration of the sugar alcohol in the pharmaceutical formulation is between about 0.5% to about 6% (w/v). In some embodiments, the concentration of the sugar alcohol in the pharmaceutical formulation is about 1% (w/v). In some embodiments, the sugar alcohol is derived from a monosaccharide. In some embodiments, the sugar alcohol is mannitol.

[0066] In some embodiments of the kit, the use, or the method provided herein, the concentration of the non-ionic surfactant in the pharmaceutical formulation is between about 0.005% to about 0.02% (w/v). In some embodiments of the kit, the use, or the method provided herein, the concentration of polysorbate 80 in the pharmaceutical formulation is about 0.01% (w/v). In some embodiments, the non-ionic surfactant is a polysorbate. In some embodiments of the kit, the use, or the method provided herein, the polysorbate is polysorbate 80. In some embodiments, the pH is between about 6.1 and about 6.9. In some embodiments, the pH is between about 6.2 and about 6.8. In some embodiments, the pH is between about 6.3 and about 6.7. In some embodiments, the pH is between about 6.4 and about 6.6. In some embodiments of the kit, the use, or the method provided herein, the pH is about 6.5.

[0067] In some embodiments of the kit, the use, or the method provided herein, the pharmaceutical formulation further comprises water. In some embodiments of the kit, the use, or the method provided herein, the water is Water for Injection, USP.

[0068] In some embodiments of the kit, the use, or the method provided herein, the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 1 g/L to about 10 g/L. In some embodiments, the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 2 g/L to about 8 g/L. In some embodiments, the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 4 g/L to about 6 g/L. In some embodiments, the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 5 g/L. In some embodiments, the pharmaceutical formulation comprises a concentration of the protein for administration of about 0.5 g/L to about 1.5 g/L. In some embodiments, the pharmaceutical formulation comprises a concentration of the protein for administration of about 0.75 g/L to about 1.25 g/L. In some embodiments, the pharmaceutical formulation comprises a concentration of the protein for administration of about 1 g/L.

[0069] In some embodiments of the kit, the use, or the method provided herein, the pharmaceutical formulation is designed to be stored at a temperature between 2°C and 8°C. In

some embodiments, the pharmaceutical formulation is a clear, colorless solution and free of visible particulates.

[0070] In some embodiments of the kit, the use, or the method provided herein, the pharmaceutical formulation has a thermal stability profile as defined by a T_{m1} of greater than about 60°C, greater than about 61°C, greater than about 62°C, greater than about 63°C, greater than about 64°C, greater than about 65°C, or greater than about 66°C; and/or a T_{m2} of greater than about 70°C, greater than about 71°C, greater than about 72°C, greater than about 73°C, greater than about 74°C, greater than about 75°C, greater than about 76°C, or greater than about 77°C, as measured by differential scanning fluorimetry. In some embodiments, the formulation has a thermal stability profile as defined by a T_{m1} of about 67.0°C and a T_{m2} of about 77.3°C.

[0071] In some embodiments of the kit, the use, or the method provided herein, the thermal stability profile of the pharmaceutical formulation, as defined by T_{m1} and/or T_{m2} is changed by less than about 2°C or less than about 1°C when the pharmaceutical formulation is incubated for 1 week at 50°C, as compared to the same pharmaceutical formulation that is incubated for 1 week at 5°C, as measured by differential scanning fluorimetry. In some embodiments, the formulation has a thermal stability profile as defined by a T_{agg} of greater than 60°C, greater than about 61°C, greater than about 62°C, greater than about 63°C, greater than about 64°C, greater than about 65°C, greater than about 66°C, or greater than about 67°C, as measured by differential scanning fluorimetry. In some embodiments, the thermal stability profile of the pharmaceutical formulation, as defined by T_{agg} is changed by less than about 2°C or less than about 1°C when the pharmaceutical formulation is incubated for 1 week at 50°C, as compared to the same pharmaceutical formulation that is incubated for 1 week at 5°C, as measured by differential scanning fluorimetry.

[0072] In some embodiments of the kit, the use, or the method provided herein, the pH of the pharmaceutical formulation does not change by more than about 0.2 or about 0.1 in pH value after the pharmaceutical formulation is incubated for 1 week at 5°C. In some embodiments, the pH of the pharmaceutical formulation does not change by more than about 0.2 or about 0.1 in pH value after the pharmaceutical formulation is incubated for 1 week at 50°C.

[0073] In some embodiments of the kit, the use, or the method provided herein, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less than about 15 nm, less than about 14 nm, less than about 13 nm, or less than about 12 nm, as measured by dynamic light scattering at 25°C. In some embodiments, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of about 11.6 nm.

[0074] In some embodiments of the kit, the use, or the method provided herein, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less than about 20 nm, less than about 19 nm, less than about 18 nm, less than about 17 nm, less than about 16 nm, or less than about 15 nm, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is incubated for 2 weeks at 50°C. In some embodiments, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of about 14.4 nm.

[0075] In some embodiments of the kit, the use, or the method provided herein, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less than about 20 nm, less than about 19 nm, less than about 18 nm, less than about 17 nm, or less than about 16 nm, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is subjected to five freeze thaw cycles. In some embodiments, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of about 15.3 nm.

[0076] In some embodiments of the kit, the use, or the method provided herein, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is less than about 0.30, less than about 0.29, less than about 0.28, or less than about 0.27, as measured by dynamic light scattering at 25°C. In some embodiments, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is about 0.26.

[0077] In some embodiments of the kit, the use, or the method provided herein, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is less than about 0.30, less than about 0.29, less than about 0.28, less than about 0.27, or less than about 0.26, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is incubated for 2 weeks at 50°C. In some embodiments, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is about 0.25.

[0078] In some embodiments of the kit, the use, or the method provided herein, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is less than about 0.40, less than about 0.35, or less than about 0.34, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is subjected to five freeze thaw cycles. In some embodiments, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is about 0.33.

[0079] In some embodiments of the kit, the use, or the method provided herein, the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage

of total detected area in a SEC-HPLC analysis, is greater than about 90%, greater than about 91%, greater than about 92%, greater than about 93%, greater than about 94%, greater than about 95%, greater than about 96%, greater than about 97%, greater than about 98%, or greater than about 99%. In some embodiments, the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is about 99.0%. In some embodiments, the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is greater than about 75%, greater than about 80%, greater than about 81%, greater than about 82%, greater than about 83%, greater than about 84%, or greater than about 85%, after the pharmaceutical formulation is incubated for 2 weeks at 50°C. In some embodiments, the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is about 85.2%.

[0080] In some embodiments of the kit, the use, or the method provided herein, the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is greater than about 90%, greater than about 91%, greater than about 92%, greater than about 93%, greater than about 94%, greater than about 95%, greater than about 96%, greater than about 97%, or greater than about 98%, after the pharmaceutical formulation is subjected to five freeze thaw cycles. In some embodiments, wherein the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is about 98.9%.

[0081] In summary, the present invention provides heterodimeric Fc-fused protein constructs of multisubunit proteins. These fusion protein constructs can exhibit a higher serum half-life compared to a native/natural multisubunit protein, improved yield during production, enhanced stability during storage, and/or improved efficacy when used as a therapeutic.

BRIEF DESCRIPTION OF THE DRAWINGS

[0082] **FIGs. 1A-1D** illustrate various features of an exemplary heterodimeric Fc-fused protein comprising a first subunit of a multisubunit protein connected by a linker to a first antibody Fc domain polypeptide, and a second, different subunit of a multisubunit protein connected by another linker to a second antibody Fc domain polypeptide, in which the subunits are connected by two disulfide bonds. **FIG. 1A** shows a general schematic diagram showing the different components of an exemplary heterodimeric Fc-fused protein. **FIG. 1B** shows an exemplary heterodimeric Fc-fused protein which includes IL12 subunits p40 and p35, linkers, and Fc domains with mutations. **FIG. 1C** shows a schematic diagram illustrating exemplary mutations that can be present in the

heterodimeric Fc-fused protein of **FIG. 1A** or **FIG. 1B**. **FIG. 1D** shows a schematic diagram illustrating exemplary disulfide bonds that can form in the heterodimeric Fc-fused protein of **FIG. 1A**, **FIG. 1B**, or **FIG. 1C**.

[0083] **FIGs. 2A-2C** are graphs showing tumor growth curves of individual mice inoculated with CT26 tumor cells and treated with recombinant mouse IL-12 (rmIL-12) (**FIG. 2A**), DF-mIL-12-Fc wt (**FIG. 2B**), DF-mIL-12-Fc si (**FIG. 2C**), or mIgG2a isotype control once a week.

[0084] **FIG. 3** is a graph showing Kaplan-Meier survival curves of mice inoculated with CT26 tumor cells and treated with rmIL-12, DF-mIL-12-Fc wt, DF-mIL-12-Fc si, or mIgG2a isotype control once a week.

[0085] **FIGs. 4A-4D** are graphs showing tumor growth curves of individual mice inoculated with CT26 tumor cells and treated with DF-mIL-12-Fc wt at a molar equivalent of 1 μ g rmIL-12 (**FIG. 4A**), DF-mIL-12-Fc si at a molar equivalent of 1 μ g rmIL-12 (**FIG. 4B**), DF-mIL-12-Fc wt at a molar equivalent of 0.1 μ g rmIL-12 (**FIG. 4C**), DF-mIL-12-Fc si at a molar equivalent of 0.1 μ g rmIL-12 (**FIG. 4D**), or mIgG2a isotype control once a week.

[0086] **FIG. 5** is a graph showing Kaplan-Meier survival curves of mice inoculated with CT26 tumor cells and treated with DF-mIL-12-Fc wt at a molar equivalent of 1 μ g rmIL-12, DF-mIL-12-Fc si at a molar equivalent of 1 μ g rmIL-12, DF-mIL-12-Fc wt at a molar equivalent of 0.1 μ g rmIL-12, DF-mIL-12-Fc si at a molar equivalent of 0.1 μ g rmIL-12, or mIgG2a isotype control once a week.

[0087] **FIGs. 6A-6C** are graphs showing tumor growth curves of individual mice inoculated with B16F10 melanoma cells and treated with rmIL-12 (**FIG. 6A**), DF-mIL-12-Fc wt (**FIG. 6B**), DF-mIL-12-Fc si (**FIG. 6C**), or mIgG2a isotype control once a week.

[0088] **FIG. 7** is a graph showing Kaplan-Meier survival curves of mice inoculated with B16F10 melanoma cells and treated with rmIL-12, DF-mIL-12-Fc wt, DF-mIL-12-Fc si, or mIgG2a isotype control once a week.

[0089] **FIGs. 8A-8D** are graphs showing tumor growth curves of individual mice inoculated with B16F10 melanoma cells and treated with DF-mIL-12-Fc wt at a molar equivalent of 0.5 μ g rmIL-12 (**FIG. 8A**), DF-mIL-12-Fc si at a molar equivalent of 0.5 μ g rmIL-12 (**FIG. 8B**), DF-mIL-12-Fc wt at a molar equivalent of 0.1 μ g rmIL-12 (**FIG. 8C**), DF-mIL-12-Fc si at a molar equivalent of 0.1 μ g rmIL-12 (**FIG. 8D**), or mIgG2a isotype control once a week.

[0090] **FIG. 9** is a graph showing Kaplan-Meier survival curves of mice inoculated with B16F10 melanoma cells and treated with DF-mIL-12-Fc wt at a molar equivalent of 0.5 μ g rmIL-12, DF-mIL-12-Fc si at a molar equivalent of 0.5 μ g rmIL-12, DF-mIL-12-Fc wt at a molar

equivalent of 0.1 µg rmIL-12, DF-mIL-12-Fc si at a molar equivalent of 0.1 µg rmIL-12, or mIgG2a isotype control once a week.

[0091] **FIG. 10A** is a graph showing IL-12 response to treatment with DF-hIL-12-Fc si (DF IL-12-Fc) or recombinant human IL-12 (rhIL-12) using a HEK-Blue IL-12 reporter assay.

[0092] **FIG. 10B** is a graph showing IFN γ production by peripheral blood mononuclear cells (PBMCs) in response to treatment with DF-hIL-12-Fc si (DF IL-12-Fc) and rhIL-12.

[0093] **FIG. 11** is a graph showing the relative plasma concentrations of DF-hIL-12-Fc si, rhIL-12, and IFN γ in cynomolgus monkey K2 EDTA plasma following a single intravenous dose of equimolar amounts of DF-hIL-12-Fc si or wild type rhIL-12 at 10 µg/kg.

[0094] **FIGs. 12A-12B** are graphs showing the PK/PD profile of rmIL-12 (**FIG. 12A**) and DF-mIL-12-Fc si (**FIG. 12B**) in naïve Balb/c mice. **FIG. 12A** shows the PK/PD profile of rmIL-12 in naïve Balb/c mice and **FIG. 12B** shows the PK/PD profile of DF-mIL-12-Fc si in naïve Balb/c mice. IL-12 and IFN γ levels in serum were analyzed by ELISA. **FIG. 12C** is a graph showing the PK/PD profile of DF-mIL-12-Fc si administered intravenously in naïve Balb/c mice. **FIG. 12D** is a graph showing the PK/PD profile of DF-mIL-12-Fc si administered intraperitoneally in naïve Balb/c mice. **FIG. 12E** is a graph showing the PK/PD profile of DF-mIL-12-Fc si administered subcutaneously in naïve Balb/c mice. Average serum levels represent the mean $\pm$ SEM.

[0095] **FIGs. 13A-13C** are graphs showing tumor growth curves of B16F10 tumor-bearing mice treated with DF-mIL-12-Fc si, anti-PD-1, or a combination thereof. Mice were treated intraperitoneally with 0.5 µg isotype control or 0.5 µg DF-mIL-12-Fc si (**FIG. 13A**), isotype control or anti-PD-1 (**FIG. 13B**), and isotype control or DF-mIL-12-Fc si/anti-PD-1 (**FIG. 13C**). Animals were injected once a week with DF-mIL-12-Fc si and twice weekly with anti-PD-1 as indicated above. Tumor growth was assessed for 60 days. Graphs show tumor growth curves of individual mice.

[0096] **FIGs. 14A-14B** are graphs showing survival and body weights of B16F10 tumor-bearing mice treated DF-mIL-12-Fc si, anti-PD-1, or a combination thereof. Mice were treated with isotype, DF-mIL-12-Fc si, anti-PD-1 or in combination of DF-mIL-12-Fc si and anti-PD-1. Animals were injected once a week with 0.5 µg DF-mIL-12-Fc si and twice weekly with 200 µg anti-PD-1 or isotype. **FIG. 14A** shows Kaplan-Meier survival curves. **FIG. 14B** shows body weights of mice as averages $\pm$ standard deviation.

[0097] **FIGs. 15A-15C** are graphs showing tumor growth curves of B16F10 tumor-bearing mice treated DF-mIL-12-Fc si, mcFAE-C26.99 TriNKETs, or a combination thereof. Mice were treated intraperitoneally with 150 µg isotype control or 0.5 µg DF-mIL-12-Fc si (**FIG. 15A**),

isotype control or 150 µg TriNKET (**FIG. 15B**), and isotype control or DF-mIL-12-Fc si/TriNKET (**FIG. 15C**). Animals were injected once a week with DF-mIL-12-Fc si and thrice weekly with TriNKET as indicated above. Tumor growth was assessed for 72 days. Graphs show tumor growth curves of individual mice.

[0098] **FIGs. 16A-16B** are graphs showing survival and body weights of B16F10 tumor-bearing mice treated with DF-mIL-12-Fc si, mcFAE-C26.99 TriNKETs, or a combination thereof. Mice were treated with isotype, DF-mIL-12-Fc si, TriNKET, or a combination of DF-mIL-12-Fc si and TriNKET. Animals were injected once a week with 0.5 µg DF-mIL-12-Fc si and thrice weekly with 150 µg TriNKET or isotype. **FIG. 16A** shows Kaplan-Meier survival curves. **FIG. 16B** shows body weights of mice as averages + standard deviation.

[0099] **FIG. 17** is a graph showing tumor growth curves of complete responder (CR) mice from the B16F10 tumor model experiment of **FIG. 15** treated with DF-mIL-12-Fc si/TriNKET combination therapy (n=3), re-challenged by engraftment with 2×10^5 B16F10 melanoma cells.

[00100] **FIG. 18A** is a graph showing tumor growth curves of individual mice inoculated with CT26 tumor cells and administered a single dose of DF-mIL-12-Fc si or mIgG2a isotype.

[00101] **FIG. 18B** is a graph showing body weights $\pm$ standard deviation of mice inoculated with CT26 tumor cells and administered a weekly dose of DF-mIL-12-Fc si, mIgG2a isotype, or rmIL-12.

[00102] **FIG. 18C** is a graph showing tumor growth curves of re-challenged individual mice that were either naïve or complete responders (CR) when previously administered a single dose of DF-mIL-12-Fc si in a CT26 tumor model.

[00103] **FIGs. 19A-19B** are graphs showing tumor growth curves of individual mice inoculated with CT26 tumor cells and administered a weekly dose of DF-mIL-12-Fc si or mIgG2a isotype either intraperitoneally (IP)(**FIG. 19A**) or subcutaneously (SC) (**FIG. 19B**).

[00104] **FIG. 20** is a graph showing tumor growth curves of individual mice inoculated with B16F10 melanoma cells and administered a single dose of DF-mIL-12-Fc si or mIgG2a isotype.

[00105] **FIGs. 21A-21B** are graphs showing tumor growth curves of individual mice inoculated with B16F10 melanoma cells and administered a weekly dose of DF-mIL-12-Fc si or mIgG2a isotype either intraperitoneally (IP) (**FIG. 21A**) or subcutaneously (SC) (**FIG. 21B**).

[00106] **FIGs. 22A-22B** are graphs showing tumor growth curves of individual mice inoculated with CT26 tumor cells and administered a single dose (**FIG. 22A**) or once weekly dose (**FIG. 22B**) of DF-mIL-12-Fc si or mIgG2A isotype intraperitoneally at a molar equivalent of 1 µg of rmIL-12.

[00107] **FIGs. 23A-23B** are graphs showing tumor growth curves of individual mice inoculated with CT26 tumor cells and administered a once weekly dose of DF-mIL-12-Fc si subcutaneously. **FIG. 23A** is a graph showing tumor growth curves of individual mice inoculated with CT26-Tyrp1 tumor cells and treated once (weekly) with either 2 µg mIgG2a isotype control or 1 µg DF-mIL-12-Fc si. **FIG. 23B** is a graph showing tumor growth curves of individual mice inoculated with CT26-Tyrp1 tumor cells and treated once (weekly) with either 2 µg mIgG2a isotype control or 2 µg DF-mIL-12-Fc si.

[00108] **FIGs. 24A-24C** are graphs showing IFN γ (**FIG. 24A**), CXCL9 (**FIG. 24B**), and CXCL10 (**FIG. 24C**) levels in blood (left) and tumor (right) samples at 72 hours following a single dose of DF-mIL-12-Fc si in C57BL/6 mice bearing B16F10 tumors.

[00109] **FIGs. 25A-25C** are line graphs showing pharmacokinetics of DF-hIL-12-Fc si in cynomolgus monkeys treated with a single subcutaneous dose of 1 µg/kg (**FIG. 25A**), 2 µg/kg (**FIG. 25B**), or 4 µg/kg (**FIG. 25C**) of DF-hIL-12-Fc si. 2240, 2241, 2740, 2741 (**FIG. 25A**); 3240, 3241, 3740, 3741 (**FIG. 25B**); 4240, 4241, 4740, 4741 (**FIG. 25C**) denote individual cynomolgus monkey subjects.

[00110] **FIGs. 26A-26F** are line graphs showing concentrations of IFN γ and IP10/CXCL10 in cynomolgus monkeys treated with a single subcutaneous dose of DF-hIL-12-Fc si. **FIGs. 26A, 26C and 26E** show IFN γ concentrations/levels of expression in cynomolgus monkeys treated with 1 µg/kg, 2 µg/kg, and 4 µg/kg of DF-hIL-12-Fc si, respectively. **FIGs. 26B, 26D and 26F** show IP10/CXCL10 concentrations/levels of expression in cynomolgus monkeys treated with 1 µg/kg, 2 µg/kg, and 4 µg/kg of DF-hIL-12-Fc si, respectively. 1240, 1740, 2240, 2241, 2740, 2741 (**FIGs. 26A-26B**); 1240, 1740, 3240, 3241, 3740, 3741 (**FIGs. 26C, 26D, 26F**); 1240, 1740, 4240, 4241, 4740, 4741 (**FIG. 26E**) denote individual cynomolgus monkey subjects.

[00111] **FIG. 27** is a graph showing tumor growth curves of individual mice inoculated with breast cancer cells and administered a weekly dose of a monotherapy (isotype control, DF-mIL-12-Fc si, Doxil® (chemotherapy), or irradiated with 10 Gy) or combination therapy (DF-mIL-12-Fc si in combination with Doxil® or radiation).

[00112] **FIG. 28A** is a graph showing tumor growth curves of individual mice inoculated with CT26-Tyrp1 tumor cells and treated (bi-weekly) either with isotype control or anti-PD-1 antibody. **FIG. 28B** is a graph showing tumor growth curve of Balb/c mice inoculated with CT26-Tyrp1 tumor cells and treated (bi-weekly) either with isotype control or anti-PD-1 antibody along with weekly treatment of 1 µg of DF-mIL-12-Fc si.

[00113] **FIG. 29A** is a graph showing tumor growth curves of treated (Tr) tumors in individual mice inoculated with CT26-Tyrp1 tumor cells and intratumorally treated once (weekly) with either isotype control or DF-mIL-12-Fc si. **FIG. 29B** is a graph showing tumor growth curves of non-treated (NT) CT26-Tyrp1 tumors in the individual mice described in **FIG. 29A**.

[00114] **FIG. 30A** is a graph showing tumor growth curves of individual mice inoculated with CT26-Tyrp1 tumor cells and treated once with either 2 μ g mIgG2a isotype control or 2 μ g DF-mIL-12-Fc si. **FIG. 30B** is a graph showing average tumor growth curves of individual mice inoculated with CT26-Tyrp1 tumor cells and treated with 2 μ g mIgG2a isotype control, 1 μ g DF-mIL-12-Fc si (weekly administration), 2 μ g DF-mIL-12-Fc si (weekly administration), or 2 μ g DF-mIL-12-Fc si (once).

[00115] **FIG. 31A** is a graph showing IFN γ production of PHA-stimulated PBMCs treated with DF hIL-12-Fc-si having L234A, L235A, and P329A mutations (LALAPA), or L234A, L235A, and P329G mutations (LALAPG). **FIG. 31B** shows flow cytometry histograms of fluorophore-conjugated hIgG1 binding to THP-1 cells in the presence or absence of DF hIL-12-Fc-si having LALAPA mutations, or LALAPG mutations.

[00116] **FIG. 32** is a process flow diagram showing steps for preparing DF hIL-12-Fc si.

[00117] **FIG. 33** is a process flow diagram showing steps for preparing a pharmaceutical formulation containing DF-hIL-12-Fc si.

[00118] **FIG. 34A** is a graph showing Ultraviolet-visible spectroscopy (UV-Vis) calculated concentrations of DF-hIL-12-Fc si in various pharmaceutical formulations containing different buffers. **FIG. 34B** is a graph showing pH of various pharmaceutical formulations containing DF-hIL-12-Fc si.

[00119] **FIGs. 35A-35B** are photographs of visual appearances of the various formulations of certain pharmaceutical formulations containing DF-hIL-12-Fc si after a 1 week incubation at 5°C (**FIG. 35A**) and 50°C (**FIG. 35B**).

[00120] **FIGs. 36A and 36B** are graphs showing the average T_{m1} (**FIG. 36A**) and T_{m2} (**FIG. 36B**) of DF-hIL-12-Fc si in various pharmaceutical formulations after a 1 week incubation at 5°C.

[00121] **FIGs. 36C and 36D** are graphs showing the average T_{m1} (**FIG. 36C**) and T_{m2} (**FIG. 36D**) of DF-hIL-12-Fc si in various pharmaceutical formulations after a 1 week incubation at 50°C.

[00122] **FIGs. 36E-36H** are graphs showing representative Differential Scanning Fluorimetry (DSF) melting curves of DF-hIL-12-Fc si in various pharmaceutical formulations after a 1 week incubation at 5°C (**FIGs. 36E-36F**) and at 50°C (**FIGs. 36G-36H**).

[00123] **FIGs. 37A-37B** are graphs showing the average T_{agg} of DF-hIL-12-Fc si in various pharmaceutical formulations after a 1 week incubation at at 5°C (**FIG. 37A**) and at 50°C (**FIG. 37B**).

[00124] **FIGs. 37C-37F** are graphs showing representative DSF aggregation curves of DF-hIL-12-Fc si in various pharmaceutical formulations after a 1 week incubation at at 5°C (**FIGs. 37C-37D**) and at 50°C (**FIGs. 37E-37F**).

[00125] **FIGs. 38A-38B** are graphs showing UV-Vis calculated concentrations of DF-hIL-12-Fc si in various pharmaceutical formulations containing different buffers after a 1 week incubation at 5°C (**FIG. 38A**) and at 50°C (**FIG. 38B**).

[00126] **FIGs. 39A-39B** are graphs showing pH of various pharmaceutical formulations containing DF-hIL-12-Fc si after a 1 week incubation at 5°C (**FIG. 39A**) and at 50°C (**FIG. 39B**).

[00127] **FIGs. 40A-40B** are graphs showing Z-average hydrodynamic diameter (**FIG. 40A**) and polydispersity index (**FIG. 40B**) of DF-hIL-12-Fc si in various pharmaceutical formulations containing different buffers after a 1 week incubation at 5°C. **FIGs. 40C-40D** are graphs showing average monomer size and average monomer % Pd of DF-hIL-12-Fc si in various pharmaceutical formulations containing different buffers after a 1 week incubation at 5°C.

[00128] **FIGs. 40E-40F** are graphs showing Z-average hydrodynamic diameter (**FIG. 40E**) and polydispersity index (**FIG. 40F**) of DF-hIL-12-Fc si in various pharmaceutical formulations containing different buffers after a 1 week incubation at 50°C. **FIGs. 40G-40H** are graphs showing average monomer size (**FIG. 40G**) and average monomer % Pd (**FIG. 40H**) of DF-hIL-12-Fc si in various pharmaceutical formulations containing different buffers after a 1 week incubation at 50°C.

[00129] **FIGs. 40I-40J** show representative Dynamic Light Scattering (DLS) traces used to calculate the data described in **FIGs. 40A-40D**. **FIGs. 40K-40L** shows representative DLS traces used to calculate the data described in **FIGs. 40E-40H**.

[00130] **FIG. 41** is a graph showing % purity of DF-hIL-12-Fc si in various pharmaceutical formulations containing different buffers after a 1 week incubation at 50°C, as measured by Size Exclusion Chromatography High-performance Liquid Chromatography (SEC-HPLC).

[00131] **FIG. 42** is a graph showing % purity of DF-hIL-12-Fc si in various pharmaceutical formulations containing different buffers after a 1 week incubation at 50°C, as measured by Capillary Electrophoresis sodium dodecyl sulfate (CE-SDS).

[00132] **FIG. 43A** is a graph showing UV-Vis calculated concentrations of DF hIL-12-Fc-si at 1mg/mL in various pharmaceutical formulations containing different buffers and excipients. Referring to **FIG. 43A**: 1 represents buffer exchange; 2 represents 2-8°C; 3 represents 50°C; and

4 represents freeze/thaw. **FIG. 43B** is a graph showing UV-Vis calculated concentrations of DF hIL-12-Fc-si at 10mg/mL in various pharmaceutical formulations containing different buffers and excipients. Referring to **FIG. 43B**: 1 represents buffer exchange; 2 represents 2-8°C; 3. Represents 50°C; and 4 represents freeze/thaw.

[00133] **FIGs. 44A-44D** are graphs showing the average T_{m1} (**FIGs. 44A** and **44B**) and T_{m2} (**FIGs. 44C** and **44D**) of DF hIL-12-Fc-si at 1 mg/mL and 10 mg/mL in various pharmaceutical formulations.

[00134] **FIGs. 45A-45D** show representative DSF melting curves of DF hIL-12-Fc-si in various pharmaceutical formulations.

[00135] **FIGs. 46A-46B** are graphs showing the average T_{agg} of DF hIL-12-Fc-si at 1mg/mL (**FIG. 46A**) and 10 mg/mL (**FIG. 46B**) in various pharmaceutical formulations.

[00136] **FIGs. 46C-46F** show representative DSF aggregation curves of DF hIL-12-Fc-si at 1mg/mL and 10 mg/mL in various pharmaceutical formulations.

[00137] **FIG. 47A-47F** show representative DLS traces showing size distribution of DF hIL-12-Fc-si at 1mg/mL and 10 mg/mL in various pharmaceutical formulations that were subjected to different stress conditions.

[00138] **FIGs. 48A-48H** show Z-average size (**FIGs. 48A-48B**), polydispersity index (PDI) (**FIGs. 48C-48D**), monomer size (**48E-48F**), and monomer %Pd (**FIGs. 48G-48H**) of DF hIL-12-Fc-si at 1 mg/mL and 10 mg/mL in various pharmaceutical formulations that were subjected to different stress conditions. In **FIGs. 48A-48H**: 1 represents 2-8°C, 2 represents 50°C, and 3 represents freeze/thaw.

[00139] **FIGs. 49A-49D** are graphs showing purity of DF hIL-12-Fc-si at 1mg/mL and 10mg/mL in various pharmaceutical formulations that were subject to different stress conditions. **FIGs. 49A-49B** show % main peak after incubation at 50°C. **FIGs. 49C-49D** show % main peak after incubation at 2-8°C or freeze-thaw cycle. Referring to **FIGs. 49C-49D**: 1 represents 2-8°C, and 2 represents freeze/thaw.

[00140] **FIGs. 50A-50H** are graphs showing particle counts by particle sizes in various pharmaceutical formulations containing DF hIL-12-Fc-si that were subject to different stress conditions, as measured by high accuracy liquid particle (HIAC) analysis. **FIG. 50A-50B** show counts for $\geq 2 \mu\text{m}$ particles, **FIGs. 50C-50D** show counts for $\geq 5 \mu\text{m}$ particles, **FIGs. 50E-50F** show counts for $\geq 10 \mu\text{m}$ particles, and **FIGs. 50G-50H** show counts for $\geq 25 \mu\text{m}$ particles. Referring to **FIGs. 50A-50H**: 1 represents 2-8°C, 2 represents 50°C, and 3 represents freeze/thaw.

[00141] **FIGs. 51A** and **51B** are schematic diagrams showing study designs for phase 1 and phase 2 where DF hIL-12-Fc-si will be used as a monotherapy (**FIG. 51A**) and in a combination therapy with Pembrolizumab (**FIG. 51B**).

[00142] **FIGs. 52A** and **52B** are schematic diagrams showing study designs for phase 1 and phase 2 where DF hIL-12-Fc-si will be used as a monotherapy (**FIG. 52A**) and in a combination therapy with Nivolumab (**FIG. 52B**).

DETAILED DESCRIPTION

[00143] The invention provides improvements on heterodimeric Fc-fused proteins, pharmaceutical formulations comprising such proteins, and therapeutic methods using such proteins and pharmaceutical formulations, including for the treatment of cancer.

[00144] To facilitate an understanding of the present invention, a number of terms and phrases are defined below.

[00145] The terms “a” and “an” as used herein mean “one or more” and include the plural unless the context is inappropriate.

[00146] As used herein, the terms “subject” and “patient” refer to an organism to be treated by the methods and compositions described herein. Such organisms preferably include, but are not limited to, mammals (*e.g.*, murines, simians, equines, bovines, porcines, canines, felines, and the like), and more preferably include humans.

[00147] As used herein, the term “effective amount” refers to the amount of a compound (*e.g.*, a compound of the present invention) sufficient to effect beneficial or desired results (*e.g.*, a desired prophylactic or therapeutic effect). An effective amount can be administered in one or more administration(s), application(s) or dosage(s) and is not intended to be limited to a particular formulation or administration route. As used herein, the term “treating” includes any effect, *e.g.*, lessening, reducing, modulating, ameliorating or eliminating, that results in the improvement of the condition, disease, disorder, and the like, or ameliorating a symptom thereof.

[00148] As used herein, the term “pharmaceutical formulation” refers to the combination of an active agent with a carrier, inert or active, making the composition especially suitable for diagnostic or therapeutic use *in vivo* or *ex vivo*.

[00149] As used herein, the term “pharmaceutically acceptable carrier” refers to any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, emulsions (*e.g.*, such as an oil/water or water/oil emulsions), and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers

and adjuvants, *see e.g.*, Martin, Remington's Pharmaceutical Sciences, 15th Ed., Mack Publ. Co., Easton, PA [1975].

[00150] As used herein, the term “pharmaceutically acceptable salt” refers to any pharmaceutically acceptable salt (*e.g.*, acid or base) of a compound of the present invention which, upon administration to a subject, is capable of providing a compound of this invention or an active metabolite or residue thereof. As is known to those of skill in the art, “salts” of the compounds of the present invention may be derived from inorganic or organic acids and bases. Exemplary acids include, but are not limited to, hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycolic, lactic, salicylic, succinic, toluene-p-sulfonic, tartaric, acetic, citric, methanesulfonic, ethanesulfonic, formic, benzoic, malonic, naphthalene-2-sulfonic, benzenesulfonic acid, and the like. Other acids, such as oxalic, while not in themselves pharmaceutically acceptable, may be employed in the preparation of salts useful as intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable acid addition salts.

[00151] Exemplary bases include, but are not limited to, alkali metal (*e.g.*, sodium) hydroxides, alkaline earth metal (*e.g.*, magnesium) hydroxides, ammonia, and compounds of formula NW_4^+ , wherein W is C_{1-4} alkyl, and the like.

[00152] Exemplary salts include, but are not limited to: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, flucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, palmoate, pectinate, persulfate, phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, undecanoate, and the like. Other examples of salts include anions of the compounds of the present invention compounded with a suitable cation such as Na^+ , NH_4^+ , and NW_4^+ (wherein W is a C_{1-4} alkyl group), and the like.

[00153] For therapeutic use, salts of the compounds of the present invention are contemplated as being pharmaceutically acceptable. However, salts of acids and bases that are non-pharmaceutically acceptable may also find use, for example, in the preparation or purification of a pharmaceutically acceptable compound.

[00154] Throughout the description, where compositions are described as having, including, or comprising specific components, or where processes and methods are described as having, including, or comprising specific steps, it is contemplated that, additionally, there are compositions

of the present invention that consist essentially of, or consist of, the recited components, and that there are processes and methods according to the present invention that consist essentially of, or consist of, the recited processing steps.

[00155] As a general matter, compositions specifying a percentage are by weight unless otherwise specified. Further, if a variable is not accompanied by a definition, then the previous definition of the variable controls.

(a) Proteins

[00156] The present invention provides Fc-fused protein constructs comprising the amino acid sequences of a multisubunit protein. These fusion protein constructs can exhibit a higher serum half-life compared to a native/natural multisubunit protein, improved yield during production, enhanced stability during storage, and/or improved efficacy when used as a therapeutic.

(i) IgG1 Fc-fused proteins

[00157] In one aspect, the present invention provides a heterodimeric IgG1 Fc-fused protein comprising: a first polypeptide comprising a first antibody IgG1 Fc domain polypeptide and a second polypeptide comprising a second antibody IgG1 Fc domain polypeptide bound to the first antibody Fc domain, in which the first polypeptide further comprises a first subunit of a multisubunit protein fused by a linker comprising the amino acid sequence of SEQ ID NO:237 or SEQ ID NO:6 to the first antibody Fc domain polypeptide; a second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide and the subunits of the multisubunit protein are bound to each other; when X₁ of SEQ ID NO: 237 or SEQ ID NO: 6 represents L and/or X₂ of SEQ ID NO: 237 or SEQ ID NO: 6 represents L, at least one of the first antibody Fc domain polypeptide and the second antibody Fc domain polypeptide comprises a Q347R mutation for promoting heterodimerization.

[00158] In some embodiments, within the heterodimeric Fc-fused protein, the linker connecting the a first subunit of a multisubunit protein to the first antibody Fc domain polypeptide consists of the amino acid sequence of SEQ ID NO:237 or SEQ ID NO:6.

[00159] In certain embodiments, the linker connecting the first subunit of a multisubunit protein to the first antibody Fc domain polypeptide further comprises a spacer peptide. In certain embodiments, the linker comprises a sequence of SEQ ID NO:237 or SEQ ID NO:6, and a spacer peptide.

[00160] In certain embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker that comprises a sequence of SEQ ID

NO:237 or SEQ ID NO:6, and a spacer peptide. In certain embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker that consists of the amino acid sequence of SEQ ID NO:237 or SEQ ID NO:6. In certain embodiments, the amino acid sequence of the linker connecting the second, different subunit of the multisubunit protein to the second antibody Fc domain polypeptide is identical to the amino acid sequence of the linker connecting the subunit of the multisubunit protein to the first antibody Fc domain polypeptide.

[00161] Any spacer peptide described under the heading “Spacer peptides” can be employed. For example, in certain embodiments, the spacer peptide comprises the amino acid sequence set forth in any one of SEQ ID NOs:107-120. In certain embodiments, the spacer peptide consists of the amino acid sequence set forth in any one of SEQ ID NOs:107-120. In certain embodiments, the linker connecting the subunit of a multisubunit protein to the first antibody Fc domain polypeptide consists of, or consists essentially of, a spacer peptide disclosed herein and a peptide having the sequence of SEQ ID NO:237 or SEQ ID NO:6. In certain embodiments, the linker connecting the second, different subunit of the multisubunit protein to the second antibody Fc domain polypeptide consists of, or consists essentially of, a spacer peptide disclosed herein and a peptide having the sequence of SEQ ID NO:237 or SEQ ID NO:6. In certain embodiments, the spacer peptide is N-terminal to the either or both of the linkers.

[00162] In some embodiments, within the heterodimeric Fc-fused protein, the linker connecting the first subunit of the multisubunit protein to the first antibody Fc domain polypeptide comprises the amino acid sequence of SEQ ID NO:239 or SEQ ID NO:9. In some embodiments, within the heterodimeric Fc-fused protein, the linker connecting the first subunit of the multisubunit protein to the first antibody Fc domain polypeptide consists of the amino acid sequence of SEQ ID NO:239 or SEQ ID NO:9.

[00163] In some embodiments, within the heterodimeric Fc-fused protein, the linker connecting the first subunit of the multisubunit protein to the first antibody Fc domain polypeptide comprises the amino acid sequence of SEQ ID NO:239. In some embodiments, within the heterodimeric Fc-fused protein, the linker connecting the first subunit of the multisubunit protein to the first antibody Fc domain polypeptide consists of the amino acid sequence SEQ ID NO:239.

[00164] In some embodiments, within the heterodimeric Fc-fused protein, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker comprising the amino acid sequence of SEQ ID NO:10 or SEQ ID NO:244. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second

antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:10 or SEQ ID NO:244.

[00165] In some embodiments, within the heterodimeric Fc-fused protein, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker comprising the amino acid sequence of SEQ ID NO:10. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:10.

[00166] In some embodiments, within the heterodimeric Fc-fused protein, the linker connecting the subunit of the multisubunit protein to the first antibody Fc domain polypeptide comprises the amino acid sequence of SEQ ID NO:238 or SEQ ID NO:7. In some embodiments, within the heterodimeric Fc-fused protein, the linker fusing the first subunit of the multisubunit protein to the first antibody Fc domain polypeptide consists of the amino acid sequence of SEQ ID NO:238 or SEQ ID NO:7.

[00167] In some embodiments, within the heterodimeric Fc-fused protein, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker comprising the amino acid sequence of SEQ ID NO:8 or SEQ ID NO:241. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:8 or SEQ ID NO:241.

[00168] In some embodiments, within the heterodimeric Fc-fused protein, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker comprising the amino acid sequence of SEQ ID NO:15 or SEQ ID NO:242. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:15 or SEQ ID NO:242.

[00169] In some embodiments, within the heterodimeric Fc-fused protein, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker comprising the amino acid sequence of SEQ ID NO:16 or SEQ ID NO:243. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:16 or SEQ ID NO:243.

[00170] In some embodiments, within the heterodimeric Fc-fused protein, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a

linker comprising the amino acid sequence of SEQ ID NO:65 or SEQ ID NO:245. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:65 or SEQ ID NO:245.

[00171] In some embodiments, within the heterodimeric Fc-fused protein, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker comprising the amino acid sequence of SEQ ID NO:66 or SEQ ID NO:246. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:66 or SEQ ID NO:246.

[00172] In some embodiments, within the heterodimeric Fc-fused protein, the linker connecting the first subunit of the multisubunit protein to the first antibody Fc domain polypeptide comprises the amino acid sequence of SEQ ID NO:11 or SEQ ID NO:240. In some embodiments, within the heterodimeric Fc-fused protein, the linker fusing the first subunit of the multisubunit protein to the first antibody Fc domain polypeptide consists of the amino acid sequence of SEQ ID NO:11 or SEQ ID NO:240.

[00173] In some embodiments, within the heterodimeric Fc-fused protein, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker comprising the amino acid sequence of SEQ ID NO:12 or SEQ ID NO:247. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:12 or SEQ ID NO:247.

[00174] In some embodiments, within the heterodimeric Fc-fused protein, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker comprising the amino acid sequence of SEQ ID NO:67 or SEQ ID NO:248. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:67 or SEQ ID NO:248.

[00175] In some embodiments, within the heterodimeric Fc-fused protein, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker comprising the amino acid sequence of SEQ ID NO:68 or SEQ ID NO:249. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second

antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:68 or SEQ ID NO:249.

[00176] In certain embodiments the Fc domain polypeptide is that of an IgG1 Fc. In some embodiments, a protein of the current invention includes, a first antibody Fc domain polypeptide and a second antibody Fc domain polypeptide, which are both mutated IgG1 Fc domain polypeptides that promote heterodimerization with each other. For example, if the Fc domain is derived from the Fc of a human IgG1, the Fc domain can comprise an amino acid sequence at least 90% identical to amino acids 234-332 of a human IgG1 antibody, and differ at one or more position(s) selected from the group consisting of Q347, Y349, L351, S354, E356, E357, K360, Q362, S364, T366, L368, K370, N390, K392, T394, D399, S400, D401, F405, Y407, K409, T411, and K439.

[00177] In some embodiments, the antibody constant domain can comprise an amino acid sequence at least 90% identical to amino acids 234-332 of a human IgG1 antibody, and differ by one or more substitution(s) selected from the group consisting of Q347E, Q347R, Y349S, Y349K, Y349T, Y349D, Y349E, Y349C, L351K, L351D, L351Y, S354C, E356K, E357Q, E357L, E357W, K360E, K360W, Q362E, S364K, S364E, S364H, S364D, T366V, T366I, T366L, T366M, T366K, T366W, T366S, L368E, L368A, L368D, K370S, N390D, N390E, K392L, K392M, K392V, K392F, K392D, K392E, T394F, D399R, D399K, D399V, S400K, S400R, D401K, F405A, F405T, Y407A, Y407I, Y407V, K409F, K409W, K409D, T411D, T411E, K439D, and K439E. All the amino acid positions in an Fc domain or hinge region disclosed herein are numbered according to EU numbering.

[00178] In some embodiments, the first antibody IgG1 Fc domain polypeptide includes one or more mutation(s) selected from K360E and K409W, and the second antibody IgG1 Fc domain polypeptide includes one or more mutation(s) selected from Q347R, D399V, and F405T. In some embodiments, the first antibody IgG1 Fc domain polypeptide includes one or more mutation(s) selected from Q347R, D399V, and F405T, and the second antibody IgG1 Fc domain polypeptide includes one or more mutation(s) selected from K360E and K409W. In some embodiments, the first antibody IgG1 Fc domain polypeptide includes mutations K360E and K409W, and the second antibody IgG1 Fc domain polypeptide includes mutations Q347R, D399V, and F405T. In some embodiments, the first antibody IgG1 Fc domain polypeptide includes mutations Q347R, D399V, and F405T, and the second antibody IgG1 Fc domain polypeptide includes mutations K360E and K409W.

[00179] In some embodiments, a heterodimeric Fc-fused protein of the present invention with an IgG1 Fc includes one or more mutation(s) to reduce binding to an FcγR (*e.g.*, FcγRI, FcγRIIA, FcγRIIB, FcγRIIA, or FcγRIIB) or a complement component (*e.g.*, C1q) in the first and/or second polypeptides. Such mutations are useful for reducing effector functions. For example, a protein of the present disclosure includes L234A and L235A mutations; L234A, L235A, and P329A mutations; L234A, L235A, and P329G mutations; or L234A, L235E, G237A, A330S, and P331S mutations.

[00180] In some embodiments, a heterodimeric Fc-fused protein according to the invention includes a first antibody IgG4 or IgG1 Fc domain polypeptide and the second antibody IgG4 or IgG1 Fc domain polypeptide each containing the mutation P329G or P329A. In specific embodiments, a heterodimeric Fc-fused protein according to the invention comprises a first antibody IgG4 or IgG1 Fc domain polypeptide and a second antibody IgG4 or IgG1 Fc domain polypeptide each comprising the mutation P329A.

[00181] In some embodiments, the first IgG1 antibody Fc domain polypeptide and the second, different IgG1 antibody Fc domain polypeptide each contain a mutation selected from A330S and P331S. In some embodiments, the first IgG1 antibody Fc domain polypeptide and the second, different IgG1 antibody Fc domain polypeptide each contain the mutations A330S and P331S.

[00182] In certain embodiments, an additional disulfide bond between IgG1 Fc monomers is introduced, which improves the stability of the heterodimer. In an exemplary embodiment, the first antibody Fc domain polypeptide fused to the first subunit of a multisubunit protein includes a Y349C substitution in the CH3 domain, which forms a disulfide bond with an S354C substitution on the second antibody Fc domain polypeptide fused to the second, different subunit of a multisubunit protein. Alternatively, the first antibody Fc domain polypeptide fused to the first subunit of a multisubunit protein includes an S354C substitution in the CH3 domain, which forms a disulfide bond with a Y349C substitution on the second antibody Fc domain polypeptide fused to the second, different subunit of a multisubunit protein.

[00183] Any of the IgG1 antibody Fc domain polypeptides provided in Table 2 below can be employed in combination with any of the IgG1 hinge sequences (which, in the current invention, is part or the entirety of a linker connecting the protein sequence of the first subunit of the multisubunit protein to the first IgG1 antibody Fc domain polypeptide, or a linker connecting the additional subunit to the second, different IgG1 antibody Fc domain polypeptide) provided in Table 1 below. Exemplary IgG1 hinge-Fc domain polypeptides are provided in Table 3 below. In certain embodiments, the first and second polypeptides of the Fc-fused protein comprise the amino acid

sequences of SEQ ID NOs: 212 and 212; 213 and 214; 215 and 216; 217 and 218; 214 and 213; 216 and 215; or 218 and 217, respectively. In certain embodiments, the first and second polypeptides of the Fc-fused protein comprise the amino acid sequences of SEQ ID NOs: 228 and 228; 229 and 230; 231 and 232; 233 and 234; 235 and 236; 230 and 229; 232 and 231; 234 and 233; 236 and 235; 228 and 250; 250 and 228; 250 and 250; 229 and 252; 252 and 229; 251 and 230; 230 and 251; 253 and 232; 232 and 253; 231 and 254; 254 and 231; 255 and 234; 234 and 255; 233 and 256; 256 and 233; 257 and 236; 236 and 257; 258 and 235; or 235 and 258, respectively.

(ii) IgG4 Fc-fused proteins

[00184] In one aspect, the current invention provides an improvement on a multisubunit protein. In one aspect the present invention provides a heterodimeric IgG4 Fc-fused protein comprising: a first polypeptide comprising a first antibody IgG4 Fc domain polypeptide and a second polypeptide comprising a second, different antibody IgG4 Fc domain polypeptide bound to the first antibody Fc domain polypeptide, in which the first polypeptide further comprises a first subunit of a multisubunit protein fused by a linker comprising the amino acid sequence of SEQ ID NO:1 to the first antibody IgG4 Fc domain polypeptide; a second, different subunit of the multisubunit protein is fused to the second antibody IgG4 Fc domain polypeptide and the subunits of the multisubunit protein are bound to each other; the first antibody Fc domain polypeptide and the second antibody IgG4 Fc domain polypeptide each contain different mutations promoting heterodimerization.

[00185] In some embodiments, within the heterodimeric IgG4 Fc-fused protein, the linker connecting the a first subunit of a multisubunit protein to the first antibody Fc domain polypeptide consists of the amino acid sequence of SEQ ID NO: 1.

[00186] In certain embodiments, the linker connecting the protein sequence of a first subunit of a multisubunit protein to the first antibody Fc domain polypeptide further comprises a spacer peptide. In certain embodiments, the linker comprises a sequence of SEQ ID NO:1 and a spacer peptide.

[00187] In certain embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker that comprises a sequence of SEQ ID NO: 1, and a spacer peptide. In certain embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker that consists of the amino acid sequence of SEQ ID NO: 1. In certain embodiments, the amino acid sequence of the linker connecting the second, different subunit of the multisubunit protein to the second

antibody Fc domain polypeptide is identical to the amino acid sequence of the linker connecting the subunit of the multisubunit protein to the first antibody Fc domain polypeptide.

[00188] Any spacer peptide described under the heading “Spacer peptides” can be employed. For example, in certain embodiments, the spacer peptide comprises the amino acid sequence set forth in any one of SEQ ID NOs: 107-120. In certain embodiments, the spacer peptide consists of the amino acid sequence set forth in any one of SEQ ID NOs: 107-120. In certain embodiments, the linker connecting the subunit of the multisubunit protein to the first antibody Fc domain polypeptide consists of, or consists essentially of, a spacer peptide disclosed herein and SEQ ID NO:1. In certain embodiments, the linker consists of, or consists essentially of, a spacer peptide disclosed herein and SEQ ID NO:1. In certain embodiments, the spacer peptide is N-terminal to the first linker and/or the second linker.

[00189] In some embodiments, within the heterodimeric Fc-fused protein, the linker connecting the subunit of the multisubunit protein to the first antibody Fc domain polypeptide comprises the amino acid sequence of SEQ ID NO:2. In some embodiments, the linker fusing the first subunit of the multisubunit protein to the first antibody Fc domain polypeptide consists of the amino acid sequence of SEQ ID NO:2.

[00190] In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker comprising the amino acid sequence of SEQ ID NO:3. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:3.

[00191] In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker comprising the amino acid sequence of SEQ ID NO:13. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:13.

[00192] In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker comprising the amino acid sequence of SEQ ID NO:14. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:14.

[00193] In some embodiments, within the heterodimeric Fc-fused protein, the linker connecting the first subunit of the multisubunit protein to the first antibody Fc domain polypeptide comprises

the amino acid sequence of SEQ ID NO:4. In some embodiments, the linker fusing the first subunit of the multisubunit protein to the first antibody Fc domain polypeptide consists of the amino acid sequence of SEQ ID NO:4.

[00194] In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker comprising the amino acid sequence of SEQ ID NO:5. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:5.

[00195] In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker comprising the amino acid sequence of SEQ ID NO:63. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:63.

[00196] In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker comprising the amino acid sequence of SEQ ID NO:64. In some embodiments, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a linker consisting of the amino acid sequence of SEQ ID NO:64.

[00197] In certain embodiments the Fc domain polypeptide is that of an IgG4 Fc. IgG4 is an unstable dimer that can undergo a Fab-arm exchange and pair with other IgG4 antibodies in the body. In certain embodiments, a S228P mutation is introduced within the hinge (which, in the current invention, is part or the entirety of a linker connecting the first subunit of the multisubunit protein to the first IgG4 antibody Fc domain polypeptide, or a linker connecting the additional subunit to the second, different IgG4 antibody Fc domain polypeptide), which increases the stability of the hinge region and reduces the chance for Fab-arm exchange. In certain embodiments, an additional disulfide bond between Fc domain polypeptide monomers is introduced, which improves the stability of the heterodimer. In an exemplary embodiment, the first antibody Fc domain polypeptide linked to the first subunit of the multisubunit protein includes a Y349C substitution in the CH3 domain, which forms a disulfide bond with an S354C substitution on the second antibody Fc domain polypeptide linked to the second, different subunit of the multisubunit protein linked to the second antibody Fc domain polypeptide. Alternatively, the first antibody Fc domain polypeptide linked to the first subunit of the multisubunit protein includes an S354C substitution in the CH3 domain, which forms a disulfide bond with a Y349C substitution on the

second antibody Fc domain polypeptide linked to the second, different subunit of the multisubunit protein.

[00198] In some embodiments, a protein of the current invention includes, a first antibody Fc domain polypeptide and a second antibody Fc domain polypeptide, which are both mutated IgG4 Fc domain polypeptides that promote heterodimerization with each other.

[00199] In some embodiments, the first antibody IgG4 Fc domain polypeptide includes one or more mutation(s) selected from K360E, K370E, and R409W, and the second antibody IgG4 Fc domain polypeptide includes one or more mutation(s) selected from E357N, Q347R, D399V, and F405T. In some embodiments, the first antibody IgG4 Fc domain polypeptide includes mutations K370E and R409W, and the second antibody IgG4 Fc domain polypeptide includes mutations E357N, D399V, and F405T. In some embodiments, the first antibody IgG4 Fc domain polypeptide includes mutations E357N, D399V, and F405T, and the second antibody IgG4 Fc domain polypeptide includes mutations K370E and R409W. In some embodiments, the first antibody IgG4 Fc domain polypeptide includes mutations K360E and R409W, and the second antibody IgG4 Fc domain polypeptide includes mutations Q347R, D399V, and F405T. In some embodiments, the first antibody IgG4 Fc domain polypeptide includes mutations Q347R, D399V, and F405T, and the second antibody IgG4 Fc domain polypeptide includes mutations K360E and R409W.

[00200] In some embodiments, a heterodimeric Fc-fused protein of the present invention with an IgG4 Fc includes one or more mutation(s) to reduce binding to an FcγR (*e.g.*, FcγRI, FcγRIIA, FcγRIIB, FcγRIIA, or FcγRIIB) or a complement component (*e.g.*, C1q) in the first and/or second polypeptide(s). Such mutations are useful for reducing effector functions. For example, a protein of the present disclosure includes S228P and L235E mutations; S228P, L235E, and P329A mutations; or S228P, L235E, and P329G mutations.

[00201] Any of the IgG4 antibody Fc domain polypeptides provided in Table 2 can be employed in combination with any of the IgG4 hinge sequences (which, in the current invention, is part or the entirety of a linker connecting the first subunit of the multisubunit protein to the first IgG4 antibody Fc domain polypeptide, or a linker connecting the second, different subunit of the multisubunit protein to the second, different IgG4 antibody Fc domain polypeptide) provided in Table 1. Exemplary IgG4 hinge-Fc domain polypeptides are provided in Table 3. In certain embodiments, the first and second polypeptides of the Fc-fused protein comprise the amino acid sequences of SEQ ID NOs:205 and 205; 206 and 207; 208 and 209; 210 and 211; 207 and 206; 209 and 208; or 211 and 210, respectively. In certain embodiments, the first and second polypeptides of the Fc-fused protein comprise the amino acid sequences of SEQ ID NOs:219 and

219; 220 and 221; 222 and 223; 224 and 225; 226 and 227; 221 and 220; 223 and 222; 225 and 224; or 227 and 226, respectively.

(b) Disulfide Bonds

[00202] Some heterodimeric Fc-fused proteins of the present invention include the native heterodimer disulfide bond between the first subunit of a multisubunit protein and the second, different subunit of the multisubunit protein. For example, in an exemplary embodiment, a heterodimeric Fc-fused protein according to the invention includes a native heterodimer disulfide bond between p35 and p40 subunits of IL-12. Such a protein includes the native disulfide bond between C74 of p35 and C177 of p40.

[00203] Some heterodimeric Fc-fused proteins of the present invention include an artificial or engineered heterodimer disulfide bond between the first subunit of a multisubunit protein and the second, different subunit of the multisubunit protein. For example, in an exemplary embodiment, a heterodimeric Fc-fused protein according to the invention includes an artificial or engineered heterodimer disulfide bond between p35 and p40 subunits of IL-12. Such a protein includes an artificial or engineered disulfide bond between V185C of p35 and Y292C of p40.

[00204] Some heterodimeric Fc-fused proteins of the present invention include the native heterodimer disulfide bond between the first subunit of a multisubunit protein and the second, different subunit of the multisubunit protein, and an artificial or engineered heterodimer disulfide bond between the first subunit of a multisubunit protein and the second, different subunit of the multisubunit protein. For example, in an exemplary embodiment, a native heterodimer disulfide bond between p35 and p40 subunits of IL-12, and includes an artificial or engineered heterodimer disulfide bond between p35 and p40 subunits of IL-12. Such a protein includes the native disulfide bond between C74 of p35 and C177 of p40, and an artificial or engineered disulfide bond between V185C of p35 and Y292C of p40.

[00205] Some heterodimeric Fc-fused proteins of the present invention are engineered to remove the native disulfide bond, and to replace it with a non-native artificial or engineered disulfide bond. For example, in an exemplary embodiment, a heterodimeric Fc-fused protein according to the invention includes p35 of IL-12 in which the native C74 is mutated to serine, and a p40 of IL-12 in which the native C177 is mutated to serine, thereby removing the native disulfide bond between p35 and p40 subunits of IL-12. To this mutated IL-12, two new mutations are introduced, V185C on p35 and Y292C on p40, thereby introducing a non-native artificial or engineered disulfide bond.

(c) Sequences of components of Fc-fused polypeptides

[00206] Exemplary heterodimeric Fc-fused proteins of the present invention are constructed with any one of the IgG1 or IgG4 Fc variant sequences and any one of the corresponding linker sequences described in the Tables 1-2 below. The fusion protein constructs of the present invention can confer a higher serum half-life compared to a native/natural multisubunit protein, improve yield of the proteins during production, enhance stability during storage, and/or improve efficacy when used as a therapeutic.

[00207] Any of the IgG4 antibody Fc variant domain polypeptides provided in Table 2 below can be employed in combination with any of the IgG4 hinge sequences provided in Table 1 below. Similarly, any of the IgG1 antibody Fc variant domain polypeptides provided in Table 2 below can be employed in combination with any of the IgG1 hinge sequences provided in Table 1 below. Exemplary IgG1 hinge-Fc domain polypeptides are provided in Table 3 below.

Table 1: Linker Variants

Hinge	Amino Acid Sequence
IgG4 hinge consensus	SEQ ID NO:1
IgG4 hinge S228P	SEQ ID NO:2
IgG4 hinge S228P/L235E	SEQ ID NO:4
IgG1 hinge consensus	SEQ ID NO:6
IgG1 hinge C220S	SEQ ID NO:7
IgG1 hinge C220S/L234A/L235A	SEQ ID NO:9
IgG1 hinge C220S/L234A/ L235E/G237A	SEQ ID NO:11
IgG1 hinge ΔE216	SEQ ID NO:237
IgG1 hinge ΔE216/C220S	SEQ ID NO:238
IgG1 hinge ΔE216/C220S/L234A/L235A	SEQ ID NO:239
IgG1 hinge ΔE216/C220S/L234A/L235E/G237A	SEQ ID NO:240

Table 2: IgG4 Fc and IgG1 Fc Wild-type Sequences; and Exemplary IgG4 Antibody Fc Variant and IgG1 Antibody Fc Variant Sequences (amino acid substitutions are indicated in bold and underline)

Fc domain	Amino Acid Sequence*
IgG4 Fc wild-type	SEQ ID NO:205
IgG4 Fc Y349C/K370E/R409W	SEQ ID NO:206
IgG4 Fc S354C/E357N/D399V/F405T	SEQ ID NO:207
IgG4 Fc Y349C/K360E/R409W	SEQ ID NO:208
IgG4 Fc Q347R/S354C/D399V/F405T	SEQ ID NO:209
IgG4 Fc P329A/Y349C/K360E/R409W	SEQ ID NO:210

Fc domain	Amino Acid Sequence*
IgG4 Fc P329A/S354C/Q347R/D399V/F405T	SEQ ID NO:211
IgG1 Fc wild-type	SEQ ID NO:212
IgG1 Fc Y349C/K360E/K409W	SEQ ID NO:213
IgG1 Fc Q347R/S354C/D399V/F405T	SEQ ID NO:214
IgG1 Fc P329A/Y349C/K360E/K409W	PSVFLFPPKPKDTLMISRTPEVTCVVVDVS HEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKAL A APIEKTISKAKGQPREPQVCTLP PSRDELTE N QVSLTCLVKGFYPSDIAVEW ESNGQPENNYKTTTPVLDSDGSFFLYS W L TVDKSRWQQGNVFS S VMHEALHNHYT QKSLSLSPG (SEQ ID NO:215)
IgG1 Fc P329A/Q347R/S354C/D399V/F405T	PSVFLFPPKPKDTLMISRTPEVTCVVVDVS HEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKAL A APIEKTISKAKGQPREP R VYTLP PCRDELTKN Q VSLTCLVKGFYPSDIAVEW ESNGQPENNYKTTTPVL V SDGSFT L YSKL TVDKSRWQQGNVFS S VMHEALHNHYT QKSLSLSPG (SEQ ID NO:216)
IgG1 Fc A330S/P331S/Y349C/K360E/ K409W	SEQ ID NO:217
IgG1 Fc A330S/P331S/Q347R/S354C/ D399V/F405T	SEQ ID NO:218

*The amino acid sequences can further comprise a lysine (K) at the C-terminus.

Table 3: S228P mutated IgG4 Hinge-Fc (wild-type); Exemplary S228P mutated IgG4 Hinge-Fc Variants or Hinge Portion-Fc Variants; C220S mutated IgG1 Hinge-Fc (wild-type); Exemplary C220S mutated IgG1 Hinge-Fc Variants or Hinge Portion-Fc Variants

Linker-Fc	Amino Acid Sequence*
IgG4 hinge-Fc S228P	SEQ ID NO:219
IgG4 hinge-Fc S228P/Y349C/K370E/R409W	SEQ ID NO:220
IgG4 hinge-Fc S228P/S354C/E357N/D399V/ F405T	SEQ ID NO:221
IgG4 hinge-Fc S228P/Y349C/K360E/R409W	SEQ ID NO:222
IgG4 hinge-Fc S228P/Q347R/S354C/D399V/ F405T	SEQ ID NO:223
IgG4 hinge-Fc S228P/L235E/Y349C/K360E/ R409W	SEQ ID NO:224
IgG4 hinge-Fc S228P/L235E/S354C/Q347R/ D399V/ F405T	SEQ ID NO:225
IgG4 hinge-Fc S228P/L235E/P329A/Y349C/ K360E/R409W	SEQ ID NO:226
IgG4 hinge-Fc S228P/L235E/ P329A/S354C/ Q347R/D399V/F405T	SEQ ID NO:227

Linker-Fc	Amino Acid Sequence*
IgG1 hinge-Fc C220S	SEQ ID NO:228
IgG1 hinge-Fc ΔE216/C220S	SEQ ID NO:250
IgG1 hinge-Fc C220S/Y349C/K360E/K409W	SEQ ID NO:229
IgG1 hinge-Fc ΔE216/C220S/Y349C/K360E/ K409W	SEQ ID NO:251
IgG1 hinge-Fc C220S/Q347R/S354C/D399V/ F405T	SEQ ID NO:230
IgG1 hinge-Fc ΔE216/C220S/Q347R/S354C/ D399V/F405T	SEQ ID NO:252
IgG1 hinge-Fc C220S/L234A/L235A/Y349C/ K360E/ K409W	SEQ ID NO:231
IgG1 hinge-Fc ΔE216/C220S/L234A/L235A/ Y349C/K360E/K409W	SEQ ID NO:253
IgG1 hinge-Fc C220S/L234A/L235A/Q347R/ S354C/D399V/F405T	SEQ ID NO:232
IgG1 hinge-Fc ΔE216/C220S/L234A/L235A/ Q347R/S354C/D399V/F405T	SEQ ID NO:254
IgG1 hinge-Fc C220S/L234A/L235A/P329A/ Y349C/K360E/K409W	SEQ ID NO:233
IgG1 hinge-Fc ΔE216/C220S/L234A/L235A/ P329A/Y349C/K360E/K409W	SEQ ID NO:255
IgG1 hinge-Fc C220S/L234A/L235A/P329A/ Q347R/S354C/D399V/F405T	SEQ ID NO:234
IgG1 hinge-Fc ΔE216/C220S/L234A/L235A/ P329A/Q347R/S354C/D399V/F405T	SEQ ID NO:256
IgG1 hinge-Fc C220S/L234A/L235E/G237A/ A330S/P331S/Y349C/K360E/K409W	SEQ ID NO:235
IgG1 hinge-Fc ΔE216/C220S/L234A/L235E/ G237A/A330S/P331S/Y349C/K360E/ K409W	SEQ ID NO:257
IgG1 hinge-Fc C220S/L234A/L235E/G237A/ A330S/P331S/Q347R/S354C/D399V/F405T	SEQ ID NO:236
IgG1 hinge-Fc ΔE216/C220S/L234A/L235E/ G237A/A330S/P331S/Q347R/S354C/ D399V/F405T	SEQ ID NO:258

*The amino acid sequences can further comprise a lysine (K) at the C-terminus.

Table 4: Exemplary Heterodimeric Fc-Fused Polypeptide Constructs

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/ substitutions
Construct 101		
SEQ ID NO:2	SEQ ID NO:129	IgG4 (IL-12 p40); Heterodimerization mutations: K370E/R409W; Substitution for stabilizing disulfide bond: Y349C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/ substitutions
SEQ ID NO:3	SEQ ID NO:130	IgG4 (IL-12 p35); Heterodimerization mutations: E357N/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P
Construct 102		
SEQ ID NO:2	SEQ ID NO:131	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; Substitution for stabilizing disulfide bond: Y349C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P
SEQ ID NO:3	SEQ ID NO:132	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P
Construct 103		
SEQ ID NO:4	SEQ ID NO:133	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; Substitution for stabilizing disulfide bond: Y349C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P and L235E
SEQ ID NO:5	SEQ ID NO:134	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P and L235E
Construct 104		
SEQ ID NO:4	SEQ ID NO:135	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; Substitution for stabilizing disulfide bond: Y349C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P, L235E, P329A
SEQ ID NO:5	SEQ ID NO:136	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P, L235E, P329A
Construct 106		

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/ substitutions
SEQ ID NO:7 OR SEQ ID NO:238	SEQ ID NO:137 OR SEQ ID NO:259	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; C220 in the upper hinge is mutated to S
SEQ ID NO:8	SEQ ID NO:138	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; C220 in the upper hinge is mutated to S
Construct 107		
SEQ ID NO:9 OR SEQ ID NO:239	SEQ ID NO:139 OR SEQ ID NO:260	IgG1FcSilent; (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; Substitutions for reducing FcγR binding: L234A, L235A; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:140	IgG1FcSilent; (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitutions for reducing FcγR binding: L234A, L235A; C220 in the upper hinge is mutated to S
Construct 108		
SEQ ID NO:9 OR SEQ ID NO:239	SEQ ID NO:141 OR SEQ ID NO:261	IgG1FcSilent; (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; Substitutions for reducing FcγR binding: L234A, L235A, P329A; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:142	IgG1FcSilent; (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitutions for reducing FcγR binding: L234A, L235A, P329A; C220 in the upper hinge is mutated to S
Construct 110		
SEQ ID NO:11 OR SEQ ID NO:240	SEQ ID NO:143 OR SEQ ID NO:262	IgG1 Fc; (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C ; Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S
SEQ ID NO:12	SEQ ID NO:144	IgG1 Fc; (IL-12 p35);

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/ substitutions
		Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S
Construct 111		
SEQ ID NO:2	SEQ ID NO:145	IgG4 Fc; (IL-12 p40); Heterodimerization mutations: K370E/R409W; Substitution for stabilizing disulfide bond: Y349C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P
SEQ ID NO:2	SEQ ID NO:146	IgG4 Fc; (IL-12 p35); Heterodimerization mutations: E357N/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P
Construct 112		
SEQ ID NO:2	SEQ ID NO:147	IgG4 Fc; (IL-12 p40); Heterodimerization mutations: K370E/R409W; Substitution for stabilizing disulfide bond: Y349C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P;
SEQ ID NO:13	SEQ ID NO:148	IgG4 Fc; (IL-12 p35); Heterodimerization mutations: E357N/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P;
Construct 113		
SEQ ID NO:2	SEQ ID NO:149	IgG4Fc; (IL-12 p40); Heterodimerization mutations: K370E/R409W; Substitution for stabilizing disulfide bond: Y349C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P
SEQ ID NO:14	SEQ ID NO:150	IgG4 Fc; (IL-12 p35); Heterodimerization mutations: E357N/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P
Construct 114		
SEQ ID NO:7	SEQ ID NO:151	IgG1 Fc;

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/ substitutions
OR SEQ ID NO:238	OR SEQ ID NO:263	(IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; C220 in the upper hinge is mutated to S
SEQ ID NO:7	SEQ ID NO:152	IgG1 Fc; (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; C220 in the upper hinge is mutated to S
Construct 115		
SEQ ID NO:7 OR SEQ ID NO:238	SEQ ID NO:153 OR SEQ ID NO:264	IgG1 Fc; (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; C220 in the upper hinge is mutated to S
SEQ ID NO:15	SEQ ID NO:154	IgG1 Fc; (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; C220 in the upper hinge is mutated to S
Construct 116		
SEQ ID NO:7 OR SEQ ID NO:238	SEQ ID NO:155 OR SEQ ID NO:265	IgG1 Fc; (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; C220 in the upper hinge is mutated to S
SEQ ID NO:16	SEQ ID NO:156	IgG1 Fc; (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; C220 in the upper hinge is mutated to S
Construct 117		
SEQ ID NO:8 OR SEQ ID NO:241	SEQ ID NO:157 OR SEQ ID NO:266	IgG1 Fc; (IL-12 p35); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; C220 in the upper hinge is mutated to S
SEQ ID NO:7	SEQ ID NO:158	IgG1 Fc; (IL-12 p40); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; C220 in the upper hinge is mutated to S
Construct 118		

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/ substitutions
SEQ ID NO:15 OR SEQ ID NO:242	SEQ ID NO:159 OR SEQ ID NO:267	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; C220 in the upper hinge is mutated to S
SEQ ID NO:15	SEQ ID NO:160	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; C220 in the upper hinge is mutated to S
Construct 119		
SEQ ID NO:15 OR SEQ ID NO:242	SEQ ID NO:161 OR SEQ ID NO:268	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted)
SEQ ID NO:15	SEQ ID NO:162	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S V185C; (native disulfide bond between subunits is deleted)
Construct 119-1		
SEQ ID NO:2	SEQ ID NO:163	IgG4 (IL-12 p40); Heterodimerization mutations: K370E/R409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); Substitution for preventing Fab-arm exchange and improving thermostability: S228P
SEQ ID NO:3	SEQ ID NO:164	IgG4 (IL-12 p35); Heterodimerization mutations: E357N/D399V/F405T; p35 substitutions: C74S V185C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P
Construct 119-2		
SEQ ID NO:2	SEQ ID NO:165	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); Substitution for preventing Fab-arm exchange and improving thermostability: S228P
SEQ ID NO:3	SEQ ID NO:166	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S V185C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/substitutions
Construct 119-3		
SEQ ID NO:4	SEQ ID NO:167	IgG4 (IL-12 p40) Heterodimerization mutations: K360E/R409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); Substitution for preventing Fab-arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P and L235E
SEQ ID NO:5	SEQ ID NO:168	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S V185C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P and L235E
Construct 119-4		
SEQ ID NO:4	SEQ ID NO:169	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); Substitution for preventing Fab-arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P, L235E, P329A
SEQ ID NO:5	SEQ ID NO:170	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S V185C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P, L235E, P329A
Construct 119-5		
SEQ ID NO:7 OR SEQ ID NO:238	SEQ ID NO:171 OR SEQ ID NO:269	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); C220 in the upper hinge is mutated to S
SEQ ID NO:8	SEQ ID NO:172	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S V185C; C220 in the upper hinge is mutated to S
Construct 119-6		
SEQ ID NO:9 OR	SEQ ID NO:173 OR SEQ ID NO:270	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitutions: C177S, Y292C;

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/ substitutions
SEQ ID NO:239		(native disulfide bond between subunits is deleted); Substitutions for reducing FcγR binding: L234A, L235A; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:174	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S V185C; Substitutions for reducing FcγR binding: L234A, L235A; C220 in the upper hinge is mutated to S
Construct 119-7		
SEQ ID NO:9 OR SEQ ID NO:239	SEQ ID NO:175 OR SEQ ID NO:271	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); Substitutions for reducing FcγR binding: L234A, L235A, P329A; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:176	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S, V185C; Substitutions for reducing FcγR binding: L234A, L235A, P329A; C220 in the upper hinge is mutated to S
Construct 119-8		
SEQ ID NO:11 OR SEQ ID NO:240	SEQ ID NO:177 OR SEQ ID NO:272	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S; C220 in the upper hinge is mutated to S
SEQ ID NO:12	SEQ ID NO:178	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S, V185C; Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S; C220 in the upper hinge is mutated to S
Construct 120		
SEQ ID NO:2	SEQ ID NO:179	IgG4 (IL-12 p40); Heterodimerization mutations: K370E/R409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-arm exchange and improving thermostability: S228P
SEQ ID NO:3	SEQ ID NO:180	IgG4 (IL-12 p35); Heterodimerization mutations: E357N/D399V/F405T;

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/ substitutions
		p35 substitution: V185C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-arm exchange and improving thermostability: S228P;
Construct 120-1		
SEQ ID NO:2	SEQ ID NO:181	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-arm exchange and improving thermostability: S228P
SEQ ID NO:3	SEQ ID NO:182	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitution: V185C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-arm exchange and improving thermostability: S228P
Construct 120-2		
SEQ ID NO:4	SEQ ID NO:183	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P and L235E
SEQ ID NO:5	SEQ ID NO:184	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitution: V185C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P and L235E
Construct 120-3		
SEQ ID NO:4	SEQ ID NO:185	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P, L235E, P329A
SEQ ID NO:5	SEQ ID NO:186	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitution: V185C (native disulfide bond between subunits is preserved);

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/substitutions
		Substitution for preventing Fab-arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P, L235E, P329A
Construct 120-4		
SEQ ID NO:7 OR SEQ ID NO:238	SEQ ID NO:187 OR SEQ ID NO:273	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); C220 in the upper hinge is mutated to S
SEQ ID NO:8	SEQ ID NO:188	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; p35 substitution: V185C (native disulfide bond between subunits is preserved); C220 in the upper hinge is mutated to S
Construct 120-5		
SEQ ID NO:9 OR SEQ ID NO:239	SEQ ID NO:189 OR SEQ ID NO:274	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitutions for reducing FcγR binding: L234A, L235A; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:190	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitution: V185C (native disulfide bond between subunits is preserved); Substitutions for reducing FcγR binding: L234A, L235A; C220 in the upper hinge is mutated to S
Construct 120-6		
SEQ ID NO:9 OR SEQ ID NO:239	SEQ ID NO:191 OR SEQ ID NO:275	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitutions for reducing FcγR binding: L234A, L235A, P329A; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:192	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitution: V185C (native disulfide bond between subunits is preserved); Substitutions for reducing FcγR binding: L234A, L235A, P329A; C220 in the upper hinge is mutated to S
Construct 120-7		

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/substitutions
SEQ ID NO:11 OR SEQ ID NO:240	SEQ ID NO:193 OR SEQ ID NO:276	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S; C220 in the upper hinge is mutated to S
SEQ ID NO:12	SEQ ID NO:194	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitution: V185C (native disulfide bond between subunits is preserved); Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S; C220 in the upper hinge is mutated to S
Construct 121		
SEQ ID NO:9 OR SEQ ID NO:239	SEQ ID NO:195 OR SEQ ID NO:277	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; Substitutions for reducing FcγR binding: L234A, L235A; Additional mutation in p40: Y292C to introduce IL-12 stabilizing disulfide bond; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:196	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitutions for reducing FcγR binding: L234A, L235A; Additional mutation in p35: V185C to introduce IL-12 stabilizing disulfide bond; C220 in the upper hinge is mutated to S
Construct 122		
SEQ ID NO:9 OR SEQ ID NO:239	SEQ ID NO:197 OR SEQ ID NO:278	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; Substitutions for reducing FcγR binding: L234A, L235A, P329A; Additional mutation Y292C to introduce IL-12 stabilizing disulfide bond; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:198	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitutions for reducing FcγR binding: L234A, L235A, P329A; Additional mutation V185C to introduce IL-12 stabilizing disulfide bond;

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/substitutions
		C220 in the upper hinge is mutated to S
Construct 123		
SEQ ID NO:11 OR SEQ ID NO:240	SEQ ID NO:199 OR SEQ ID NO:279	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S; Additional mutation Y292C to introduce IL-12 stabilizing disulfide bond; C220 in the upper hinge is mutated to S
SEQ ID NO:12	SEQ ID NO:200	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S; Additional mutation V185C to introduce IL-12 stabilizing disulfide bond; C220 in the upper hinge is mutated to S

*The amino acid sequences can further comprise a lysine (K) at the C-terminus.

Table 5: Exemplary Dimeric Fc-fused Proteins

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/substitutions
Construct 1		
SEQ ID NO:2	SEQ ID NO:17	IgG4 (IL-12 p40); Heterodimerization mutations: K370E/R409W; Substitution for stabilizing disulfide bond: Y349C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P
SEQ ID NO:3	SEQ ID NO:18	IgG4 (IL-12 p35); Heterodimerization mutations: E357N/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P
Construct 2		
SEQ ID NO:2	SEQ ID NO:19	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; Substitution for stabilizing disulfide bond: Y349C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P
SEQ ID NO:3	SEQ ID NO:20	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T;

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/ substitutions
		Substitution for stabilizing disulfide bond: S354C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P
Construct 3		
SEQ ID NO:4	SEQ ID NO:21	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; Substitution for stabilizing disulfide bond: Y349C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P and L235E
SEQ ID NO:5	SEQ ID NO:22	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitution for preventing Fab-arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P and L235E
Construct 4		
SEQ ID NO:4	SEQ ID NO:23	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; Substitution for stabilizing disulfide bond: Y349C; Substitution for preventing Fab-Arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P, L235E, P329A
SEQ ID NO:5	SEQ ID NO:24	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitution for preventing Fab-Arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P, L235E, P329A
Construct 5		
SEQ ID NO:4	SEQ ID NO:25	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; Substitution for stabilizing disulfide bond: Y349C; Substitution for preventing Fab-Arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P, L235E, P329G
SEQ ID NO:5	SEQ ID NO:26	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitution for preventing Fab-Arm exchange and improving thermostability: S228P;

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/substitutions
		Substitutions for reducing FcγR binding: S228P, L235E, P329G
Construct 6		
SEQ ID NO:7	SEQ ID NO:27	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; C220 in the upper hinge is mutated to S
SEQ ID NO:8	SEQ ID NO:28	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; C220 in the upper hinge is mutated to S
Construct 7		
SEQ ID NO:9	SEQ ID NO:29	IgG1FcSilent; (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; Substitutions for reducing FcγR binding: L234A, L235A; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:30	IgG1FcSilent; (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitutions for reducing FcγR binding: L234A, L235A; C220 in the upper hinge is mutated to S
Construct 8		
SEQ ID NO:9	SEQ ID NO:31	IgG1FcSilent; (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; Substitutions for reducing FcγR binding: L234A, L235A, P329A; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:32	IgG1FcSilent; (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitutions for reducing FcγR binding: L234A, L235A, P329A; C220 in the upper hinge is mutated to S
Construct 9		
SEQ ID NO:9	SEQ ID NO:33	IgG1FcSilent; (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C;

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/substitutions
		Substitutions for reducing FcγR binding: L234A/L235A/P329G; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:34	IgG1FcSilent; (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitutions for reducing FcγR binding: L234A, L235A, P329G; C220 in the upper hinge is mutated to S
Construct 10		
SEQ ID NO:11	SEQ ID NO:35	IgG1 Fc; (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S
SEQ ID NO:12	SEQ ID NO:36	IgG1 Fc; (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S
Construct 11		
SEQ ID NO:2	SEQ ID NO:37	IgG4 Fc; (IL-12 p40); Heterodimerization mutations: K370E/R409W; Substitution for stabilizing disulfide bond: Y349C; Substitution for preventing Fab-Arm exchange and improving thermostability: S228P
SEQ ID NO:2	SEQ ID NO:38	IgG4 Fc; (IL-12 p35); Heterodimerization mutations: E357N/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitution for preventing Fab-Arm exchange and improving thermostability: S228P
Construct 12		
SEQ ID NO:2	SEQ ID NO:39	IgG4 Fc; (IL-12 p40); Heterodimerization mutations: K370E/R409W; Substitution for stabilizing disulfide bond: Y349C; Substitution for preventing Fab-Arm exchange and improving thermostability: S228P
SEQ ID NO:13	SEQ ID NO:40	IgG4 Fc; (IL-12 p35);

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/ substitutions
		Heterodimerization mutations: E357N/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitution for preventing Fab-Arm exchange and improving thermostability: S228P
Construct 13		
SEQ ID NO:2	SEQ ID NO:41	IgG4 Fc; (IL-12 p40); Heterodimerization mutations: K370E/R409W; Substitution for stabilizing disulfide bond: Y349C; Substitution for preventing Fab-Arm exchange and improving thermostability: S228P
SEQ ID NO:14	SEQ ID NO:42	IgG4 Fc; (IL-12 p35); Heterodimerization mutations: E357N/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitution for preventing Fab-Arm exchange and improving thermostability: S228P
Construct 14		
SEQ ID NO:7	SEQ ID NO:43	IgG1 Fc; (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; C220 in the upper hinge is mutated to S
SEQ ID NO:7	SEQ ID NO:44	IgG1 Fc; (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; C220 in the upper hinge is mutated to S
Construct 15		
SEQ ID NO:7	SEQ ID NO:45	IgG1 Fc; (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; C220 in the upper hinge is mutated to S
SEQ ID NO:15	SEQ ID NO:46	IgG1 Fc; (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; C220 in the upper hinge is mutated to S
Construct 16		
SEQ ID NO:7	SEQ ID NO:47	IgG1 Fc; (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; C220 in the upper hinge is mutated to S

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/ substitutions
SEQ ID NO:16	SEQ ID NO:48	IgG1 Fc; (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; C220 in the upper hinge is mutated to S
Construct 17		
SEQ ID NO:8	SEQ ID NO:49	IgG1 Fc; (IL-12 p35); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; C220 in the upper hinge is mutated to S
SEQ ID NO:7	SEQ ID NO:50	IgG1 Fc; (IL-12 p40); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; C220 in the upper hinge is mutated to S
Construct 18		
SEQ ID NO:15	SEQ ID NO:51	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; C220 in the upper hinge is mutated to S
SEQ ID NO:15	SEQ ID NO:52	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; C220 in the upper hinge is mutated to S
Construct 19		
SEQ ID NO:15	SEQ ID NO:53	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted)
SEQ ID NO:15	SEQ ID NO:54	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S V185C; (native disulfide bond between subunits is deleted)
Construct 19-1		
SEQ ID NO:2	SEQ ID NO:69	IgG4 (IL-12 p40); Heterodimerization mutations: K370E/R409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); Substitution for preventing Fab-Arm exchange and improving thermostability: S228P
SEQ ID NO:3	SEQ ID NO:70	IgG4 (IL-12 p35); Heterodimerization mutations: E357N/D399V/F405T; p35 substitutions: C74S V185C;

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/ substitutions
		Substitution for preventing Fab-Arm exchange and improving thermostability: S228P
Construct 19-2		
SEQ ID NO:2	SEQ ID NO:71	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); Substitution for preventing Fab-Arm exchange and improving thermostability: S228P
SEQ ID NO:3	SEQ ID NO:72	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S V185C; Substitution for preventing Fab-Arm exchange and improving thermostability: S228P
Construct 19-3		
SEQ ID NO:4	SEQ ID NO:73	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); Substitution for preventing Fab-Arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P and L235E
SEQ ID NO:5	SEQ ID NO:74	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S V185C; Substitution for preventing Fab-Arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P and L235E
Construct 19-4		
SEQ ID NO:4	SEQ ID NO:75	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); Substitution for preventing Fab-Arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P, L235E, P329A
SEQ ID NO:5	SEQ ID NO:76	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S V185C; Substitution for preventing Fab-Arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P, L235E, P329A
Construct 19-5		

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/substitutions
SEQ ID NO:4	SEQ ID NO:77	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); Substitution for preventing Fab-Arm exchange and improving thermostability: S228P; Substitutions for reducing Fc γ R binding: S228P, L235E, P329G
SEQ ID NO:5	SEQ ID NO:78	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S V185C; Substitution for preventing Fab-Arm exchange and improving thermostability: S228P; Substitutions for reducing Fc γ R binding: S228P, L235E, P329G
Construct 19-6		
SEQ ID NO:7	SEQ ID NO:79	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); C220 in the upper hinge is mutated to S
SEQ ID NO:8	SEQ ID NO:80	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S V185C; C220 in the upper hinge is mutated to S
Construct 19-7		
SEQ ID NO:9	SEQ ID NO:81	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); Substitutions for reducing Fc γ R binding: L234A, L235A; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:82	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S V185C; Substitutions for reducing Fc γ R binding: L234A, L235A; C220 in the upper hinge is mutated to S
Construct 19-8		
SEQ ID NO:9	SEQ ID NO:83	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); Substitutions for reducing Fc γ R binding: L234A, L235A, P329A; C220 in the upper hinge is mutated to S

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/ substitutions
SEQ ID NO:10	SEQ ID NO:84	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S, V185C; Substitutions for reducing FcγR binding: L234A, L235A, P329A; C220 in the upper hinge is mutated to S
Construct 19-9		
SEQ ID NO:9	SEQ ID NO:85	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); Substitutions for reducing FcγR binding: L234A/L235A/P329G; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:86	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S V185C; Substitutions for reducing FcγR binding: L234A, L235A, P329G; C220 in the upper hinge is mutated to S
Construct 19-10		
SEQ ID NO:11	SEQ ID NO:87	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitutions: C177S, Y292C; (native disulfide bond between subunits is deleted); Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S; C220 in the upper hinge is mutated to S
SEQ ID NO:12	SEQ ID NO:88	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitutions: C74S, V185C; Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S; C220 in the upper hinge is mutated to S
Construct 20		
SEQ ID NO:2	SEQ ID NO:55	IgG4 (IL-12 p40); Heterodimerization mutations: K370E/R409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-Arm exchange and improving thermostability: S228P
SEQ ID NO:3	SEQ ID NO:56	IgG4 (IL-12 p35); Heterodimerization mutations: E357N/D399V/F405T; p35 substitution: V185C (native disulfide bond between subunits is preserved);

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/substitutions
		Substitution for preventing Fab-Arm exchange and improving thermostability: S228P
Construct 20-1		
SEQ ID NO:2	SEQ ID NO:89	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-Arm exchange and improving thermostability: S228P
SEQ ID NO:3	SEQ ID NO:90	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitution: V185C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-Arm exchange and improving thermostability: S228P
Construct 20-2		
SEQ ID NO:4	SEQ ID NO:91	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-Arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P and L235E
SEQ ID NO:5	SEQ ID NO:92	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitution: V185C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-Arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P and L235E
Construct 20-3		
SEQ ID NO:4	SEQ ID NO:93	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-Arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P, L235E, P329A
SEQ ID NO:5	SEQ ID NO:94	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitution: V185C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-Arm exchange and improving thermostability: S228P;

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/substitutions
		Substitutions for reducing FcγR binding: S228P, L235E, P329A
Construct 20-4		
SEQ ID NO:4	SEQ ID NO:95	IgG4 (IL-12 p40); Heterodimerization mutations: K360E/R409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-Arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P, L235E, P329G
SEQ ID NO:5	SEQ ID NO:96	IgG4 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitution: V185C (native disulfide bond between subunits is preserved); Substitution for preventing Fab-Arm exchange and improving thermostability: S228P; Substitutions for reducing FcγR binding: S228P, L235E, P329G
Construct 20-5		
SEQ ID NO:7	SEQ ID NO:97	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); C220 in the upper hinge is mutated to S
SEQ ID NO:8	SEQ ID NO:98	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; p35 substitution: V185C (native disulfide bond between subunits is preserved); C220 in the upper hinge is mutated to S
Construct 20-6		
SEQ ID NO:9	SEQ ID NO:99	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitutions for reducing FcγR binding: L234A, L235A; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:100	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitution: V185C (native disulfide bond between subunits is preserved); Substitutions for reducing FcγR binding: L234A, L235A; C220 in the upper hinge is mutated to S
Construct 20-7		

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/substitutions
SEQ ID NO:9	SEQ ID NO:101	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitutions for reducing FcγR binding: L234A, L235A, P329A; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:102	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitution: V185C (native disulfide bond between subunits is preserved); Substitutions for reducing FcγR binding: L234A, L235A, P329A; C220 in the upper hinge is mutated to S
Construct 20-8		
SEQ ID NO:9	SEQ ID NO:103	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitutions for reducing FcγR binding: L234A, L235A, P329G; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:104	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitution: V185C (native disulfide bond between subunits is preserved); Substitutions for reducing FcγR binding: L234A, L235A, P329G; C220 in the upper hinge is mutated to S
Construct 20-9		
SEQ ID NO:11	SEQ ID NO:105	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; p40 substitution: Y292C (native disulfide bond between subunits is preserved); Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S; C220 in the upper hinge is mutated to S
SEQ ID NO:12	SEQ ID NO:106	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; p35 substitution: V185C (native disulfide bond between subunits is preserved); Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S; C220 in the upper hinge is mutated to S
Construct 21		

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/substitutions
SEQ ID NO:9	SEQ ID NO:57	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; Substitutions for reducing FcγR binding: L234A, L235A; Additional mutation in p40: Y292C to introduce IL-12 stabilizing disulfide bond; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:58	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitutions for reducing FcγR binding: L234A, L235A; Additional mutation in p35: V185C to introduce IL-12 stabilizing disulfide bond; C220 in the upper hinge is mutated to S
Construct 22		
SEQ ID NO:9	SEQ ID NO:59	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; Substitutions for reducing FcγR binding: L234A, L235A, P329A; Additional mutation Y292C to introduce IL-12 stabilizing disulfide bond; C220 in the upper hinge is mutated to S
SEQ ID NO:10	SEQ ID NO:60	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitutions for reducing FcγR binding: L234A, L235A, P329A; Additional mutation V185C to introduce IL-12 stabilizing disulfide bond; C220 in the upper hinge is mutated to S
Construct 23		
SEQ ID NO:11	SEQ ID NO:61	IgG1 (IL-12 p40); Heterodimerization mutations: K360E/K409W; Substitution for stabilizing disulfide bond: Y349C; Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S; Additional mutation Y292C to introduce IL-12 stabilizing disulfide bond; C220 in the upper hinge is mutated to S
SEQ ID NO:12	SEQ ID NO:62	IgG1 (IL-12 p35); Heterodimerization mutations: Q347R/D399V/F405T; Substitution for stabilizing disulfide bond: S354C; Substitutions for reducing FcγR and C1q binding: L234A, L235E, G237A, A330S, P331S;

Linker Sequence	Sequences of Heterodimeric Fc-fused Proteins*	Type of IgG (IL-12 subunit); mutations/substitutions
		Additional mutation V185C to introduce IL-12 stabilizing disulfide bond; C220 in the upper hinge is mutated to S

*The amino acid sequences can further comprise a lysine (K) at the C-terminus.

(d) IL-12 subunits

[00208] IL-12 is a multisubunit protein including a p40 subunit and a p35 subunit. The amino acid sequence of mature wild-type IL-12 p40 is amino acids 23-328 of the GenBank Accession No. NP_002178.2, set forth in SEQ ID NO:127 below. The amino acid sequence of mature wild-type IL-12 p35 is amino acids 57-253 of GenBank Accession No. NP_000873.2, set forth in SEQ ID NO:128 below. The numbering of amino acid residues of p40 and p35 used herein corresponds to the mature wild-type protein sequences. As used herein, an IL-12 p40 subunit comprises an amino acid sequence at least 90% (*e.g.*, at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) identical to SEQ ID NO:127. As used herein, an IL-12 p35 subunit comprises an amino acid sequence at least 90% (*e.g.*, at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) identical to SEQ ID NO:128.

[00209] In certain embodiments of any one of the foregoing aspects, the p40 and p35 subunits of IL-12 comprise the amino acid sequences of SEQ ID NOs: 121 and 122; 127 and 128; 201 and 202; 203 and 204; 123 and 124; or 125 and 126, respectively. In certain embodiments, the first polypeptide comprises the amino acid sequence of a p40 subunit of IL-12, and the second polypeptide comprises the amino acid sequence of a p35 subunit of IL-12. In certain embodiments, the first polypeptide comprises the amino acid sequence of a p35 subunit of IL-12, and the second polypeptide comprises the amino acid sequence of a p40 subunit of IL-12.

[00210] In certain embodiments, the present disclosure includes a heterodimeric Fc-fused protein comprising: a first polypeptide comprising a first antibody Fc domain polypeptide and a second polypeptide comprising a second antibody Fc domain polypeptide, wherein the first polypeptide further comprises a first subunit of IL-12 fused to the first antibody Fc domain polypeptide by a linker; and a second, different subunit of IL-12 is fused to the second antibody Fc domain polypeptide, wherein the first and second, different subunits of IL-12 are bound to each other, wherein the first antibody Fc domain polypeptide and the second antibody Fc domain polypeptide each contain different mutations promoting heterodimerization, wherein the first antibody Fc domain polypeptide and the second antibody Fc domain polypeptide are bound to each other, and

wherein the first subunit of IL-12 is a p40 subunit with a Y292C substitution, and the second, different subunit of IL-12 is a p35 subunit with a V185C substitution. In certain embodiments, the first subunit and second, different subunit of IL-12 comprise the amino acid sequences of SEQ ID NOs: 125 and 126, respectively.

[00211] The first subunit and second, different subunit of IL-12 can be fused to any of the antibody Fc domain polypeptides via any linkers disclosed herein to form Fc-fused proteins having sequences including but not limited to Constructs 120, 120-1, 120-2, 120-3, 120-4, 120-5, 120-6, and 120-7 as described in Table 4 and Constructs 20, 20-1, 20-2, 20-3, 20-4, 20-5, 20-6, 20-7, 20-8, and 20-9 as described in Table 5.

[00212] In certain embodiments, the p40 subunit of IL-12 further comprises a replacement of C177, and the p35 subunit of IL-12 further comprises a replacement of C74. In certain embodiments, C177 in the p40 subunit of IL-12 is replaced by S, and C74 in the p35 subunit of IL-12 is replaced by S. In certain embodiments, the p40 and p35 subunits of IL-12 comprise the amino acid sequences of SEQ ID NOs: 123 and 124, respectively.

[00213] The first subunit and second, different subunit of IL-12 can be fused to any of the antibody Fc domain polypeptides via any linkers disclosed herein to form Fc-fused proteins having sequences including but not limited to Constructs 119, 119-1, 119-2, 119-3, 119-4, 119-5, 119-6, 119-7, and 119-8 as described in Table 4, and Constructs 19, 19-1, 19-2, 19-3, 19-4, 19-5, 19-6, 19-7, 19-8, 19-9, and 19-10 as described in Table 5.

Table 6: Human IL-12 p40 and p35 amino acid sequences

IL-12 p40	IL-12 p35
Human IL-12 p40 wild-type IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEED GITWTLDQSSEVLGSGKTLTIQVKEFGDAGQYTC HKGGEVLSHSLLLHKKEDGIWSTDILKDQKEPK NKTFLRCEAKNYSGRFTCWWLTTISTDLTFSVKS SRGSSDPQGVTCGAATLSAERVVRGDNKEYEYSVE CQEDSACPAAEESLP I EVMVDAVHKLKYENYTSS FFIRDI IKPDPPKNLQLKPLKNSRQVEVSWEYDP TWSTPHSYFSLTFCVQVQGKSKREKKDRVFTDKT SATVICRKNASISVRAQDRYYSSSWSEWASVPCS (SEQ ID NO:127)	Human IL-12 p35 wild-type RNLFPVATPDPGMFPCLHHSQNLLRAVSNNMLQKAR QTLEFYPTCTSEEIDHEDITKDKTSTVEACLPLEL TKNESCLNSRETSFITNGSCLASRKTSFMMALCL SSIYEDLKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEDPFYKTK IKLCILLHAFRIRAVTIDRVMSYLNAS (SEQ ID NO:128)
Human IL-12 p40 C177S Y292C SEQ ID NO:201	Human IL-12 p35 C74S V185C SEQ ID NO:202
Human IL-12 p40 Y292C SEQ ID NO:203	Human IL-12 p35 V185C SEQ ID NO:204
Human IL-12 p40 Q56R SEQ ID NO:121	Human IL-12 p35 E50V SEQ ID NO:122
Human IL-12 p40 Q56R C177S Y292C	Human IL-12 p35 E50V C74S V185C

SEQ ID NO:123	SEQ ID NO:124
Human IL-12 p40 Q56R Y292C	Human IL-12 p35 E50V V185C
SEQ ID NO:125	SEQ ID NO:126

(e) Spacer peptides

[00214] Exemplary spacer peptide sequences are provided in Table 7, and exemplary full length linker sequences are provided in Tables 4 and 5.

[00215] Within the first polypeptide of the present invention, a first subunit of a multisubunit protein is fused via a linker to a first antibody Fc domain polypeptide (*e.g.*, an IgG4 antibody Fc variant sequence or an IgG1 antibody Fc variant sequence, as disclosed in Table 2), in an amino-to-carboxyl direction. And within the second polypeptide of the present invention, a second, different subunit of a multisubunit protein is fused via a linker to a second antibody Fc domain polypeptide (*e.g.*, an IgG4 antibody Fc variant sequence or an IgG1 antibody Fc variant sequence, as disclosed in Table 2), in an amino-to-carboxyl direction.

[00216] In some embodiments, the first subunit of a multisubunit protein of the present invention is fused via a linker to a first antibody Fc domain sequence, wherein the linker comprises or consists of a spacer peptide L₁ and the amino acid sequence of SEQ ID NO:1, 2, 4, 6, 7, 9, 11, 237, 238, 239, or 240. In some embodiments, the second, different subunit of the multisubunit protein is fused to a second antibody Fc domain polypeptide via a linker, wherein the linker comprises or consists of a spacer peptide L₂ and the amino acid sequence of SEQ ID NO:1, 2, 4, 6, 7, 9, 11, 237, 238, 239, or 240.

[00217] In certain embodiments, L₁ and L₂ are peptide linkers, for example, L₁ and/or L₂ include(s) 4-50 amino acid residues. In certain embodiments, L₁ consists of 4-50 amino acid residues. In certain embodiments, L₁ consists of 4-20 amino acid residues. In certain embodiments, L₂ consists of 4-50 amino acid residues. In certain embodiments, L₂ consists of about 4-20 amino acid residues. In certain embodiments, L₁ and L₂ each independently consist of about 4-50 amino acid residues. In certain embodiments, L₁ and L₂ each independently consist of 4-20 amino acid residues.

[00218] In some embodiments, L₁ and L₂ have an optimized length and/or amino acid composition. In some embodiments, L₁ and L₂ are of the same length and have the same amino acid composition. In other embodiments, L₁ and L₂ are different.

[00219] In certain embodiments, L₁ is of equal number of amino acids to L₂; in certain embodiments L₁ is longer (*i.e.*, more in the number of amino acids) than L₂; in certain embodiments L₁ is shorter (*i.e.*, fewer number of amino acids) than L₂.

[00220] In certain embodiments, L₁ and/or L₂ are “short,” *e.g.*, consist of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 amino acid residues. Thus, in certain instances, the spacer peptides consist of about 12 or fewer amino acid residues. In the case of 0 amino acid residues, the spacer peptide is a peptide bond. In certain embodiments, L₁ and/or L₂ are “long,” *e.g.*, consist of 15, 20 or 25 amino acid residues. In some embodiments, the spacer peptides consist of about 3 to about 15, for example 8, 9 or 10 contiguous amino acid residues. Regarding the amino acid composition of L₁ and L₂, peptides are selected with properties that confer flexibility to first and the second polypeptides of the proteins of the present invention, do not interfere with the binding of the first and the second, different subunits to each other, as well as resist cleavage from proteases. For example, glycine and serine residues generally provide protease resistance. The spacer peptides suitable for linking the first subunit of the multisubunit protein to the amino acid sequence of SEQ ID NO:1, 2, 4, 6, 7, 9, 11, 237, 238, 239, or 240, and/or suitable for linking the second, different subunit of the multisubunit protein to the amino acid sequence of SEQ ID NO:1, 2, 4, 6, 7, 9, 11, 237, 238, 239, or 240 may include, as part of a linker, a (GS)_n, (GGS)_n, (GGGS)_n, (GGSG)_n, (GGSGG)_n, and (GGGGS)_n sequence, wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20. In some embodiments, L₁ and/or L₂ independently include a (GGGGS)₄ (SEQ ID NO:107) or (GGGGS)₃ (SEQ ID NO:108) sequence as part of a linker. In other embodiments, L₁ and/or L₂ independently include a peptide sequence, as part of a linker, as set forth in the sequences selected from: SEQ ID NO:111, 112, 113, 114, 115, 116, 117, 118, 119, and 120, as listed in Table 7. In some embodiments, L₁ and/or L₂ are independently SEQ ID NO:108, SEQ ID NO:109, or SEQ ID NO:110.

Table 7: Linkers

Linker	SEQ ID NOs
G/S Linker	SEQ ID NOs: 111, 112, 113, 114, 115, 116, 117, 118, 119, and 120

[00221] In certain embodiments, L₁ includes a sequence, as part of a linker, SEQ ID NO:108, and L₂ includes, as part of a linker, SEQ ID NO:109, or SEQ ID NO:110. In certain embodiments, L₂ includes a sequence, as part of a linker, SEQ ID NO:108, and L₁ includes, as part of a linker, SEQ ID NO:109, or SEQ ID NO:110 sequence. In certain embodiments, L₁, as part of a linker, does not include a sequence as set forth in SEQ ID NO:107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, or 120.

[00222] In certain embodiments, only L₂, as part of a linker, includes a sequence as set forth in SEQ ID NO:107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, or 120. In certain

embodiments, neither L₁ nor L₂, as part of a linker sequence, includes a sequence as set forth in SEQ ID NO:107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, or 120.

[00223] Some heterodimeric Fc-fused proteins of the present invention comprise a first polypeptide comprising a first subunit of a multisubunit protein and a first antibody Fc domain polypeptide, in which a linker comprising SEQ ID NO:118 connects the first subunit of a multisubunit protein to the first antibody Fc domain polypeptide, for example an Fc domain polypeptide of an IgG4 antibody or an Fc domain polypeptide of an IgG1 antibody. Some heterodimeric Fc-fused proteins of the present invention comprise a second polypeptide comprising a second, different subunit of a multisubunit protein and a second antibody Fc domain polypeptide, in which a linker comprising SEQ ID NO:118 connects the second, different subunit of a multisubunit protein to the second antibody Fc domain polypeptide, for example an Fc domain polypeptide of an IgG4 antibody or an Fc domain polypeptide of an IgG1 antibody.

[00224] In certain embodiments, some heterodimeric Fc-fused proteins of the present invention comprise a first polypeptide comprising a first subunit of a multisubunit protein and a first antibody Fc domain polypeptide, in which a linker comprising SEQ ID NO:118; connects the first subunit of a multisubunit protein to the Fc domain polypeptide, and a second polypeptide comprising a second, different subunit of a multisubunit protein and a second antibody Fc domain polypeptide, in which the additional subunit is connected to the second antibody Fc domain polypeptide with a linker that does not comprise SEQ ID NO:118.

[00225] In certain embodiments, some heterodimeric Fc-fused proteins of the present invention comprise a first polypeptide comprising a first subunit of a multisubunit protein and a first antibody Fc domain polypeptide, in which a linker that does not comprise SEQ ID NO:118 connects the first subunit of a multisubunit protein to the Fc domain polypeptide, and a second polypeptide comprising a second, different subunit of a multisubunit protein and a second antibody Fc domain polypeptide, in which the second, different subunit of a multisubunit protein is connected to the second antibody Fc domain polypeptide with a linker comprising SEQ ID NO:118.

[00226] Some heterodimeric Fc-fused proteins of the present invention comprise a first polypeptide comprising a first subunit of a multisubunit protein and a first antibody Fc domain polypeptide, in which a linker comprising SEQ ID NO:109 connects the first subunit of a multisubunit protein to the first antibody Fc domain polypeptide, for example an Fc domain polypeptide of an IgG4 antibody or an Fc domain polypeptide of an IgG1 antibody. Some heterodimeric Fc-fused proteins of the present invention comprise a second polypeptide comprising a second, different subunit of a multisubunit protein and a second antibody Fc domain

polypeptide, in which a linker comprising SEQ ID NO:109 connects the second, different subunit of a multisubunit protein to the second antibody Fc domain polypeptide, for example an Fc domain polypeptide of an IgG4 antibody or an Fc domain polypeptide of an IgG1 antibody.

[00227] Some heterodimeric Fc-fused proteins of the present disclosure include a linker comprising SEQ ID NO:109, which connects a first subunit of a multisubunit protein to a first antibody Fc domain polypeptide, for example an Fc domain polypeptide of an IgG4 antibody or an Fc domain polypeptide of an IgG1 antibody, and connects a second, different subunit of a multisubunit protein to a second antibody Fc domain polypeptide, for example an Fc domain polypeptide of an IgG4 antibody or an Fc domain polypeptide of an IgG1 antibody.

[00228] In certain embodiments, some heterodimeric Fc-fused proteins of the present invention comprise a first polypeptide comprising a first subunit of a multisubunit protein and a first antibody Fc domain polypeptide, in which a linker comprising SEQ ID NO:109 connects the first subunit of a multisubunit protein to the Fc domain polypeptide, and a second polypeptide comprising a second, different subunit of a multisubunit protein and a second antibody Fc domain polypeptide, in which additional subunit of a multisubunit protein is connected to the second antibody Fc domain polypeptide with a linker that does not comprise SEQ ID NO:109.

[00229] In certain embodiments, some heterodimeric Fc-fused proteins of the present invention comprise a first polypeptide comprising a first subunit of a multisubunit protein and a first antibody Fc domain polypeptide, in which a linker that does not comprise SEQ ID NO:109 connects the first subunit of a multisubunit protein to the Fc domain polypeptide, and a second polypeptide comprising a second, different subunit of a multisubunit protein and a second antibody Fc domain polypeptide, in which the second, different subunit of a multisubunit protein is connected to the second antibody Fc domain polypeptide with a linker comprising SEQ ID NO:109.

[00230] Some heterodimeric Fc-fused proteins of the present invention comprise a first polypeptide comprising a first subunit of a multisubunit protein and a first antibody Fc domain polypeptide, in which a linker comprising SEQ ID NO:110 connects the first subunit of a multisubunit protein to the first antibody Fc domain polypeptide, for example an Fc domain polypeptide of an IgG4 antibody or an Fc domain polypeptide of an IgG1 antibody. Some heterodimeric Fc-fused proteins of the present invention comprise a second polypeptide comprising a second, different subunit of a multisubunit protein and a second antibody Fc domain polypeptide, in which a linker comprising SEQ ID NO:110 connects the second, different subunit of a multisubunit protein to the second antibody Fc domain polypeptide, for example an Fc domain polypeptide of an IgG4 antibody or an Fc domain polypeptide of an IgG1 antibody.

[00231] Some heterodimeric Fc-fused proteins of the present disclosure include a linker comprising SEQ ID NO:110, which connects a first subunit of a multisubunit protein to a first antibody Fc domain polypeptide, for example an Fc domain polypeptide of an IgG4 antibody or an Fc domain polypeptide of an IgG1 antibody, and connects a second, different subunit of a multisubunit protein to a second antibody Fc domain polypeptide, for example an Fc domain polypeptide of an IgG4 antibody or an Fc domain polypeptide of an IgG1 antibody.

[00232] In certain embodiments, some heterodimeric Fc-fused proteins of the present invention comprise a first polypeptide comprising a first subunit of a multisubunit protein and a first antibody Fc domain polypeptide, in which a linker comprising SEQ ID NO:110 connects the first subunit of a multisubunit protein to the Fc domain polypeptide, and a second polypeptide comprising a second, different subunit of a multisubunit protein and a second antibody Fc domain polypeptide, in which the second, different subunit of a multisubunit protein is connected to the second antibody Fc domain polypeptide with a linker that does not comprise SEQ ID NO:110.

[00233] In certain embodiments, some heterodimeric Fc-fused proteins of the present invention comprise a first polypeptide comprising a first subunit of a multisubunit protein and a first antibody Fc domain polypeptide, in which a linker that does not comprise SEQ ID NO:110 connects the first subunit of a multisubunit protein to the Fc domain polypeptide, and a second polypeptide comprising a second, different subunit of a multisubunit protein and a second antibody Fc domain polypeptide, in which the second, different subunit of a multisubunit protein is connected to the second antibody Fc domain polypeptide with a linker comprising SEQ ID NO:110.

[00234] In certain embodiments, some heterodimeric Fc-fused proteins of the present invention comprise a first polypeptide comprising a first subunit of a multisubunit protein and a first antibody Fc domain polypeptide, in which a linker comprising SEQ ID NO:110 sequence connects the first subunit of a multisubunit protein to the Fc domain polypeptide, and a second polypeptide comprising a second, different subunit of a multisubunit protein and a second antibody Fc domain polypeptide, in which the second, different subunit of a multisubunit protein is connected to the second antibody Fc domain polypeptide with a linker comprising SEQ ID NO:109 sequence.

[00235] In certain embodiments, some heterodimeric Fc-fused proteins of the present invention comprise a first polypeptide comprising a first subunit of a multisubunit protein and a first antibody Fc domain polypeptide, in which a linker comprising SEQ ID NO:109 sequence connects the first subunit of a multisubunit protein to the Fc domain polypeptide, and a second polypeptide comprising a second, different subunit of a multisubunit protein and a second antibody Fc domain

polypeptide, in which the second, different subunit of a multisubunit protein is connected to the second antibody Fc domain polypeptide with a linker comprising SEQ ID NO:110 sequence.

(f) Fc domain and substitutions for promoting heterodimerization

[00236] The assembly of proteins of the present invention can be accomplished by expressing a first polypeptide comprising a first subunit of a multisubunit protein sequence fused to a first antibody Fc domain polypeptide (*e.g.*, an IgG4 antibody Fc variant sequence or an IgG1 antibody Fc variant sequence, as disclosed in Table 2), and a second polypeptide comprising a second, different subunit of a multisubunit protein sequence fused to a second antibody Fc domain polypeptide (*e.g.*, an IgG4 antibody Fc variant sequence or an IgG1 antibody Fc variant sequence, as disclosed in Table 2) in the same cell, which leads to the assembly of a heterodimeric Fc-fused protein according to the invention. The assembled proteins have heterodimeric Fc domain polypeptides with the first antibody Fc domain polypeptide and the second antibody Fc domain polypeptide bound to each other. Promoting the preferential assembly of heterodimers of the Fc can be accomplished by incorporating different mutations in the CH3 domain of each antibody heavy chain constant region as shown in US13/494870, US16/028850, US11/533709, US12/875015, US13/289934, US14/773418, US12/811207, US13/866756, US14/647480, and US14/830336. For example, mutations can be made in the CH3 domain based on human IgG1 and incorporating distinct pairs of amino acid substitutions within a first antibody Fc domain polypeptide and a second antibody Fc domain polypeptide that allow these two chains to selectively heterodimerize with each other. The positions of amino acid substitutions illustrated below are all numbered according to the EU index as in Kabat.

[00237] In one scenario, an amino acid substitution in the first antibody Fc domain polypeptide replaces the original amino acid with a larger amino acid, selected from arginine (R), phenylalanine (F), tyrosine (Y) or tryptophan (W), and at least one amino acid substitution in the second antibody Fc domain polypeptide replaces the original amino acid(s) with a smaller amino acid(s), chosen from alanine (A), serine (S), threonine (T), or valine (V), such that the larger amino acid substitution (a protuberance) fits into the surface of the smaller amino acid substitutions (a cavity). For example, one antibody Fc domain polypeptide can incorporate a T366W substitution, and the other can incorporate three substitutions including T366S, L368A, and Y407V.

[00238] A first polypeptide comprising a first subunit of a multisubunit protein sequence or a second polypeptide comprising a second, different subunit of a multisubunit protein sequence of the invention can optionally be coupled to an amino acid sequence at least 90% (*e.g.*, at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) identical to an antibody constant region, such as

an IgG constant region including hinge, CH2 and CH3 domains with or without a CH1 domain. In some embodiments, the amino acid sequence of the constant region is at least 90% (*e.g.*, at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) identical to a human antibody constant region, such as a human IgG1 constant region, an IgG2 constant region, an IgG3 constant region, or an IgG4 constant region. In some other embodiments, the amino acid sequence of the constant region is at least 90% (*e.g.*, at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) identical to an antibody constant region from another mammal, such as rabbit, dog, cat, mouse, or horse. One or more mutation(s) can be incorporated into the constant region as compared to the human IgG1 constant region, for example at Q347, Y349, L351, S354, E356, E357, K360, Q362, S364, T366, L368, K370, N390, K392, T394, D399, S400, D401, F405, Y407, K409, T411 and/or K439. Exemplary substitutions include, for example, Q347E, Q347R, Y349S, Y349K, Y349T, Y349D, Y349E, Y349C, T350V, L351K, L351D, L351Y, S354C, E356K, E357Q, E357L, E357W, K360E, K360W, Q362E, S364K, S364E, S364H, S364D, T366V, T366I, T366L, T366M, T366K, T366W, T366S, L368E, L368A, L368D, K370S, N390D, N390E, K392L, K392M, K392V, K392F, K392D, K392E, T394F, T394W, D399R, D399K, D399V, S400K, S400R, D401K, F405A, F405T, Y407A, Y407I, Y407V, K409F, K409W, K409D, T411D, T411E, K439D, and K439E.

[00239] In certain embodiments, mutations that can be incorporated into the CH1 of a human IgG1 constant region may be at amino acids V125, F126, P127, T135, T139, A140, F170, P171, and/or V173. In certain embodiments, mutations that can be incorporated into the C κ of a human IgG1 constant region may be at amino acids E123, F116, S176, V163, S174, and/or T164.

[00240] Amino acid substitutions could be selected from the following sets of substitutions shown in Table 8.

Table 8: Amino Acid Substitutions

	First Polypeptide	Second Polypeptide		First Polypeptide	Second Polypeptide
Set 1	S364E/F405A	Y349K/T394F	Set 9	L368D/K370S	S364K
Set 2	S364H/D401K	Y349T/T411E	Set 10	L368E/K370S	S364K
Set 3	S364H/T394F	Y349T/F405A	Set 11	K360E/Q362E	D401K
Set 4	S364E/T394F	Y349K/F405A	Set 12	L368D/K370S	S364K/E357L
Set 5	S364E/T411E	Y349K/D401K	Set 13	K370S	S364K/E357Q
Set 6	S364D/T394F	Y349K/F405A	Set 14	F405L	K409R
Set 7	S364H/F405A	Y349T/T394F	Set 15	K409R	F405L
Set 8	S364K/E357Q	L368D/K370S			

[00241] Alternatively, amino acid substitutions could be selected from the following sets of substitutions shown in Table 9.

Table 9: Amino Acid Substitutions

	First Polypeptide	Second Polypeptide
Set 1	K409W	D399V/F405T
Set 2	Y349S	E357W
Set 3	K360E	Q347R
Set 4	K360E/K409W	Q347R/D399V/F405T
Set 5	Q347E/K360E/K409W	Q347R/D399V/F405T
Set 6	Y349S/K409W	E357W/D399V/F405T

[00242] Alternatively, amino acid substitutions could be selected from the following set of substitutions shown in Table 10.

Table 10: Amino Acid Substitutions

	First Polypeptide	Second Polypeptide
Set 1	T366K/L351K	L351D/L368E
Set 2	T366K/L351K	L351D/Y349E
Set 3	T366K/L351K	L351D/Y349D
Set 4	T366K/L351K	L351D/Y349E/L368E
Set 5	T366K/L351K	L351D/Y349D/L368E
Set 6	E356K/D399K	K392D/K409D

[00243] Alternatively, at least one amino acid substitution in each polypeptide chain could be selected from Table 11.

Table 11: Amino Acid Substitutions

First Polypeptide	Second Polypeptide
L351Y, D399R, D399K, S400K, S400R, Y407A, Y407I, Y407V	T366V, T366I, T366L, T366M, N390D, N390E, K392L, K392M, K392V, K392F, K392D, K392E, K409F, K409W, T411D and T411E

[00244] Alternatively, at least one amino acid substitutions could be selected from the following set of substitutions in Table 12, where the position(s) indicated in the First Polypeptide column is replaced by any known negatively-charged amino acid, and the position(s) indicated in the Second Polypeptide Column is replaced by any known positively-charged amino acid.

Table 12: Amino Acid Substitutions

First Polypeptide	Second Polypeptide
K392, K370, K409, or K439	D399, E356, or E357

[00245] Alternatively, at least one amino acid substitutions could be selected from the following set of in Table 13, where the position(s) indicated in the First Polypeptide column is replaced by any known positively-charged amino acid, and the position(s) indicated in the Second Polypeptide Column is replaced by any known negatively-charged amino acid.

Table 13: Amino Acid Substitutions

First Polypeptide	Second Polypeptide
D399, E356, or E357	K409, K439, K370, or K392

[00246] Alternatively, amino acid substitutions could be selected from the following set in Table 14.

Table 14: Amino Acid Substitutions

First Polypeptide	Second Polypeptide
T350V, L351Y, F405A, and Y407V	T350V, T366L, K392L, and T394W

[00247] Alternatively, or in addition, the structural stability of a heterodimeric Fc-fused protein according to the invention may be increased by introducing S354C on either of the first or second polypeptide chain, and Y349C on the opposing polypeptide chain, which forms an artificial disulfide bond within the interface of the two polypeptides.

[00248] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at position T366, and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from T366, L368 and Y407.

[00249] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from T366, L368 and Y407, and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at position T366.

[00250] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from E357, K360, Q362, S364, L368, K370, T394, D401, F405, and T411 and

the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from Y349, E357, S364, L368, K370, T394, D401, F405 and T411.

[00251] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from Y349, E357, S364, L368, K370, T394, D401, F405 and T411 and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from E357, K360, Q362, S364, L368, K370, T394, D401, F405, and T411.

[00252] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from L351, D399, S400 and Y407 and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from T366, N390, K392, K409 and T411.

[00253] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from T366, N390, K392, K409 and T411 and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from L351, D399, S400 and Y407.

[00254] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from Q347, Y349, K360, and K409, and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from Q347, E357, D399 and F405.

[00255] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from Q347, E357, D399 and F405, and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from Y349, K360, Q347 and K409.

[00256] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from K370, K392, K409 and K439, and the amino acid sequence of the other

polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from D356, E357 and D399.

[00257] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from D356, E357 and D399, and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from K370, K392, K409 and K439.

[00258] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from L351, E356, T366 and D399, and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from Y349, L351, L368, K392 and K409.

[00259] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from Y349, L351, L368, K392 and K409, and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more position(s) selected from L351, E356, T366 and D399.

[00260] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by an S354C substitution and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by a Y349C substitution.

[00261] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by a Y349C substitution and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by an S354C substitution.

[00262] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by K360E and K409W substitutions and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by O347R, D399V and F405T substitutions.

[00263] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by O347R, D399V

and F405T substitutions and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by K360E and K409W substitutions.

[00264] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by a T366W substitutions and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by T366S, T368A, and Y407V substitutions.

[00265] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by T366S, T368A, and Y407V substitutions and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by a T366W substitution.

[00266] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by T350V, L351Y, F405A, and Y407V substitutions and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by T350V, T366L, K392L, and T394W substitutions.

[00267] In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by T350V, T366L, K392L, and T394W substitutions and the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by T350V, L351Y, F405A, and Y407V substitutions.

[00268] A skilled person in the art would appreciate that during production and/or storage of proteins, N-terminal glutamate (E) or glutamine (Q) can be cyclized to form a lactam (*e.g.*, spontaneously or catalyzed by an enzyme present during production and/or storage). Accordingly, in some embodiments where the N-terminal residue of an amino acid sequence of a polypeptide is E or Q, a corresponding amino acid sequence with the E or Q replaced with pyroglutamate is also contemplated herein.

[00269] A skilled person in the art would also appreciate that during protein production and/or storage, the C-terminal lysine (K) of a protein can be removed (*e.g.*, spontaneously or catalyzed by an enzyme present during production and/or storage). Such removal of K is often observed with proteins that comprise a Fc domain at its C-terminus. Accordingly, in some embodiments where

the C-terminal residue of an amino acid sequence of a polypeptide (*e.g.*, a Fc domain sequence) is K, a corresponding amino acid sequence with the K removed is also contemplated herein.

(g) Mutations for reducing effector functions

[00270] In one aspect, the present invention provides a heterodimeric Fc-fused protein comprising (a) a first polypeptide comprising a first antibody Fc domain polypeptide and a first subunit of a multisubunit protein; and (b) a second polypeptide comprising a second antibody Fc domain polypeptide and a second, different subunit of the multisubunit protein, wherein the first and second antibody Fc domain polypeptides each comprise different mutations promoting heterodimerization, wherein the first and/or second antibody Fc domain polypeptides comprise one or more mutation(s) that reduce(s) an effector function of an Fc, and wherein the first subunit and second, different subunit of the multisubunit protein are bound to each other. In certain embodiments, a heterodimeric Fc-fused protein disclosed herein comprising one or more mutation(s) that reduce(s) an effector function of an Fc has an increased activity to inhibit tumor growth than its counterpart without the Fc mutation(s) that reduce(s) the effector function. The mutations contemplated herein include substitution, insertion, and deletion of amino acid residues. All the amino acid positions in an Fc domain or hinge region disclosed herein are numbered according to EU numbering.

[00271] In certain embodiments, the first and/or second antibody Fc domain polypeptides comprise one or more mutation(s) that reduce(s) the ability of the Fc domain polypeptide to induce antibody-dependent cellular cytotoxicity (ADCC) and/or antibody-dependent cellular phagocytosis (ADCP). ADCC and ADCP are typically mediated by an Fc receptor. For example, in certain embodiments, the first and second antibody Fc domain polypeptides are human IgG (*e.g.*, human IgG1, human IgG2, human IgG3, or human IgG4) antibody sequences. The Fc receptors of human IgG, also called Fc gamma receptors (FcγRs), include but are not limited to activating Fc gamma receptors FcγRI (CD64), FcγRIIA (CD32A), FcγRIIA (CD16 or CD16A), and FcγRIIB (CD16B), and inhibitor Fc gamma receptor FcγRIIB (CD32B). Accordingly, in some embodiments, a heterodimeric Fc-fused protein of the present invention includes one or more mutation(s) to reduce binding to an activating FcγR (*e.g.*, FcγRI, FcγRIIA, FcγRIIA, or FcγRIIB) in the first and/or second polypeptides. In some embodiments, a heterodimeric Fc-fused protein of the present invention includes one or more mutation(s) to increase binding to an inhibitory FcγR (*e.g.*, FcγRIIB) in the first and/or second polypeptides.

[00272] Fc mutations that reduce binding to an activating FcγR and/or increase binding to an inhibitory FcγR are known in the art. For example, within the hinge and Fc regions, CD16 binding

is mediated by the hinge region and the CH2 domain. For example, within human IgG1, the interaction with CD16 is primarily focused on amino acid residues Asp 265 – Glu 269, Asn 297 – Thr 299, Ala 327 – Ile 332, Leu 234 – Ser 239, and carbohydrate residue N-acetyl-D-glucosamine in the CH2 domain (*see*, Sondermann *et al*, Nature, 406 (6793):267-273). Based on the known domains, mutations can be selected to enhance or reduce the binding affinity to CD16, such as by using phage-displayed libraries or yeast surface-displayed cDNA libraries, or can be designed based on the known three-dimensional structure of the interaction.

[00273] As reviewed in Want *et al.*, Protein Cell (2018) 9(1):63-73, the regions including amino acid positions 232-239, 265-270, 296-299, and 325-332 are implicated in activating FcγR binding according to a crystal structure of human IgG1 Fc. Wang *et al.* also discloses that L235E and F234A/L235A mutations of human IgG4, L234A/L235A mutations of human IgG1, and N297 mutations (*e.g.*, N297A, N297Q, N297G, or N297D) of IgG antibodies reduce activating FcγR binding. As disclosed in U.S. Patent No. 8,969,526, mutation at position 329 (*e.g.*, P329A, P329G, or P329R) also reduces activating FcγR binding. Additional amino acid positions and mutations (*e.g.*, E233P mutation) implicated in activating FcγR binding are disclosed in U.S. Patent. No. 7,943,743 and Isaacs *et al.*, J. Immunol. (1998) 161:3862-69.

[00274] Accordingly, in certain embodiments, the first and second antibody Fc domain polypeptides comprise a mutation (*e.g.*, substitution relative to wild-type human IgG1) at one or more of positions selected from 233, 234, 235, 297, and 329. In certain embodiments, the first and second antibody Fc domain polypeptides are human IgG1 antibody Fc domain polypeptides comprising mutation(s) E233P; L234A (human IgG1) or F234A (human IgG4); L235A or L235E; N297A, N297Q, N297G, or N297D; and/or P329A, P329G, or P329R. In certain embodiments, the first and second antibody Fc domain polypeptides are human IgG1 antibody Fc domain polypeptides comprising mutations L234A and L235A. In certain embodiments, the first and second antibody Fc domain polypeptides are human IgG1 antibody Fc domain polypeptides comprising mutations L234A, L235A, and P329A. In certain embodiments, the first and second antibody Fc domain polypeptides are human IgG4 antibody Fc domain polypeptides comprising mutation L235E. In certain embodiments, the first and second antibody Fc domain polypeptides are human IgG1 antibody Fc domain polypeptides comprising mutations L235E and P329A.

[00275] In certain embodiments, the first and/or second antibody Fc domain polypeptides comprise one or more mutation(s) that reduce(s) the ability of the Fc domain polypeptide to induce complement dependent cytotoxicity (CDC). CDC is typically mediated by a complement component (*e.g.*, C1q). Accordingly, in certain embodiments, a heterodimeric Fc-fused protein of

the present invention includes one or more mutation(s) to reduce binding to a complement component (*e.g.*, C1q) in the first and/or second polypeptides.

[00276] Fc mutations that reduce binding to C1q are known in the art. For example, as disclosed in U.S. Patent Nos. 5,648,260 and 5,624,821, the amino acid residues of Fc at positions 234, 235, 236, 237, 297, 318, 320, and 322 are implicated in C1q binding. As disclosed in Tao *et al.*, J. Exp. Med. (1993) 178:661-667 and Brekke *et al.*, Eur. J. Immunol. (1994) 24:2542-47, residue Pro at position 331 is implicated in C1q binding. As disclosed in Idusogie *et al.*, J. Immunol. (2000) 164:4178-84, mutations of Fc at positions 270 (*e.g.*, D270A), 322 (K322A), 329 (*e.g.*, P329A), and 331 (*e.g.*, P331A, P331S, or P331G) reduced C1q binding.

[00277] Accordingly, in certain embodiments, the first and second antibody Fc domain polypeptides comprise a mutation (*e.g.*, substitution relative to wild-type human IgG1) at one or more of positions selected from 234, 235, 236, 237, 270, 297, 318, 320, 322, 329, and 331. In certain embodiments, the first and second antibody Fc domain polypeptides are human IgG1 antibody Fc domain polypeptides comprising mutation(s) G237A, A330S, P331S, and/or P329A. In certain embodiments, the first and second antibody Fc domain polypeptides are human IgG1 antibody Fc domain polypeptides comprising mutations G237A, A330S, and P331S. In certain embodiments, the first and second antibody Fc domain polypeptides are human IgG1 antibody Fc domain polypeptides comprising mutation P329A.

[00278] The mutations that reduce ADCC and/or ADCP and the mutations that reduce CDC can be combined. In certain embodiments, the first and/or second antibody Fc domain polypeptides comprise one or more mutation(s) that reduce(s) the ability of the Fc domain polypeptide to induce ADCC and/or ADCP and further comprise one or more mutation(s) that reduce(s) the ability of the Fc domain polypeptide to induce CDC. In certain embodiments, the first and second antibody Fc domain polypeptides each comprise one or more mutation(s) that reduce(s) the ability of the Fc domain polypeptide to induce ADCC and/or ADCP and further comprise one or more mutation(s) that reduce(s) the ability of the Fc domain polypeptide to induce CDC.

[00279] In some embodiments, a heterodimeric Fc-fused protein of the present invention with an IgG4 Fc includes one or more mutation(s) to reduce binding to an FcγR (*e.g.*, FcγRI, FcγRIIA, FcγRIIB, FcγRIIA, or FcγRIIB) or a complement component (*e.g.*, C1q) in the first and/or second polypeptides. Such mutations are useful for reducing effector functions. For example, a protein of the present disclosure can include S228P and L235E mutations; S228P, L235E, and P329A mutations; or S228P, L235E, and P329G mutations.

[00280] In some embodiments, a heterodimeric Fc-fused protein of the present invention with an IgG1 Fc includes one or more mutation(s) to reduce binding to an FcγR (*e.g.*, FcγRI, FcγRIIA, FcγRIIB, FcγRIIA, or FcγRIIB) or a complement component (*e.g.*, C1q) in the first and/or second polypeptides. Such mutations are useful for reducing effector functions. For example, a protein of the present disclosure can include L234A and L235A mutations; L234A, L235A, and P329A mutations; L234A, L235A, and P329G mutations; or L234A, L235E, G237A, A330S, and P331S mutations.

[00281] In some embodiments, a heterodimeric Fc-fused protein according to the invention includes a first antibody IgG4 or IgG1 Fc domain polypeptide and a second antibody IgG4 or IgG1 Fc domain polypeptide each containing the mutation P329G or P329A.

[00282] In some embodiments, the first antibody Fc domain polypeptide and the second antibody Fc domain polypeptide each contain a mutation selected from A330S and P331S.

[00283] In some embodiments, the first antibody Fc domain polypeptide and the second antibody Fc domain polypeptide each contain the mutations A330S and P331S.

[00284] In certain embodiments, in the first polypeptide of the heterodimeric Fc-fused protein of the present invention, the first subunit of the multisubunit protein is fused to the first antibody Fc domain polypeptide by a first linker. In certain embodiments, in the second polypeptide of the heterodimeric Fc-fused protein of the present invention, the second, different subunit of the multisubunit protein is fused to the second antibody Fc domain polypeptide by a second linker. Amino acid sequences of linkers suitable for such use are described under the headings “IgG4 constructs” and “IgG1 constructs.” Additional linker sequences suitable for use in the first and/or second polypeptides include but are not limited to wild-type IgG (*e.g.*, human IgG1, human IgG2, human IgG3, or human IgG4) hinge sequences and mutant forms thereof. For example, in certain embodiments, the first and second linkers each comprise amino acid sequence ESKYGPPCPPCPAPEFXGG, wherein X is L or E (SEQ ID NO:280) or SKYGPPCPPCPAPEFXGG, wherein X is L or E (SEQ ID NO:281). In certain embodiments, the first and second linkers each comprise amino acid sequence of SEQ ID NO:282 or of SEQ ID NO:283. In certain embodiments, the first and second linkers each comprise amino acid sequence of SEQ ID NO:284 or of SEQ ID NO:285.

(h) Serum Half-life

[00285] Heterodimeric Fc-fused proteins according to the invention have pharmacokinetic properties suitable for therapeutic use. For example, in certain embodiments, a heterodimeric Fc-fused protein according to the invention has a serum half-life of at least about 50 hours. In certain

embodiments, a heterodimeric Fc-fused protein according to the invention has a serum half-life of at least about 100 hours.

[00286] In certain embodiments, 50 hours after intravenous administration to a subject, the serum concentration of the heterodimeric Fc-fused protein according to the invention is at least 10% of the serum concentration of the protein of the present invention 1 hour after the administration in said subject.

[00287] In certain embodiments, a heterodimeric Fc-fused protein according to the invention has a serum half-life that is at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% longer than the multisubunit protein not fused to Fc domain polypeptides. In certain embodiments, a heterodimeric Fc-fused protein comprising a protein sequence of a multisubunit protein according to the present invention has a serum half-life that is at least 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 15-fold, or 20-fold longer than the multisubunit protein not fused to Fc domain polypeptides.

(i) Tumor Retention

[00288] Heterodimeric Fc-fused proteins of the invention can optionally incorporate additional features to enhance retention of the proteins at the tumor site. For example, in certain embodiments of the present invention, the heterodimeric Fc-fused protein further comprises a proteoglycan-binding domain, a collagen-binding domain, and/or a hyaluronic acid-binding domain. In certain embodiments, the heterodimeric Fc-fused protein further comprises a proteoglycan-binding domain that binds one or more proteoglycans (*e.g.*, proteoglycans known in the art, *e.g.*, as disclosed in Lizzo *et al.*, Matrix Bio (2015) 42:11-55; and Nikitovic *et al.*, Frontiers in Endocrinology (2018) 9:69) that are present in a tumor (*e.g.*, on the surface of a tumor cell, in a pericellular matrix in a tumor, or in an extracellular matrix in a tumor). In certain embodiments, the collagen-binding domain binds one or more collagens that are present in a tumor (*e.g.*, on the surface of a tumor cell, in a pericellular matrix in a tumor, or in an extracellular matrix in a tumor). In certain embodiments, the heterodimeric Fc-fused protein further comprises a hyaluronic acid-binding domain that binds to one or more hyaluronic acid that are present in a tumor. Such heterodimeric Fc-fused proteins have enhanced retention in tumors and may be administered to a subject intratumorally at a lower dose and/or frequency.

[00289] In certain embodiments, the proteoglycan-binding domain comprised in the heterodimeric Fc-fused protein binds one or more proteoglycans that are specifically expressed in a tumor (*e.g.*, on the surface of a tumor cell, in a pericellular matrix in a tumor, or in an extracellular matrix in a tumor). In certain embodiments, the collagen-binding domain comprised in the

heterodimeric Fc-fused protein binds one or more collagens that are specifically expressed in a tumor (*e.g.*, on the surface of a tumor cell, in a pericellular matrix in a tumor, or in an extracellular matrix in a tumor). Such heterodimeric Fc-fused proteins may be enriched in tumors after administration (*e.g.*, intravenous, subcutaneous, or pulmonary administration) and have enhanced tumor retention, thereby allowing administration at a lower dose and/or frequency.

[00290] In certain embodiments, the heterodimeric Fc-fused protein of the present invention further comprises a proteoglycan-binding domain that binds one or more proteoglycans selected from syndecan, chondroitin sulfate proteoglycan 4 (CSPG4), betaglycan, phosphacan, glypican, perlecan, agrin, collagen (*e.g.*, collagen IX, XII, XV, or XVIII), hyalactan, aggrecan, versican, neurocan, brevican, and a small leucine-rich proteoglycan (SLRP). Proteoglycans implicated in cancer include but are not limited to collagen, syndecan (*e.g.*, syndecan-1 or syndecan-2), serglycin, CSPG4, betaglycan, glypican (*e.g.*, glypican-1 or glypican-3), perlecan, versican, brevican, and SLPR (*e.g.*, decorin, biglycan, asporin, fibrodulin, and lumican). Accordingly, in certain embodiments, the proteoglycan-binding domain comprised in the heterodimeric Fc-fused protein binds one or more proteoglycans selected from syndecan (*e.g.*, syndecan-1 or syndecan-2), serglycin, CSPG4, betaglycan, glypican (*e.g.*, glypican-1 or glypican-3), perlecan, versican, brevican, and a SLPR. In certain embodiments, the proteoglycan-binding domain comprised in the heterodimeric Fc-fused protein binds one or more SLPRs selected from decorin, biglycan, asporin, fibrodulin, and lumican.

[00291] The proteoglycan-binding domain comprised in the heterodimeric Fc-fused protein can be a protein (*e.g.*, an antibody or an antigen-binding fragment thereof), a peptide (*e.g.*, a portion of a proteoglycan-binding protein or a variant thereof), an aptamer, a small molecule, or a combination thereof. Proteoglycan-binding domains are also known in the art. For example, syndecan-binding domains are disclosed in U.S. Patent Nos. 6,566,489, 8,647,828, and 10,124,038; U.S. Patent Application Publication No. 2009/0297479; and PCT Patent Application Publication No. WO2018199176A1. CSPG4-binding domains are disclosed in U.S. Patent Nos. 9,801,928 and 10,093,745; and U.S. Patent Application Publication Nos. 2016/0032007, 2017/0342151, and 2018/0072811. β -glycan-binding domains are disclosed in U.S. Patent No. 7,455,839. Glypican-binding domains are disclosed in U.S. Patent No. 7,919,086, 7,776,329, 8,680,247, 8,388,937, 9,260,492, 9,394,364, 9,790,267, 9,522,940, and 9,409,994; U.S. Patent Application Publication Nos. 2004/0236080, 2011/0123998, 2018/0244805, 2018/0230230, and 2018/0346592; European Patent No. 2270509; and PCT Patent Application Publication No. WO2017053619A1, WO2018026533A1, WO2018165344A1, and WO2018199318A1. Perlecan-

binding domains are disclosed in U.S. Patent No. 10,166,304. Decorin-binding domains are disclosed in U.S. Patent No. 6,517,838 and PCT Patent Application Publication No. WO2000021989A1, WO2000077041A2, and WO2000078800A2.

[00292] In certain embodiments, the heterodimeric Fc-fused protein of the present invention further comprises a collagen-binding domain. Collagen is a class of proteins having at least 28 different types identified in vertebrates. Each type of collagen has its unique structural characteristics and distribution pattern, as disclosed in Fang *et al.*, Tumor Biol. (2014) 35:2871-82 and Xiong *et al.*, J. Cancer Metasta. Treat. (2016) 2:357-64. Various types of collagens are implicated in cancer, including but not limited to Col3A1, Col5A2, Col6, Col7A1, Col15A1, Col19A1, and Col22A1. The collagen-binding domain can be a protein (*e.g.*, an antibody or an antigen-binding fragment thereof), a peptide (*e.g.*, a portion of a collagen-binding protein or a variant thereof), an aptamer, a small molecule, or a combination thereof. Collagen-binding domains are known in the art, and are disclosed in, for example, U.S. Patent Nos. 5,788,966, 5,587,360, 5,851,794, 5,741,670, 5,849,701, 6,288,214, 6,387,663, 6,908,994, 7,169,902, 7,488,792, 7,820,401, 8,956,612, 8,642,728, and 8,906,649, and U.S. Patent Application Publication Nos. 2007/0161062, 2009/0142345, and 2012/0100106.

[00293] In certain embodiments, the heterodimeric Fc-fused protein of the present invention further comprises a hyaluronic acid-binding domain. The hyaluronic acid-binding domain can be a protein (*e.g.*, an antibody or an antigen-binding fragment thereof), a peptide (*e.g.*, a portion of a hyaluronic acid-binding protein or a variant thereof), an aptamer, a small molecule, or a combination thereof. Hyaluronic acid-binding domains are known in the art, and are disclosed in, for example, U.S. Patent Nos. 6,864,235, 8,192,744, 8,044,022, 8,163,498, 8,034,630, 9,217,016, 9,795,686, and 9,751,919, and U.S. Patent Application Publication Nos. 2002/0055488 and 2007/0259380.

[00294] A proteoglycan-binding domain, collagen-binding domain, and/or hyaluronic acid-binding domain, if present, can be at any position of the heterodimeric Fc-fused protein. For example, in certain embodiments, where the IL-12 subunits are positioned N-terminal to the antibody Fc domain polypeptides, a proteoglycan-binding domain, a collagen-binding domain, and/or a hyaluronic acid-binding domain as disclosed herein can be fused to the C-terminus of the first antibody Fc domain polypeptide and/or to the C-terminus of the second antibody Fc domain polypeptide. In certain embodiments, where the IL-12 subunits are positioned C-terminal to the antibody Fc domain polypeptides, a proteoglycan-binding domain, a collagen-binding domain, and/or a hyaluronic acid-binding domain as disclosed herein can be fused to the N-terminus of the

first antibody Fc domain polypeptide and/or to the N-terminus of the second antibody Fc domain polypeptide.

[00295] A proteoglycan-binding domain, collagen-binding domain, and/or hyaluronic acid-binding domain, if present, can be fused to the rest of the heterodimeric Fc-fused protein through a linker. In certain embodiments, the proteoglycan-binding domain is fused to the rest of the heterodimeric Fc-fused protein through a peptide linker. In certain embodiments, the peptide linker includes a spacer peptide disclosed herein.

Exemplary heterodimeric Fc-fused proteins

[00296] In certain embodiments, a heterodimeric Fc-fused protein of the present invention comprises a first polypeptide comprising the amino acid sequence of SEQ ID NO:290 and a second polypeptide comprising the amino acid sequence of SEQ ID NO:291. In certain embodiments, the heterodimeric Fc-fused protein of the present invention comprising SEQ ID NO:290 and SEQ ID NO:291 comprises a Y349C mutation in the CH3 domain of the first antibody Fc domain polypeptide and a S354C mutation in the CH3 domain of the second antibody Fc domain polypeptide. In certain embodiments, the heterodimeric Fc-fused protein of the present invention comprising SEQ ID NO:290 and SEQ ID NO:291 comprise different mutations in the respective Fc domain polypeptide sequences for promoting heterodimerization between the Fc domains.

[00297] In certain embodiments, the first polypeptide sequence comprises a first antibody Fc domain polypeptide (human IgG1) sequence comprising K360E and K409W substitutions. In certain embodiments, the second polypeptide sequence comprises a second antibody Fc domain polypeptide (human IgG1) sequence comprising Q347R, D399V, and F405T substitutions. In certain embodiments, the first polypeptide and second polypeptide amino acid sequences comprise one or more mutations for reducing effector functions. In certain embodiments, the heterodimeric Fc-fused protein of the present invention comprises L234A, L235A, and P329A mutations.

[00298] In certain embodiments, in the first polypeptide of the heterodimeric Fc-fused protein of the present invention (SEQ ID NO:290), the p40 subunit of human IL-12 is fused to the first antibody Fc domain polypeptide by a first linker comprising a first amino acid sequence, and in the second polypeptide of the heterodimeric Fc-fused protein of the present invention (SEQ ID NO:291), the p35 subunit of human IL-12 is fused to the second antibody Fc domain polypeptide by a second linker comprising a second amino acid sequence.

[00299] SEQ ID NO:290 is a sequence of p40 subunit of human IL-12 (underlined amino acids) fused to human IgG1 Fc domain polypeptide. Mutations are shown in bold.

IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKTLTIQVKEF
GDAGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDILKDQKEPKNKTFLRCEAKNYSGRFTC
WWLTITISTDLTFSVKSSRGSSDPQGVTCGAATLSAERVRGDNKEYEYSVEQCQEDSACPAA
EESLPIEVMVDAVHKLKYENYTSSFFIRDIIKPDPPKNLQLKPLKNSRQVEVSWEYPDTW
STPHSYFSLTFCVQVQGKSKREKKDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEW
ASVPCSPKSSDKTHTCPPCPAPE**AA**GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALAA
PIEKTISKAKGQPREPQV**CT**LPPSRDEL**T**ENQVSLTCLVKGFPSDIAVEWESNGQPENN
YKTTPPVLDSGSFFLYS**W**LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
 (SEQ ID NO:290)

[00300] SEQ ID NO:291 is a sequence of p35 subunit of human IL-12 (underlined amino acids) fused to human IgG1 Fc domain polypeptide. Mutations are shown in bold.

RNLPVATPDPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPCTSEEIDHEDITKDKTST
VEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVEFKT
MNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSETVPQKSSLEEDFYKTKIKLCILL
HAFRIRAVTIDRVMSYLNASGGGGSGGGGSGGGGSEPKSSDKTHTCPPCPAPE**AA**GGPS
VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS
TYRVVSVLTVLHQDWLNGKEYKCKVSNKALAAPIEKTISKAKGQPREP**R**VYTLPP**C**RDE
LTKNQVSLTCLVKGFPSDIAVEWESNGQPENNYKTTPPVL**V**SDGSF**T**LYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO:291)

[00301] The first and second polypeptides represented by amino acid sequences SEQ ID NO:290 and SEQ ID NO:291, respectively, form a disulfide bond due to a Y349C mutation in the CH3 domain of the first antibody Fc domain polypeptide sequence (human IgG1) in SEQ ID NO:290 (bolded and underlined) and a S354C mutation in the CH3 domain of the second antibody Fc domain polypeptide sequence (human IgG1) in SEQ ID NO:291 (bolded and underlined), which imparts stability to the heterodimeric Fc-fused protein (Fc numbering according to the EU system).

[00302] For promoting heterodimerization between the two Fc domain polypeptides of the heterodimeric Fc-fused protein, the first antibody Fc domain polypeptide sequence (human IgG1) in SEQ ID NO:290 includes K360E and K409W substitutions in the CH3 domain, and the second, different Fc domain polypeptide sequence (human IgG1) in SEQ ID NO:291 includes Q347R, D399V, and F405T substitutions in the CH3 domain (Fc numbering according to the EU system).

[00303] The first antibody Fc domain polypeptide sequence and the second, different Fc domain polypeptide sequence (human IgG1) in SEQ ID NO:290 and SEQ ID NO:291 also include L234A, L235A, and P329A (LALAPA) mutations for reducing effector functions.

[00304] This heterodimeric Fc-fused protein is herein referred to as DF-hIL-12-Fc si.

(j) Methods of Preparation and Production

[00305] The proteins of the present invention can be made using recombinant DNA technology well known to a skilled person in the art. For example, a first nucleic acid sequence encoding a

first polypeptide comprising a first subunit of a multisubunit protein sequence fused to a first antibody Fc domain polypeptide can be cloned into a first expression vector; a second nucleic acid sequence encoding a second polypeptide comprising a second, different subunit of a multisubunit protein sequence fused to a second antibody Fc domain polypeptide can be cloned into a second expression vector; and the first and the second expression vectors can be stably transfected together into host cells to produce the multimeric proteins.

[00306] To achieve the highest yield of the protein, different ratios of the first and second expression vectors can be explored to determine the optimal ratio for transfection into the host cells. After transfection, single clones can be isolated for cell bank generation using methods known in the art, such as limited dilution, ELISA, FACS, microscopy, or Clonepix.

[00307] Clones can be cultured under conditions suitable for bio-reactor scale-up and maintained expression of the proteins of the present invention. The proteins can be isolated and purified using methods known in the art including centrifugation, depth filtration, cell lysis, homogenization, freeze-thawing, affinity purification, gel filtration, ion exchange chromatography, hydrophobic interaction exchange chromatography, and mixed-mode chromatography.

(i) Drug Substance Preparation

[00308] In some embodiments, a heterodimeric Fc-fused protein of the present disclosure, *e.g.* DF hIL12-Fc si, is produced in a eukaryotic cell, *e.g.*, a Chinese Hamster Ovary (CHO) cell. In certain embodiments, a heterodimeric Fc-fused protein of the present disclosure, *e.g.* DF hIL12-Fc si, is produced in a CHO cell in suspension culture (*e.g.*, in a shake flask). In certain embodiments, a vial of CHO cells is thawed and passaged more than one time before the protein is produced (*e.g.*, twice, thrice, four times, five times, 6 times). In certain embodiments, a vial of CHO cells is thawed and passaged four times before the protein is produced. In certain embodiments, CHO cells from the fourth passage are used to inoculate a culture in a first bioreactor. In certain embodiments, the first bioreactor has a volume of about 40 L, about 45 L, about 50 L, about 55 L, or about 60 L. In certain embodiments, the first bioreactor has a volume of about 50 L. In certain embodiments, the CHO cells from the culture of the first bioreactor are used to inoculate a culture in a production bioreactor. In certain embodiments, the production bioreactor has a volume of about 180 L, about 185 L, about 190 L, about 195 L, about 200 L, about 205 L, about 210 L, about 215 L, or about 220 L. In certain embodiments, the final culture volume in the production bioreactor is about 180 L. In certain embodiments, the CHO cells are grown in growth media supplemented with L-glutamine (*e.g.*, 6 mM L-glutamine). In certain embodiments, the CHO cells

are grown at a temperature of about 37°C. In certain embodiments, culture conditions are monitored daily (*e.g.*, for glucose, for lactate, for pH).

[00309] In some embodiments, the production bioreactor regulates dissolved oxygen in the culture with air and oxygen supplementation. In some embodiments, the production bioreactor regulates pH with addition of carbon dioxide gas and/or sodium carbonate base. In some embodiments, the production bioreactor is sampled daily for cell density and viability until a target cell viability is met. In certain embodiments, the target cell viability is about 10×10^6 viable cells/mL, about 11×10^6 viable cells/mL, about 12×10^6 viable cells/mL, about 13×10^6 viable cells/mL, about 14×10^6 viable cells/mL, about 15×10^6 viable cells/mL, about 16×10^6 viable cells/mL, about 17×10^6 viable cells/mL, about 18×10^6 viable cells/mL, about 19×10^6 viable cells/mL, or about 20×10^6 viable cells/mL. In certain embodiments, the target cell viability is greater than about 14×10^6 viable cells/mL. In certain embodiments, after target cell viability is met, the temperature is shifted from about 37°C to about 33°C for culture harvest. In certain embodiments, the culture is harvested when the viability is higher than about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%. In certain embodiments, the culture is harvested when the viability is higher than about 85%. In certain embodiments, culture conditions are monitored daily during the culture period (*e.g.*, for glucose, for lactate, for pH). In certain embodiments, titer of DF hIL12-Fc si is monitored in the culture, starting at about Day 8 (*e.g.*, Day 6, Day 7, Day 8, Day 9, or Day 10). In certain embodiments, the culture is supplemented with concentrated nutrient feeds, concentrated glucose solution, and/or antifoam. In some embodiments, the cells are cultured for about 7 – about 21 days, about 8 – about 20 days, about 9 – about 19 days, about 10 – about 18 days, about 11 – about 17 days, about 12 – about 16 days, or about 11 – about 15 days. In certain embodiments, the cells are cultured for about 14 days.

[00310] In some embodiments, the production bioreactor is clarified by depth filtration prior to purification of a protein of the present disclosure, *e.g.*, DF hIL12-Fc si. In certain embodiments, a two-stage single-use depth filtration system consisting of DOHC and XOHC filters is used for the clarification. In certain embodiments, before filtration, the production bioreactor temperature is adjusted to about 18°C and the dissolved oxygen setpoint is increased to about 70% of saturation. In certain embodiments, the harvest filters are rinsed with water for injection (WFI) and then equilibrated with buffer. In some embodiments, the cell suspension is passed through the harvest filters using a peristaltic pump and the filters are flushed to collect the product. In certain embodiments, pressure is monitored and maintained at about less than 25 psig (*e.g.*, about less than

25 psig, about less than 20 psig, or about less than 15 psig). The filtrate is then filtered through a 0.45/0.2 μ m membrane into storage, *e.g.*, in a sterile bag.

[00311] In some embodiments, purification of a heterodimeric Fc-fused protein of the present disclosure, *e.g.* DF hIL12-Fc si, comprises or consists of three chromatography steps and two virus clearance steps. In certain embodiments, the three chromatography steps comprise or consist of Protein A Capture Chromatography, Mixed Mode Chromatography, and Cation Exchange Chromatography. In certain embodiments, clarified harvest comprising the heterodimeric Fc-fused protein of the present disclosure, *e.g.* DF hIL12-Fc si, is captured by Protein A capture chromatography (*e.g.*, using a Protein A resin column). In certain embodiments, the Protein A capture chromatography removes process-related impurities (*e.g.*, DNA, host cell proteins), media additives, and allows for volume reduction. In certain embodiments, the Protein A resin column is first equilibrated with a buffer comprising 20 mM Tris, 150 mM NaCl, at a pH of about 7.5. In certain embodiments, after loading, the column is washed with equilibration buffer to remove unbound or loosely bound impurities. In certain embodiments, after the first wash, the column is washed a second time with a buffer comprising 50 mM acetate at a pH of about 5.4. In certain embodiments, the second wash lowers the pH and prepares the column for elution. In certain embodiments, DF hIL12-Fc si is eluted with a buffer comprising 50 mM acetate, 100 mM arginine at a pH of about 3.7. In certain embodiments, DF hIL12-Fc si is collected by 280 nm UV wavelength starting at 1.25 AU/cm ascending and then ending at 1.25 AU/cm descending. In certain embodiments, the eluate is collected in one pool, and each column cycle is individually processed by low pH virus inactivation.

[00312] In certain embodiments, the virus clearance steps comprise low pH inactivation and nanofiltration. In certain embodiments, the protein A eluate is incubated at low pH to inactivate potentially present viruses. In certain embodiments, pH of the eluate is adjusted with acetic acid, *e.g.*, 0.5 M acetic acid, and incubated for at least 30 minutes, at least 35 minutes, at least 40 minutes, at least 45 minutes, at least 50 minutes, at least 55 minutes, at least 60 minutes, at least 65 minutes, at least 70 minutes, at least 75 minutes, at least 80 minutes, at least 85 minutes, or at least 90 minutes. In certain embodiments, pH of the eluate is adjusted with acetic acid, *e.g.*, 0.5 M acetic acid, and incubated for at least 60 minutes. In certain embodiments, the acetic acid adjusts the pH to about 3.55 to 3.75, *e.g.*, about 3.60 to 3.70, or about 3.65. In certain embodiments, the acetic acid adjusts the pH to about 3.65. In certain embodiments, after low pH incubation, pH is raised, *e.g.*, with Tris base, *e.g.*, with 2 M Tris base. In certain embodiments, pH is raised to a neutralization pH of about 5.1, about 5.2, or about 5.3. In certain embodiments, the protein A

eluate is filtered through a 0.2 μm filtration assembly. In certain embodiments, the low pH inactivation precedes the nanofiltration. In certain embodiments, the nanofiltration precedes the low pH inactivation.

[00313] In some embodiments, after the virus clearance steps, the pool is filtered through an intermediate depth filter, *e.g.*, X0SP intermediate depth filter. In certain embodiments, DF hIL12-Fc si is loaded at a range of about 500 – about 1000 g/m^2 (*e.g.*, about 400 – about 1100 g/m^2 , about 450 – about 1050 g/m^2 , about 500 – about 1000 g/m^2). In certain embodiments, the X0SP pool conductivity is adjusted to less than 6.0 mS/cm with acetate, *e.g.* 50 mM acetate pH of about 5.2, prior to Mixed Mode Chromatography.

[00314] In some embodiments, Mixed Mode Chromatography is performed to remove high molecular weight (HMW) species. In certain embodiments, the column is equilibrated, *e.g.*, with a buffer comprising 50 mM Acetate at pH of about 5.2, and loaded. In certain embodiments, after loading, the column is washed, *e.g.*, with a buffer comprising 50 mM Acetate and 250 mM NaCl at pH of about 5.2. In certain embodiments, collection is initiated by 280 nm UV detection at 0.625 AU/cm ascending and ended at 1.50 AU/cm descending. In certain embodiments, after collection, each cycle is passed through a filter train containing a terminal 0.2 μm filter.

[00315] In some embodiments, Cation Exchange Chromatography is performed to remove product-related impurities (*e.g.*, HMW species, low molecular weight (LMW) species) and process-related impurities. In some embodiments, multiples cycles of Cation Exchange Chromatography are performed (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more cycles) for each product lot. In certain embodiments, the cycles are pooled and diluted, *e.g.*, with a buffer comprising 50 mM Tris at pH 7.4. In certain embodiments, the pooled samples are adjusted to pH of about 7.3, about 7.4, about 7.5, about 7.6, or about 7.7 with a base solution, *e.g.*, Tris, *e.g.*, 2 M Tris base. In certain embodiments, the pooled samples are adjusted to pH of about 7.5. In certain embodiments, the column is equilibrated with a buffer comprising 50 mM Tris at pH 7.4. In certain embodiments, elution comprises a gradient of 50 mM Tris at pH of about 7.4 (Buffer A) and 50 mM Tris and 0.5 M NaCl at pH of about 7.4 (Buffer B). In certain embodiments, product collection is initiated by 280 nm UV detection starting at 2.5 AU/cm ascending and ending at 4.5 AU/cm descending. In certain embodiments, after collection, each cycle is passed through a filter train containing a terminal 0.2 μm filter.

[00316] In some embodiments, nanofiltration of the cycles from the Cation Exchange Chromatography removes viruses. In certain embodiments, the eluate first passes through a prefilter and a nominal filter (*e.g.*, a nominal filter of about 20 nm). In certain embodiments, the

system is equilibrated with a buffer, *e.g.*, a buffer comprising 50 mM Tris and 265 mM NaCl at pH of about 7.4. In certain embodiments, after loading, the system is rinsed with equilibration buffer, *e.g.*, a buffer comprising 50 mM Tris and 265 mM NaCl at of about pH 7.4. In certain embodiments, the filtrate is filtered through a membrane, *e.g.*, a 0.2 μ m membrane.

[00317] In some embodiments, the filtrate undergoes ultrafiltration and diafiltration (UF/DF). In certain embodiments, ultrafiltration and diafiltration are performed using a molecular weight cut-off membrane of about 20 kDa, about 25 kDa, about 30 kDa, about 35 kDa, or about 40 kDa. In certain embodiments, ultrafiltration and diafiltration are performed using a molecular weight cut-off membrane of about 30 kDa. In certain embodiments, the system is equilibrated with a buffer, *e.g.*, a buffer comprising 50 mM Tris and 265 mM NaCl at pH of about 7.4. In certain embodiments, the viral filtrate pool is concentrated to a target of about 5.0 g/L. In certain embodiments, buffer exchange is performed against at least 7 diavolumes (*e.g.*, 7, 8, or 9 diavolumes) of buffer comprising 20 mM Citrate at pH of about 6.5. In some embodiments, after diafiltration, a second concentration step is performed to target about 11.0 g/L. In certain embodiments, the product is diluted to a final target concentration of about 7.5 g/L in diafiltration buffer.

[00318] In some embodiments, a 20 mM Citrate, 18 % (w/v) Sucrose, 3 % (w/v) Mannitol, 0.03% (w/v) polysorbate-80, pH 6.5 stock solution is spiked into the UF/DF pool to target a final concentration of 20 mM Citrate, 6 % (w/v) Sucrose, 1 % (w/v) Mannitol, 0.01 % (w/v) polysorbate-80 in the drug substance.

[00319] In certain embodiments, the formulated retentate is filtered, *e.g.*, through a 0.2 μ m membrane, into the final drug substance storage containers. In certain embodiments, the final fill volume is about 1.0 L. In certain embodiments, the final substance storage containers comprise 2 L polycarbonate bottles with polypropylene closures. In certain embodiments, each bottle is aseptically sampled, labeled, and frozen at less than -65°C (*e.g.*, -65°C, -70°C, -75°C, -80°C, or lower).

(ii) Drug Product Preparation

[00320] In some embodiments, frozen drug substance comprising a heterodimeric Fc-fused protein of the present disclosure, *e.g.* DF hIL12-Fc si, is thawed for more than 96 hours (*e.g.*, 96 hours, 120 hours, 144 hours, 168 hours, or more), at about 2-8°C, in the dark. In certain embodiments, a buffer consisting of 20 mM citrate, 6% (w/v) sucrose, 1% (w/v) mannitol, 0.01% polysorbate 80 (w/v) at pH 6.0 is prepared. In certain embodiments, the buffer is prepared by adding solid sodium citrate dihydrate, citric acid monohydrate, sucrose, and mannitol to water for

injection (WFI) and mixing until dissolution. In certain embodiments, citrate in the drug product comprises or consists of solid sodium citrate dihydrate and/or citric acid monohydrate. In some embodiments, a polysorbate 80 stock solution is prepared in WFI and added to the buffer. In certain embodiments, acceptance pH of the buffer is about 6.5 ± 0.4 (*e.g.*, pH 6.1, pH 6.2, pH 6.3, pH 6.4, pH 6.5, pH 6.6, pH 6.7, pH 6.8, or pH 6.9). In certain embodiments, the buffer is diluted with WFI and tested for acceptance pH of about 6.5 ± 0.4 (*e.g.*, pH 6.1, pH 6.2, pH 6.3, pH 6.4, pH 6.5, pH 6.6, pH 6.7, pH 6.8, or pH 6.9) and osmolality. In certain embodiments, the buffer is filtered through a membrane, *e.g.*, a 0.2 μm membrane.

[00321] In some embodiments, the weight of drug substance is used to calculate a target batch volume. In certain embodiments, the drug substance is added to the buffer in a carboy (*e.g.*, a 10 L carboy) to approximately 80% of the calculated batch volume and mixed. In certain embodiments, the 80% drug product solution is tested for acceptance pH acceptance of about 6.5 ± 0.3 (*e.g.*, pH 6.2, pH 6.3, pH 6.4, pH 6.5, pH 6.6, pH 6.7, or pH 6.8). In certain embodiments, the 80% drug product solution is tested for protein concentration by absorbance at 280 nm using an Extinction coefficient of 1.44 L/(g*cm).

[00322] In some embodiments, the buffer components are designed to yield a pH of about 6.5. In certain embodiments, at the buffer steps, a titration with 1N sodium hydroxide or 1N hydrochloric acid may be performed to bring the pH within the acceptance pH of about 6.5 ± 0.4 (*e.g.*, pH 6.1, pH 6.2, pH 6.3, pH 6.4, pH 6.5, pH 6.6, pH 6.7, pH 6.8, or pH 6.9). In certain embodiments, at the 80% bulk drug product steps, a titration with 1N sodium hydroxide or 1N hydrochloric acid may be performed to bring the pH within the acceptance pH of about 6.5 ± 0.3 (*e.g.*, pH 6.2, pH 6.3, pH 6.4, pH 6.5, pH 6.6, pH 6.7, or pH 6.8).

[00323] In some embodiments, the target concentration of a heterodimeric Fc-fused protein of the present disclosure, *e.g.* DF hIL12-Fc si, is about 1 mg/mL. In some embodiments, the protein concentration is verified by absorbance at 280 nm with acceptance criteria 1.0 ± 0.2 mg/mL (*e.g.*, 0.8 mg/mL, 0.9 mg/mL, 1.0 mg/mL, 1.1 mg/mL, 1.2 mg/mL). In some embodiments, samples are taken to confirm the acceptance pH of about 6.5 ± 0.3 (*e.g.*, pH 6.2, pH 6.3, pH 6.4, pH 6.5, pH 6.6, pH 6.7, or pH 6.8) and osmolality.

[00324] In some embodiments, the compounded bulk drug product solution is passed through a filter, *e.g.*, a sterile 0.2 μm filter, into a carboy, *e.g.*, a 10 L carboy, for bioburden reduction, and held until sterile filtration and filling.

[00325] In some embodiments, the bulk drug product is filtered through two filter capsules in series, each filter capsule consisting of a 0.45 μm polyethersulfone (PES) pre-filter membrane and

a 0.2 μm PES sterilizing membrane. In certain embodiments, the drug product is filtered into a sterile, disposable fill bag inside a controlled Grade B area of the filling suite. In certain embodiments, both sterilizing filter capsules are tested for integrity by bubble point after filtration, with acceptance criteria greater than 3200 mbar, using WFI.

[00326] In some embodiments, the bulk drug product solution is filled from the disposable bag residing immediately outside of the restricted access barrier system (RABS). In certain embodiments, the product is filled into vials, *e.g.*, ready-to-use 2R borosilicate type I vials.

[00327] In certain embodiments, the vials are stoppered, *e.g.*, with sterilized, 13 mm serum stoppers and capped with 13 mm aluminum overseals. In certain embodiments, the fill volume of the vial is $1.3 \text{ mL} \pm 5\%$ (*i.e.*, from 1.235 mL to 1.365 mL). In certain embodiments, vials are moved to 2-8°C storage. In certain embodiments, vials are stored at 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, or 8°C.

(k) Pharmaceutical Formulation

[00328] The present disclosure also features pharmaceutical compositions that contain an effective amount of a protein described herein. The composition can be formulated for use in a variety of drug delivery systems. One or more physiologically acceptable excipient(s) or carrier(s) can also be included in the composition for proper formulation. The term “excipient,” as used herein, means any non-therapeutic agent added to the formulation to provide a desired physical or chemical property, for example, pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption, or penetration.

(i) Excipients and pH

[00329] The one or more excipients in the pharmaceutical formulation of the present invention comprises a buffering agent. The term “buffering agent,” as used herein, refers to one or more components that when added to an aqueous solution is able to protect the solution against variations in pH when adding acid or alkali, or upon dilution with a solvent. In addition to phosphate buffers, glycinate, carbonate, citrate, histidine buffers and the like can be used, in which case, sodium, potassium or ammonium ions can serve as counterion.

[00330] In certain embodiments, the buffer or buffer system comprises at least one buffer that has a buffering range that overlaps fully or in part with the range of pH 5.5 - 7.4. In certain embodiments, the buffer has a pKa of about 6.5 ± 0.5 . In certain embodiments, the buffer comprises a citrate buffer. In specific embodiments, the citrate buffer comprises sodium citrate dihydrate and citric acid monohydrate. In certain embodiments, the citrate is present at a

concentration of about 5 to about 100 mM, about 10 to about 100 mM, about 15 to about 100 mM, about 20 to about 100 mM, about 5 to about 50 mM, about 10 to about 50 mM, about 15 to about 100 mM, about 20 to about 100 mM, about 5 to about 25 mM, about 10 to about 25 mM, about 15 to about 25 mM, about 20 to about 25 mM, about 5 to about 20 mM, about 10 to about 20 mM, or about 15 to about 20 mM. In certain embodiments, the citrate is present at a concentration of about 5 mM, about 10 mM, about 15 mM, about 20 mM, about 25 mM, or about 50 mM. In certain embodiments, the citrate is present at a concentration of 20 mM.

[00331] The pharmaceutical formulation of the present invention may have a pH of 6.0 to 7.0. For example, in certain embodiments, the pharmaceutical formulation has a pH of 6.0 to 7.0 (*i.e.*, 6.5 ± 0.5), 6.1 to 6.9 (*i.e.*, 6.5 ± 0.4), 6.2 to 6.8 (*i.e.*, 6.5 ± 0.3), 6.3 to 6.7 (*i.e.*, 6.5 ± 0.2), 6.4 to 6.6 (*i.e.*, 6.5 ± 0.1), or 6.45 to 6.65 (*i.e.*, 6.5 ± 0.05). In certain embodiments, the pharmaceutical formulation has a pH of about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, about 6.6, about 6.7, about 6.8, about 6.9, or about 7.0. In certain embodiments, the pharmaceutical formulation has a pH of 6.5. Under the rules of scientific rounding, a pH greater than or equal to 6.45 and smaller than or equal to 6.55 is rounded as 6.0.

[00332] In certain embodiments, the buffer system of the pharmaceutical formulation comprises citrate at 10 to 25 mM, at a pH of 6.5 ± 0.2 . In certain embodiments, the buffer system of the pharmaceutical formulation comprises citrate at 20 mM, at a pH of 6.5 ± 0.2 . In certain embodiments, the buffer system of the pharmaceutical formulation comprises citrate at 10 to 25 mM, at a pH of 6.5 ± 0.05 . In certain embodiments, the buffer system of the pharmaceutical formulation comprises citrate at 20 mM, at a pH of 6.5 ± 0.05 .

[00333] The one or more excipients in the pharmaceutical formulation of the present invention further comprises a sugar or sugar alcohol. Sugars and sugar alcohols are useful in pharmaceutical formulations as a thermal stabilizer. In certain embodiments, the pharmaceutical formulation comprises a sugar, for example, a monosaccharide (*e.g.*, glucose, xylose, or erythritol), a disaccharide (*e.g.*, sucrose, trehalose, maltose, or galactose), or an oligosaccharide (*e.g.*, stachyose). In specific embodiments, the pharmaceutical formulation comprises sucrose. In certain embodiments, the pharmaceutical formulation comprises a sugar alcohol, for example, a sugar alcohol derived from a monosaccharide (*e.g.*, mannitol, sorbitol, or xylitol), a sugar alcohol derived from a disaccharide (*e.g.*, lactitol or maltitol), or a sugar alcohol derived from an oligosaccharide. In specific embodiments, the pharmaceutical formulation comprises mannitol.

[00334] The amount of the sugar or sugar alcohol contained within the formulation can vary depending on the specific circumstances and intended purposes for which the formulation is used.

In certain embodiments, the pharmaceutical formulation comprises 0% w/v – about 12% w/v, about 1% w/v – about 11% w/v, about 2% w/v – about 10% w/v, about 3% w/v – about 9% w/v, about 3% w/v - about 12% w/v, about 4% w/v – about 8% w/v, or about 5% w/v – about 7% w/v of the sugar or sugar alcohol. In certain embodiments, the pharmaceutical formulation comprises 0% w/v – about 2% w/v, about 0.5% w/v – about 1.5% w/v, about 0.6% w/v – about 1.4% w/v, about 0.7% w/v – about 1.3% w/v, about 0.8% w/v – about 1.2% w/v, or about 0.9% w/v – about 1.1% w/v of the sugar or sugar alcohol. In certain embodiments, the pharmaceutical formulation comprises about 0% w/v, about 0.5% w/v, about 1% w/v, about 2% w/v, about 3% w/v, about 4% w/v, about 5% w/v, about 6% w/v, about 7% w/v, about 8% w/v, about 9% w/v, or about 10% w/v of the sugar or sugar alcohol. In specific embodiments, the pharmaceutical formulation comprises about 6% w/v of the sugar or sugar alcohol (*e.g.*, sucrose). In specific embodiments, the pharmaceutical formulation comprises about 1% w/v of the sugar or sugar alcohol (*e.g.*, mannitol). In specific embodiments, the pharmaceutical formulation comprises about 6% w/v of the sugar or sugar alcohol (*e.g.*, sucrose) and about 1% w/v of a second sugar or sugar alcohol (*e.g.*, mannitol).

[00335] The one or more excipients in the pharmaceutical formulation disclosed herein further comprises a surfactant. The term “surfactant,” as used herein, refers to a surface active molecule containing both a hydrophobic portion (*e.g.*, alkyl chain) and a hydrophilic portion (*e.g.*, carboxyl and carboxylate groups). Surfactants are useful in pharmaceutical formulations for reducing aggregation of a therapeutic protein. Surfactants suitable for use in the pharmaceutical formulations are generally non-ionic surfactants and include, but are not limited to, polysorbates (*e.g.*, polysorbates 20 or 80); poloxamers (*e.g.*, poloxamer 188); sorbitan esters and derivatives; Triton; sodium laurel sulfate; sodium octyl glycoside; lauryl-, myristyl-, linoleyl-, or stearyl-sulfobetadine; lauryl-, myristyl-, linoleyl- or stearyl-sarcosine; linoleyl-, myristyl-, or cetyl-betaine; lauramidopropyl-cocamidopropyl-, linoleamidopropyl-, myristamidopropyl-, palmidopropyl-, or isostearamidopropylbetaine (*e.g.*, lauroamidopropyl); myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-dimethylamine; sodium methyl cocoyl-, or disodium methyl oleyl-taurate; and the MONAQUATTM series (Mona Industries, Inc., Paterson, N.J.), polyethylene glycol, polypropyl glycol, and copolymers of ethylene and propylene glycol (*e.g.*, Pluronics, PF68 etc.). In certain embodiments, the surfactant is a polysorbate. In certain embodiments, the surfactant is polysorbate 80.

[00336] The amount of a non-ionic surfactant contained within the pharmaceutical formulation of the present invention may vary depending on the specific properties desired of the formulation, as well as the particular circumstances and purposes for which the formulations are intended to be

used. In certain embodiments, the pharmaceutical formulation comprises 0.005% to about 0.5%, about 0.005% to about 0.2%, about 0.005% to about 0.1%, about 0.005% to about 0.05%, about 0.005% to about 0.02%, about 0.005% to about 0.01%, about 0.01% to about 0.5%, about 0.01% to about 0.2%, about 0.01% to about 0.1%, about 0.01% to about 0.05%, or about 0.01% to about 0.02% of the non-ionic surfactant (*e.g.*, polysorbate 80). In certain embodiments, the pharmaceutical formulation comprises about 0.005%, about 0.01%, about 0.02%, about 0.03%, about 0.04%, about 0.05%, about 0.06%, about 0.07%, about 0.08%, about 0.09%, about 0.1%, about 0.15%, about 0.2%, about 0.25%, about 0.3%, about 0.35%, about 0.4%, about 0.45%, or about 0.5% of the non-ionic surfactant (*e.g.*, polysorbate 80).

[00337] The pharmaceutical formulation of the present invention may further comprise one or more other substances, such as a bulking agent or a preservative. A “bulking agent” is a compound which adds mass to a lyophilized mixture and contributes to the physical structure of the lyophilized cake (*e.g.*, facilitates the production of an essentially uniform lyophilized cake which maintains an open pore structure). Illustrative bulking agents include mannitol, glycine, polyethylene glycol and sorbitol. The lyophilized formulations of the present invention may contain such bulking agents. A preservative reduces bacterial action and may, for example, facilitate the production of a multi-use (multiple-dose) formulation.

(ii) Exemplary Formulations

[00338] In certain embodiments, the pharmaceutical formulation of the present invention comprises the heterodimeric Fc-fused protein, citrate, a sugar (*e.g.*, sucrose), a sugar alcohol (*e.g.*, mannitol), and a polysorbate (*e.g.*, polysorbate 80), at pH 6.0 to 7.0.

[00339] In certain embodiments, the pharmaceutical formulation comprises 0.5 to 1.5 mg/mL of the heterodimeric Fc-fused protein, 10 to 30 mM of citrate, 4% w/v to 8% w/v of a sugar (*e.g.*, sucrose), 0.5% w/v to 1.5% w/v of a sugar alcohol (*e.g.*, mannitol), and 0.005% to 0.05% of a polysorbate (*e.g.*, polysorbate 80), at pH 6.5 to 7.5. In certain embodiments, the pharmaceutical formulation comprises 0.5 to 1.5 mg/mL of the heterodimeric Fc-fused protein, 20 mM of citrate, 6% w/v of a sugar (*e.g.*, sucrose), 0.5% w/v to 1.5% w/v of a sugar alcohol (*e.g.*, mannitol), and 0.01% of a polysorbate (*e.g.*, polysorbate 80), at pH 6.0 to 7.0. In certain embodiments, the pharmaceutical formulation comprises 0.5 to 1.5 mg/mL of the heterodimeric Fc-fused protein, 20 mM of citrate, 6% w/v of a sugar (*e.g.*, sucrose), 1% w/v of a sugar alcohol (*e.g.*, mannitol), and 0.01% of a polysorbate (*e.g.*, polysorbate 80), at pH 6.3 to 6.7. In certain embodiments, the pharmaceutical formulation comprises 0.5 to 1.5 mg/mL of the heterodimeric Fc-fused protein, 20

mM of citrate, 6% w/v of a sugar (*e.g.*, sucrose), 1% w/v of a sugar alcohol (*e.g.*, mannitol), and 0.01% of a polysorbate (*e.g.*, polysorbate 80), at pH 6.45 to 6.55.

(iii) Stability of the Heterodimeric Fc-fused Protein and Formulation

[00340] The pharmaceutical formulations of the present invention exhibit high levels of stability. A pharmaceutical formulation is stable when the heterodimeric Fc-fused protein within the formulation retains an acceptable degree of physical property, chemical structure, and/or biological function after storage under defined conditions. In certain embodiments, the pharmaceutical formulation is a clear liquid, free of visible particulates. In certain embodiments, the thermal stability is tested at 5°C, 50°C, and following freeze-thaw cycles (*e.g.*, 5 freeze-thaw cycles).

[00341] Stability can be measured by determining the percentage of the heterodimeric Fc-fused protein in the formulation that remains in a native conformation after storage for a defined amount of time at a defined temperature. The percentage of a protein in a native conformation can be determined by, for example, size exclusion chromatography (*e.g.*, size exclusion high performance liquid chromatography, SEC-HPLC), where a protein in the native conformation is not aggregated (eluted in a high molecular weight fraction) or degraded (eluted in a low molecular weight fraction). In certain embodiments, more than about 95%, more than about 96%, more than about 97%, more than about 98%, or more than about 99% of the heterodimeric Fc-fused protein has native conformation, as determined by size-exclusion chromatography, after incubation at 2-8°C for 2 weeks. In certain embodiments, more than about 95%, more than about 96%, more than about 97%, more than about 98%, or more than about 99% of the heterodimeric Fc-fused protein has native conformation, as determined by size-exclusion chromatography, after freeze-thaw. In certain embodiments, more than about 75%, more than about 76%, more than about 77%, more than about 78%, more than about 79%, more than about 80%, more than about 81%, more than about 82%, more than about 83%, more than about 84%, or more than about 85% of the heterodimeric Fc-fused protein has native conformation, as determined by size-exclusion chromatography, after incubation at 50°C for 2 weeks. In certain embodiments, less than about 0.5%, less than about 0.6%, less than about 0.7%, less than about 0.8%, less than about 0.9%, or less than about 1% of the heterodimeric Fc-fused protein forms a high molecular weight complex (*i.e.*, having a higher molecular weight than the native protein), as determined by size-exclusion chromatography, after incubation at 2-8°C for 2 weeks. In certain embodiments, less than about 40%, less than about 30%, less than about 20%, less than about 10%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1% of the heterodimeric Fc-

fused protein form a high molecular weight complex (*i.e.*, having a higher molecular weight than the native protein), as determined by size-exclusion chromatography, after incubation at 50°C for 2 weeks. In certain embodiments, less than about 0.5%, less than about 0.6%, less than about 0.7%, less than about 0.8%, less than about 0.9%, or less than about 1% of the heterodimeric Fc-fused protein forms a high molecular weight complex (*i.e.*, having a higher molecular weight than the native protein), as determined by size-exclusion chromatography, after freeze-thaw. In certain embodiments, less than about 0.1%, less than about 0.2%, less than about 0.3%, less than about 0.4%, less than about 0.5%, less than about 0.6%, less than about 0.7%, less than about 0.8%, less than about 0.9%, or less than about 1% of the heterodimeric Fc-fused protein is degraded (*i.e.*, having a lower molecular weight than the native protein), as determined by size-exclusion chromatography, after incubation at 2-8°C for 2 weeks. In certain embodiments, less than about 1%, less than about 1.5%, less than about 2%, less than about 2.5%, or less than about 3% of the heterodimeric Fc-fused protein is degraded (*i.e.*, having a lower molecular weight than the native protein), as determined by size-exclusion chromatography, after incubation at 50°C for 2 weeks. In certain embodiments, less than about 0.1%, less than about 0.2%, less than about 0.3%, less than about 0.4%, less than about 0.5%, less than about 0.6%, less than about 0.7%, less than about 0.8%, less than about 0.9%, or less than about 1% of the heterodimeric Fc-fused protein is degraded (*i.e.*, having a lower molecular weight than the native protein), as determined by size-exclusion chromatography, after freeze-thaw.

[00342] SEC-HPLC can provide a measure of the purity of a pharmaceutical formulation by the percentage of protein, *e.g.*, the heterodimeric Fc-fused protein, in the main peak. A purity profile is determined by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis. In some embodiments, the purity profile of the pharmaceutical formulation, is greater than about 90%, greater than about 91%, greater than about 92%, greater than about 93%, greater than about 94%, greater than about 95%, greater than about 96%, greater than about 97%, greater than about 98%, or greater than about 99%. In certain embodiments, the purity profile of the pharmaceutical formulation, is about 99.0%. In some embodiments, the purity profile of the pharmaceutical formulation, is greater than about 75%, greater than about 80%, greater than about 81%, greater than about 82%, greater than about 83%, greater than about 84%, or greater than about 85%, after the pharmaceutical formulation is incubated for 2 weeks at 50°C. In certain embodiments, the purity profile of the pharmaceutical formulation, is about 85.2%. In some embodiments, the purity profile of the pharmaceutical formulation, is greater than about 90%, greater than about 91%, greater than about 92%, greater than about 93%, greater than about 94%,

greater than about 95%, greater than about 96%, greater than about 97%, greater than about 98%, or greater than about 98.5% after the pharmaceutical formulation is subjected to five freeze thaw cycles. In certain embodiments, the purity profile of the pharmaceutical formulation, is about 98.9%.

[00343] Stability can also be measured by determining the parameters of a protein solution by dynamic light scattering. The Z-average and polydispersity index (PDI) values indicate the average diameter of particles in a solution and these measures increase when aggregates are present in the solution. The monomer %Pd value indicates the spread of different monomers detected, where lower values indicate a monodisperse solution, which is preferred. The monomer size detected by DLS is useful in confirming that the main population is monomer and to characterize any higher order aggregates that may be present. In certain embodiments, the Z-average value of the pharmaceutical formulation does not increase by more than 5%, 10%, or 15% after incubation at 2-8°C for 2 weeks. In certain embodiments, the Z-average value of the pharmaceutical formulation does not increase by more than 2-fold, 3-fold, 4-fold, or 5-fold after freeze-thaw. In certain embodiments, the Z-average value of the pharmaceutical formulation does not increase by more than about 10%, more than about 20%, more than about 30%, more than about 40%, more than about 50%, more than about 60%, more than about 70%, more than about 80%, more than about 90%, more than about 100%, more than about 150%, or more than about 200% after incubation at 50°C for 2 weeks. In some embodiments, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less than about 15 nm, less than about 14 nm, less than about 13 nm, or less than about 12 nm, as measured by dynamic light scattering at 25°C. In specific embodiments, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less than about 11.6 nm as measured by dynamic light scattering at 25°C. In some embodiments, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less than about 20 nm, less than about 19 nm, less than about 18 nm, less than about 17 nm, less than about 16 nm, less than about 15.5, less than about 15 nm, or less than about 14.5, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is incubated for 2 weeks at 50°C. In specific embodiments, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of about 14.4 nm. In some embodiments, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less than about 20 nm, less than about 19 nm, less than about 18 nm, less than about 17 nm, less than about 16.5 nm, less than about 16 nm, or less than about 15.5 nm as measured by dynamic light

scattering at 25°C, after the pharmaceutical formulation is subjected to five freeze thaw cycles. In certain embodiments, the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of about 15.3 nm.

[00344] In certain embodiments, the PDI value of the pharmaceutical formulation does not increase by more than about 2-fold, about 3-fold, about 4-fold, or about 5-fold after incubation at 2-8°C for 2 weeks. In certain embodiments, the PDI value of the pharmaceutical formulation does not increase by more than about 2-fold, about 3-fold, about 4-fold, about 5-fold, or about 6-fold after freeze-thaw. In certain embodiments, the PDI value of the pharmaceutical formulation does not increase by more than about 2-fold, about 3-fold, about 4-fold, about 5-fold, or about 6-fold after incubation at 50°C for 2 weeks. In some embodiments, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is less than about 0.30, less than about 0.29, less than about 0.28, less than about 0.27, less than about 0.26, or less than about 0.25 as measured by dynamic light scattering at 25°C. In certain embodiments, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is about 0.26. In some embodiments, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is less than about 0.30, less than about 0.29, less than about 0.28, less than about 0.27, or less than about 0.26 as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is incubated for 2 weeks at 50°C. In certain embodiments, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is about 0.25. In some embodiments, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is less than about 0.40, less than about 0.35, or less than about 0.34, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is subjected to five freeze thaw cycles. In certain embodiments, the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is about 0.33.

[00345] Stability can also be measured by determining the thermal stability of a protein solution by differential scanning fluorimetry (DSF). DSF allows for quantification of changes in the thermal denaturation temperature and stability of a protein under varying test conditions, *e.g.*, buffer or pH. In some embodiments, DSF provides two thermal unfolding temperatures (also known as melting temperatures), T_{m1} and T_{m2} . In certain embodiments, the T_{m1} of the pharmaceutical formulation is greater than about 60°C, greater than about 61°C, greater than about 62°C, greater than about 63°C, greater than about 64°C, greater than about 65°C, or greater than about 66°C. In certain embodiments, the T_{m1} of the pharmaceutical formulation is greater than about 70°C, greater than about 71°C, greater than about 72°C, greater than about 73°C, greater

than about 74°C, greater than about 75°C, greater than about 76°C, or greater than about 77°C. In specific embodiments, the pharmaceutical formulation has a thermal stability profile defined by a T_{m1} of about 67.0°C and a T_{m2} of about 77.3°C. In certain embodiments, the T_{m1} and/or T_{m2} is changed by less than 2°C, less than 1.5°C, or less than 1°C when the pharmaceutical formulation is incubated for 1 week at 50°C, as compared to the same pharmaceutical formulation that is incubated for 1 week at 5°C. In specific embodiments, the T_{m1} is changed by less than 1°C when the pharmaceutical formulation is incubated for 1 week at 50°C, as compared to the same pharmaceutical formulation that is incubated for 1 week at 5°C. In specific embodiments, the T_{m2} is changed by less than 1°C when the pharmaceutical formulation is incubated for 1 week at 50°C, as compared to the same pharmaceutical formulation that is incubated for 1 week at 5°C.

[00346] In some embodiments, DSF provides the temperature at which protein aggregation begins to occur, T_{agg} . In some embodiments, the T_{agg} of the pharmaceutical formulation is greater than 60°C, greater than about 61°C, greater than about 62°C, greater than about 63°C, greater than about 64°C, greater than about 65°C, greater than about 66°C, or greater than about 67°C. In certain embodiments, the T_{agg} is changed by less than about 2°C, less than 1.5°C, or less than about 1°C when the pharmaceutical formulation is incubated for 1 week at 50°C, as compared to the same pharmaceutical formulation that is incubated for 1 week at 5°C. In certain embodiments, the T_{agg} is changed by less than about 2°C, less than about 1.5°C, or less than about 1°C when the pharmaceutical formulation is incubated for 1 week at 50°C, as compared to the same pharmaceutical formulation that is incubated for 1 week at 5°C. In certain embodiments, the T_{agg} is changed by less than about 1°C when the pharmaceutical formulation is incubated for 1 week at 50°C, as compared to the same pharmaceutical formulation that is incubated for 1 week at 5°C.

[00347] In some embodiments, pH is used to determine stability of the pharmaceutical formulation. In some embodiments, the pH of the pharmaceutical formulation does not change by more than about 0.25, about 0.2, about 0.15, or about 0.1 in pH value after the pharmaceutical formulation is incubated for 1 week at 5°C. In certain embodiments, the pH of the pharmaceutical formulation does not change by more than about 0.2 or about 0.1 in pH value after the pharmaceutical formulation is incubated for 1 week at 5°C. In some embodiments, the pH of the pharmaceutical formulation does not change by more than about 0.25, about 0.2, about 0.15, or about 0.1 in pH value after the pharmaceutical formulation is incubated for 1 week at 50°C. In certain embodiments, the pH of the pharmaceutical formulation does not change by more than about 0.2 or about 0.1 in pH value after the pharmaceutical formulation is incubated for 1 week at 50°C.

[00348] Exemplary methods to determine stability of the heterodimeric Fc-fused protein in the pharmaceutical formulation are described in Example 24 of the present disclosure.

(iv) Dosage Forms

[00349] The pharmaceutical formulation can be prepared and stored as a liquid formulation or a lyophilized form. In certain embodiments, the pharmaceutical formulation is a clear, colorless solution, free of visible particulates. In certain embodiments, the pharmaceutical formulation is a liquid formulation for storage at 2-8°C (*e.g.*, 4°C), a frozen formulation for storage at -20°C or lower, or a frozen formulation for storage at -65°C or lower. The sugar and/or sugar alcohol in the formulation are used as lyoprotectants.

[00350] Prior to pharmaceutical use, the pharmaceutical formulation can be diluted or reconstituted in an aqueous carrier suitable for the route of administration. Other exemplary carriers include sterile water for injection (SWFI), bacteriostatic water for injection (BWFI), a pH buffered solution (*e.g.*, phosphate-buffered saline), sterile saline solution, Ringer's solution, or dextrose solution. For example, when the pharmaceutical formulation is prepared for administration, the pharmaceutical formulation can be diluted in a 0.9% sodium chloride (NaCl) solution. In specific embodiments, the pharmaceutical formulation the pharmaceutical formulation is diluted in a 0.9% sodium chloride (NaCl) solution comprising 0.01% polysorbate 80. In certain embodiments, the diluted pharmaceutical formulation is isotonic and suitable for administration by subcutaneous injection.

[00351] The pharmaceutical formulation comprises the heterodimeric Fc-fused protein at a concentration suitable for storage. In certain embodiments, the pharmaceutical formulation comprises the heterodimeric Fc-fused protein at a concentration of about 0.1 - about 2 mg/mL, about 0.2- about 1.8 mg/mL, about 0.3- about 1.7 mg/mL, about 0.4- about 1.6 mg/mL, about 0.5- about 1.5 mg/mL, about 0.6- about 1.4 mg/mL, about 0.7- about 1.3 mg/mL, about 0.8- about 1.2 mg/mL, or about 0.9- about 1.1 mg/mL. In certain embodiments, the pharmaceutical formulation comprises the heterodimeric Fc-fused protein at a concentration of about 0.1 mg/mL, about 0.2 mg/mL, about 0.3 mg/mL, about 0.4 mg/mL, about 0.5 mg/mL, about 0.6 mg/mL, about 0.7 mg/mL, about 0.8 mg/mL, about 0.9 mg/mL, about 1 mg/mL, about 2 mg/mL, about 2.5 mg/mL, about 3 mg/mL, about 3.5 mg/mL, about 4 mg/mL, about 4.5 mg/mL, about 5 mg/mL, or about 10 mg/mL.

[00352] In certain embodiments, the pharmaceutical formulation comprises the heterodimeric Fc-fused protein at a bulk concentration of about 1 g/L to about 10 g/L, about 2 g/L to about 8 g/L, about 4 g/L to about 6 g/L, or about 5 g/L. In certain embodiments, the pharmaceutical formulation

comprises the heterodimeric Fc-fused protein at a bulk concentration of about 1 g/L, about 2 g/L, about 3 g/L, about 4 g/L, about 5 g/L, about 6 g/L, about 7 g/L, about 8 g/L, about 9 g/L, or about 10 g/L. In specific embodiments, the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 5 g/L. In certain embodiments, the pharmaceutical formulation comprises a concentration for administration of heterodimeric Fc-fused protein of about 0.5 g/L - about 2 g/L, about 0.75 g/L – about 1.5 g/L, or about 0.9 g/L – about 1.1 g/L. In certain embodiments, the pharmaceutical formulation comprises a concentration for administration of heterodimeric Fc-fused protein of about 0.5 g/L, about 0.6 g/L, about 0.7 g/L, about 0.8 g/L, about 0.9 g/L, about 1 g/L, about 1.1 g/L, about 1.2 g/L, about 1.3 g/L, about 1.4 g/L, about 1.5 g/L, or about 2 g/L. In specific embodiments, the pharmaceutical formulation comprises a concentration for administration of heterodimeric Fc-fused protein of about 1 g/L.

[00353] In certain embodiments, the pharmaceutical formulation is packaged in a container (*e.g.*, a vial, bag, pen, or syringe). In certain embodiments, the formulation may be a lyophilized formulation or a liquid formulation. In certain embodiments, the amount of heterodimeric Fc-fused protein in the container is suitable for administration as a single dose. In certain embodiments, the amount of heterodimeric Fc-fused protein in the container is suitable for administration in multiple doses. In certain embodiments, the pharmaceutical formulation comprises the heterodimeric Fc-fused protein at an amount of about 0.1 to about 10 mg. In certain embodiments, the pharmaceutical formulation comprises the heterodimeric Fc-fused protein at an amount of about 0- about 2 mg, about 0.2- about 1.8 mg, about 0.3- about 1.7 mg, about 0.4- about 1.6 mg, about 0.5- about 1.5 mg, about 0.6- about 1.4 mg, about 0.7- about 1.3 mg, about 0.8- about 1.2 mg, or about 0.9- about 1.1 mg. In certain embodiments, the pharmaceutical formulation comprises the heterodimeric Fc-fused protein at an amount of about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, or about 10 mg. In specific embodiments, the pharmaceutical formulation comprises the heterodimeric Fc-fused protein, *e.g.*, DF-hIL-12-Fc si, at an amount of about 1 mg.

(l) Dosage Regimens and Therapeutic Uses

[00354] In another aspect, the present disclosure provides a method for treating cancer, the method comprising administering to a subject in need thereof a heterodimeric Fc-fused protein disclosed herein (*e.g.*, DF-hIL-12-Fc si) in an initial three-week treatment cycle on Day 1. In certain embodiments, the heterodimeric Fc-fused protein is administered to the subject only Day 1 in the initial three-week treatment cycle.

[00355] In certain embodiments, the method comprises administering to the subject in need thereof the heterodimeric Fc-fused protein in combination with an anti-PD-1 antibody, *e.g.*, pembrolizumab, in an initial three-week treatment cycle on Day 1. In certain embodiments, the heterodimeric Fc-fused protein and anti-PD-1 antibody are administered to the subject only Day 1 in the initial three-week treatment cycle. In certain embodiments, administration of the PD-1 antibody precedes administration of the heterodimeric Fc-fused protein.

[00356] In certain embodiments, the method further comprises administering to the subject, after the initial treatment cycle, the heterodimeric Fc-fused protein in one or more subsequent three-week treatment cycles, wherein the heterodimeric Fc-fused protein is administered on Day 1 in each subsequent treatment cycle. The subsequent treatment cycles, in which the subject receives administration of the heterodimeric Fc-fused protein once every three weeks or once every four weeks, are designed to maintain a certain level of the heterodimeric Fc-fused protein in the subject. In certain embodiments, the subject receives administration of the heterodimeric Fc-fused protein once every three weeks, once every four weeks, once every five weeks, or once every six weeks. In certain embodiments, the subject receives administration of the heterodimeric Fc-fused protein once every six weeks, *i.e.*, once every other treatment cycle. In certain embodiments, the subject receives at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 subsequent treatment cycles. In certain embodiments, the subject receives subsequent treatment cycles until regression of the cancer (*i.e.*, a complete response). In certain embodiments, the subject has advanced (*i.e.*, unresectable or metastatic) melanoma. In certain embodiments, the subject has advanced (*i.e.*, unresectable or metastatic) renal cell carcinoma.

[00357] In certain embodiments, the method further comprises administering to the subject, after the initial treatment cycle, the heterodimeric Fc-fused protein in one or more subsequent three-week treatment cycles, in combination with an anti-PD-1 antibody, *e.g.*, pembrolizumab, wherein the heterodimeric Fc-fused protein and anti-PD-1 antibody are administered on Day 1 in each subsequent treatment cycle. The subsequent treatment cycles, in which the subject receives administration of the heterodimeric Fc-fused protein and anti-PD-1 antibody once every three weeks, are designed to maintain a certain level of the heterodimeric Fc-fused protein and anti-PD-1 antibody in the subject. In certain embodiments, the subject receives at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 subsequent treatment cycles. In certain embodiments, administration of the anti-PD-1 antibody precedes administration of the heterodimeric Fc-fused protein. In certain embodiments, the subject receives subsequent treatment cycles until regression of the cancer (*i.e.*,

a complete response). In certain embodiments, the subject has advanced (*i.e.*, unresectable or metastatic) urothelial carcinoma.

[00358] In certain embodiments, one or more doses in the initial and subsequent treatment cycles comprise the heterodimeric Fc-fused protein at an amount of 0.01- about 3 µg/kg, about 0.01- about 0.02 µg/kg, about 0.01- about 0.05 µg/kg, about 0.05- about 0.1 µg/kg, about 0.05- about 0.5 µg/kg, about 0.05- about 0.75 µg/kg, about 0.05- about 1 µg/kg, about 0.05- about 1.5 µg/kg, about 0.05- about 2 µg/kg, about 0.05- about 2.5 µg/kg, about 0.05- about 3 µg/kg, about 0.1- about 3 µg/kg, about 0.1- about 1 µg/kg, about 0.5- about 1 µg/kg, about 0.1- about 2 µg/kg, about 0.5- about 2 µg/kg, about 0.1- about 0.5 µg/kg, about 0.1- about 0.25 µg/kg, about 0.2- about 1 µg/kg, about 0.2- about 2 µg/kg, about 1- about 1.2 µg/kg, about 1- about 1.5 µg/kg, about 1- about 2 µg/kg, about 1- about 2.5 µg/kg, about 0.5- about 2.5 µg/kg, about 1- about 3 µg/kg, or about 0.5- about 3 µg/kg. In certain embodiments, one or more doses in the initial and subsequent treatment cycles comprise the heterodimeric Fc-fused protein at an amount selected from the group consisting of about 0.01 µg/kg, about 0.02 µg/kg, about 0.03 µg/kg, about 0.04 µg/kg, about 0.05 µg/kg, about 0.1 µg/kg, about 0.15 µg/kg, about 0.2 µg/kg, about 0.25 µg/kg, about 0.3 µg/kg, about 0.35 µg/kg, about 0.4 µg/kg, about 0.45 µg/kg, about 0.5 µg/kg, about 0.6 µg/kg, about 0.7 µg/kg, about 0.8 µg/kg, about 0.9 µg/kg, about 1 µg/kg, about 1.2 µg/kg, about 1.25 µg/kg, about 1.3 µg/kg, about 1.4 µg/kg, about 1.5 µg/kg, about 1.75 µg/kg, about 2 µg/kg, about 2.5 µg/kg, about 3 µg/kg, about 4 µg/kg, or about 5 µg/kg.

[00359] In certain embodiments, each of the doses in the initial and subsequent treatment cycles comprises the heterodimeric Fc-fused protein at an amount of 0.01- about 3 µg/kg, about 0.01- about 0.02 µg/kg, about 0.01- about 0.05 µg/kg, about 0.05- about 0.1 µg/kg, about 0.05- about 0.5 µg/kg, about 0.05- about 0.75 µg/kg, about 0.05- about 1 µg/kg, about 0.05- about 1.5 µg/kg, about 0.05- about 2 µg/kg, about 0.05- about 2.5 µg/kg, about 0.05- about 3 µg/kg, about 0.1- about 3 µg/kg, about 0.1- about 1 µg/kg, about 0.5- about 1 µg/kg, about 0.1- about 2 µg/kg, about 0.5- about 2 µg/kg, about 0.1- about 0.5 µg/kg, about 0.1- about 0.25 µg/kg, about 0.2- about 1 µg/kg, about 0.2- about 2 µg/kg, about 1- about 1.2 µg/kg, about 1- about 1.5 µg/kg, about 1- about 2 µg/kg, about 1- about 2.5 µg/kg, about 0.5- about 2.5 µg/kg, about 1- about 3 µg/kg, or about 0.5- about 3 µg/kg. In certain embodiments, each of the doses in the initial and subsequent treatment cycles comprises the heterodimeric Fc-fused protein at a same amount selected from the group consisting of about 0.01 µg/kg, about 0.02 µg/kg, about 0.03 µg/kg, about 0.04 µg/kg, about 0.05 µg/kg, about 0.1 µg/kg, about 0.15 µg/kg, about 0.2 µg/kg, about 0.25 µg/kg, about 0.3 µg/kg, about 0.35 µg/kg, about 0.4 µg/kg, about 0.45 µg/kg, about 0.5 µg/kg, about 0.6 µg/kg, about 0.7

μg/kg, about 0.8 μg/kg, about 0.9 μg/kg, about 1 μg/kg, about 1.2 μg/kg, about 1.25 μg/kg, about 1.3 μg/kg, about 1.4 μg/kg, about 1.5 μg/kg, about 1.75 μg/kg, about 2 μg/kg, about 2.5 μg/kg, about 3 μg/kg, about 4 μg/kg, or about 5 μg/kg.

[00360] In certain embodiments, each of the doses in the initial and subsequent treatment cycles comprises the heterodimeric Fc-fused protein at an amount 0.01- about 3 μg/kg, about 0.01- about 0.02 μg/kg, about 0.01- about 0.05 μg/kg, about 0.05- about 0.1 μg/kg, about 0.05- about 0.5 μg/kg, about 0.05- about 0.75 μg/kg, about 0.05- about 1 μg/kg, about 0.05- about 1.5 μg/kg, about 0.05- about 2 μg/kg, about 0.05- about 2.5 μg/kg, about 0.05- about 3 μg/kg, about 0.1- about 3 μg/kg, about 0.1- about 1 μg/kg, about 0.5- about 1 μg/kg, about 0.1- about 2 μg/kg, about 0.5- about 2 μg/kg, about 0.1- about 0.5 μg/kg, about 0.1- about 0.25 μg/kg, about 0.2- about 1 μg/kg, about 0.2- about 2 μg/kg, about 1- about 1.2 μg/kg, about 1- about 1.5 μg/kg, about 1- about 2 μg/kg, about 1- about 2.5 μg/kg, about 0.5- about 2.5 μg/kg, about 1- about 3 μg/kg, or about 0.5- about 3 μg/kg. In certain embodiments, each of the doses in the initial and subsequent treatment cycles comprises the heterodimeric Fc-fused protein at a same amount selected from the group consisting of about 0.01 μg/kg, about 0.02 μg/kg, about 0.03 μg/kg, about 0.04 μg/kg, about 0.05 μg/kg, about 0.1 μg/kg, about 0.15 μg/kg, about 0.2 μg/kg, about 0.25 μg/kg, about 0.3 μg/kg, about 0.35 μg/kg, about 0.4 μg/kg, about 0.45 μg/kg, about 0.5 μg/kg, about 0.6 μg/kg, about 0.7 μg/kg, about 0.8 μg/kg, about 0.9 μg/kg, about 1 μg/kg, about 1.2 μg/kg, about 1.25 μg/kg, about 1.3 μg/kg, about 1.4 μg/kg, about 1.5 μg/kg, about 1.75 μg/kg, about 2 μg/kg, about 2.5 μg/kg, about 3 μg/kg, about 4 μg/kg, or about 5 μg/kg.

[00361] In certain embodiments, the heterodimeric Fc-fused protein is administered subcutaneously. For example, in certain embodiments, the heterodimeric Fc-fused protein is administered by subcutaneous injection, *e.g.*, with a prefilled pen or a prefilled syringe. In certain embodiments, the heterodimeric Fc-fused protein is administered in a volume of about 0.1 mL, about 0.2 mL, about 0.3 mL, about 0.4 mL, about 0.5 mL, about 0.6 mL, about 0.7 mL, about 0.8 mL, about 0.9 mL, about 1 mL, about 1.1 mL, or about 1.2 mL. In certain embodiments, the heterodimeric Fc-fused protein is administered in a volume of about 1 mL. In certain embodiments, the heterodimeric Fc-fused protein is administered in a maximum of 2 injection sites (*e.g.*, 1 injection site, or 2 injection sites). In specific embodiments, the heterodimeric Fc-fused protein is administered in a single injection. In specific embodiments, the heterodimeric Fc-fused protein is administered in two injections. In specific embodiments, the heterodimeric Fc-fused protein is administered in two injections, and a second injection is completed within 10 minutes after a first injection.

[00362] In certain embodiments, the anti-PD-1 antibody, *e.g.*, pembrolizumab, is administered intravenously. In certain embodiments, the anti-PD-1 antibody is administered intravenously preceding administration of the heterodimeric Fc-fused protein. In certain embodiments, the anti-PD-1 antibody is administered intravenously no more than 1 hour (*e.g.*, 5 mins, 10 mins, 15 mins, 20 mins, 25 mins, 30 mins, 35 mins, 40 mins, 45 mins, 50 mins, 55 mins, 1 hour) prior to administration of the heterodimeric Fc-fused protein. In certain embodiments, the PD-1 antibody is administered intravenously concurrently with administration of the heterodimeric Fc-fused protein.

[00363] The types of cancer that can be treated with the heterodimeric Fc-fused protein or pharmaceutical formulation disclosed herein include but are not limited to melanoma, non-small cell lung cancer (NSCLC), small-cell lung cancer (SCLC), head and neck squamous cell carcinoma (HNSCC), classical Hodgkin lymphoma, primary mediastinal large B-Cell lymphoma, bladder cancer, urothelial carcinoma, micro-satellite instability high cancer, colorectal cancer, gastric cancer, oesophageal cancer, cervical cancer, hepatocellular carcinoma, Merkel cell carcinoma, renal cell carcinoma (RCC), endometrial carcinoma, cutaneous T cell lymphoma, or triple negative breast cancer. In certain embodiments, the cancer is a solid tumor. In certain embodiments, the cancer is a locally advanced or metastatic solid tumor. In certain embodiments, the cancer is melanoma. In certain embodiments, the cancer is renal cell carcinoma. In certain embodiments, the cancer is urothelial bladder cancer. In certain embodiments, the subject has clinical or radiological evidence of disease. In certain embodiments, the subject has measurable disease, as determined by the Response Evaluation Criteria for Solid Tumors (RECIST), version 1.1. In certain embodiments, the pharmaceutical formulation disclosed herein is administered as a monotherapy. In certain embodiments, the pharmaceutical formulation disclosed herein is administered as a combination therapy. In certain embodiments, a subject who has a confirmed complete response (CR) is treated with the pharmaceutical formulation for at least 12 months after confirmation, unless a criterion for discontinuation is met. In certain embodiments, the total duration of the multi-dose therapy is equal to or less than 24 months (*e.g.*, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 12 months, 18 months, 24 months). In certain embodiments, the total duration of the multi-dose therapy is more than 24 months.

[00364] In certain embodiments, the subject treated by the method disclosed herein has advanced melanoma. In certain embodiments, the subject has received treatment with an anti PD-1 antibody for at least 6 weeks and has confirmed disease progression. In certain embodiments, the subject has a BRAF activating mutation, has received a BRAF inhibitor, and has disease progression after

the last line of treatment. In certain embodiments, progressive disease is confirmed by radiological or clinical observation. In certain embodiments, the subject does not have a BRAF activating mutation.

[00365] In certain embodiments, the subject treated by the method disclosed herein has advanced renal clear cell carcinoma (RCC). In certain embodiments, the subject has a clear cell histology component. In certain embodiments, the subject has received treatment with a checkpoint inhibitor, *e.g.*, an anti PD-1/PD-L1 antibody, or a VEGF therapy as a monotherapy. In certain embodiments, the subject has received treatment with a checkpoint inhibitor, *e.g.*, an anti PD-1/PD-L1 antibody and a VEGF therapy in combination. In certain embodiments, the subject has received treatment with a checkpoint inhibitor, *e.g.*, an anti PD-1/PD-L1 antibody, and a platinum-based chemotherapy in combination. In certain embodiments, the subject has not received treatment with a checkpoint inhibitor, *e.g.*, an anti PD-1/PD-L1 antibody. In certain embodiments, the subject has received more than 3 prior lines of therapy.

[00366] In certain embodiments, the subject treated by the method disclosed herein has advanced urothelial carcinoma. In certain embodiments, the advanced urothelial carcinoma is metastatic or unresectable. In certain embodiments, the subject has histologically or cytologically documented locally advanced or metastatic transitional cell carcinoma of the urothelium (including but not limited to the renal pelvis, ureters, urinary urothelial, and urethra). In certain embodiments, the subject has received only one platinum-containing regimen (*e.g.*, platinum plus another agent, such as gemcitabine, methotrexate, vinblastine, doxorubicin, *etc.*). In some embodiments, the subject has not received more than one platinum-containing regimen for inoperable locally advanced or metastatic urothelial carcinoma with radiographic progression or with recurrence within 6 months after the last administration of the platinum-containing regimen as an adjuvant. In certain embodiments, the subject has not received treatment with a checkpoint inhibitor (CPI) (*e.g.*, anti-PD-1 or anti-PD-L1) as a monotherapy, or in combination with a platinum based chemotherapy. In certain embodiments, the subject has received ≤ 2 prior lines of therapy. In certain embodiments, the urothelial carcinoma is considered failure of a first-line, platinum-containing regimen.

[00367] In some embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth comprises administration of the heterodimeric Fc-fused protein at a dose of about 0.01- about 3 $\mu\text{g/kg}$, about 0.01- about 0.02 $\mu\text{g/kg}$, about 0.01- about 0.05 $\mu\text{g/kg}$, about 0.05- about 0.1 $\mu\text{g/kg}$, about 0.05- about 0.5 $\mu\text{g/kg}$, about 0.05- about 0.75 $\mu\text{g/kg}$, about 0.05- about 1 $\mu\text{g/kg}$, about 0.05- about 1.5 $\mu\text{g/kg}$, about 0.05- about 2 $\mu\text{g/kg}$, about 0.05- about 2.5

μg/kg, about 0.05- about 3 μg/kg, about 0.1- about 3 μg/kg, about 0.1- about 1 μg/kg, about 0.5- about 1 μg/kg, about 0.1- about 2 μg/kg, about 0.5- about 2 μg/kg, about 0.1- about 0.5 μg/kg, about 0.1- about 0.25 μg/kg, about 0.2- about 1 μg/kg, about 0.2- about 2 μg/kg, about 1- about 1.2 μg/kg, about 1- about 1.5 μg/kg, about 1- about 2 μg/kg, about 1- about 2.5 μg/kg, about 0.5- about 2.5 μg/kg, about 1- about 3 μg/kg, or about 0.5- about 3 μg/kg. In certain embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth comprises administration of the heterodimeric Fc-fused protein at a dose selected from the group consisting of about 0.01 μg/kg, about 0.02 μg/kg, about 0.03 μg/kg, about 0.04 μg/kg, about 0.05 μg/kg, about 0.1 μg/kg, about 0.15 μg/kg, about 0.2 μg/kg, about 0.25 μg/kg, about 0.3 μg/kg, about 0.35 μg/kg, about 0.4 μg/kg, about 0.45 μg/kg, about 0.5 μg/kg, about 0.6 μg/kg, about 0.7 μg/kg, about 0.8 μg/kg, about 0.9 μg/kg, about 1 μg/kg, about 1.2 μg/kg, about 1.25 μg/kg, about 1.3 μg/kg, about 1.4 μg/kg, about 1.5 μg/kg, about 1.75 μg/kg, about 2 μg/kg, about 2.5 μg/kg, about 3 μg/kg, about 4 μg/kg, or about 5 μg/kg. In certain embodiments, the dose administered is based on the subject's weight. In certain embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth further comprises administering an anti-PD-1 antibody, *e.g.*, pembrolizumab or nivolumab.

[00368] In some embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth comprises the heterodimeric Fc-fused protein at an amount of about 0- about 2 mg, about 0.2- about 1.8 mg, about 0.3- about 1.7 mg, about 0.4- about 1.6 mg, about 0.5- about 1.5 mg, about 0.6- about 1.4 mg, about 0.7- about 1.3 mg, about 0.8- about 1.2 mg, or about 0.9- about 1.1 mg. In certain embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth comprises the heterodimeric Fc-fused protein at an amount selected from the group consisting of about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, or about 10 mg. In certain embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth further comprises administering an anti-PD-1 antibody, *e.g.*, pembrolizumab or nivolumab. In specific embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth further comprises administering 200 mg of an anti-PD-1 antibody, *e.g.*, pembrolizumab or nivolumab.

[00369] In some embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth in a subject with advanced melanoma is administered at a dose of about 0.01- about 3 μg/kg, about 0.01- about 0.02 μg/kg, about 0.01- about 0.05 μg/kg, about 0.05- about 0.1 μg/kg, about 0.05- about 0.5 μg/kg, about 0.05- about 0.75 μg/kg, about 0.05- about

1 µg/kg, about 0.05- about 1.5 µg/kg, about 0.05- about 2 µg/kg, about 0.05- about 2.5 µg/kg, about 0.05- about 3 µg/kg, about 0.1- about 3 µg/kg, about 0.1- about 1 µg/kg, about 0.5- about 1 µg/kg, about 0.1- about 2 µg/kg, about 0.5- about 2 µg/kg, about 0.1- about 0.5 µg/kg, about 0.1- about 0.25 µg/kg, about 0.2- about 1 µg/kg, about 0.2- about 2 µg/kg, about 1- about 1.2 µg/kg, about 1- about 1.5 µg/kg, about 1- about 2 µg/kg, about 1- about 2.5 µg/kg, about 0.5- about 2.5 µg/kg, about 1- about 3 µg/kg, or about 0.5- about 3 µg/kg. In certain embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth in a subject with advanced melanoma is administered a dose selected from the group consisting of about 0.01 µg/kg, about 0.02 µg/kg, about 0.03 µg/kg, about 0.04 µg/kg, about 0.05 µg/kg, about 0.1 µg/kg, about 0.15 µg/kg, about 0.2 µg/kg, about 0.25 µg/kg, about 0.3 µg/kg, about 0.35 µg/kg, about 0.4 µg/kg, about 0.45 µg/kg, about 0.5 µg/kg, about 0.6 µg/kg, about 0.7 µg/kg, about 0.8 µg/kg, about 0.9 µg/kg, about 1 µg/kg, about 1.2 µg/kg, about 1.25 µg/kg, about 1.3 µg/kg, about 1.4 µg/kg, about 1.5 µg/kg, about 1.75 µg/kg, about 2 µg/kg, about 2.5 µg/kg, about 3 µg/kg, about 4 µg/kg, or about 5 µg/kg.

[00370] In some embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth in a subject with advanced melanoma comprises the heterodimeric Fc-fused protein at an amount of about 0- about 2 mg, about 0.2- about 1.8 mg, about 0.3- about 1.7 mg, about 0.4- about 1.6 mg, about 0.5- about 1.5 mg, about 0.6- about 1.4 mg, about 0.7- about 1.3 mg, about 0.8- about 1.2 mg, or about 0.9- about 1.1 mg. In certain embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth in a subject with advanced melanoma comprises the heterodimeric Fc-fused protein at an amount selected from the group consisting of about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, or about 10 mg.

[00371] In some embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth in a subject with advanced RCC is administered at a dose of about 0.01- about 3 µg/kg, about 0.01- about 0.02 µg/kg, about 0.01- about 0.05 µg/kg, about 0.05- about 0.1 µg/kg, about 0.05- about 0.5 µg/kg, about 0.05- about 0.75 µg/kg, about 0.05- about 1 µg/kg, about 0.05- about 1.5 µg/kg, about 0.05- about 2 µg/kg, about 0.05- about 2.5 µg/kg, about 0.05- about 3 µg/kg, about 0.1- about 3 µg/kg, about 0.1- about 1 µg/kg, about 0.5- about 1 µg/kg, about 0.1- about 2 µg/kg, about 0.5- about 2 µg/kg, about 0.1- about 0.5 µg/kg, about 0.1- about 0.25 µg/kg, about 0.2- about 1 µg/kg, about 0.2- about 2 µg/kg, about 1- about 1.2 µg/kg, about 1- about 1.5 µg/kg, about 1- about 2 µg/kg, about 1- about 2.5 µg/kg, about 0.5- about 2.5 µg/kg,

about 1- about 3 $\mu\text{g/kg}$, or about 0.5- about 3 $\mu\text{g/kg}$. In certain embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth in a subject with advanced RCC is administered a dose selected from the group consisting of about 0.01 $\mu\text{g/kg}$, about 0.02 $\mu\text{g/kg}$, about 0.03 $\mu\text{g/kg}$, about 0.04 $\mu\text{g/kg}$, about 0.05 $\mu\text{g/kg}$, about 0.1 $\mu\text{g/kg}$, about 0.15 $\mu\text{g/kg}$, about 0.2 $\mu\text{g/kg}$, about 0.25 $\mu\text{g/kg}$, about 0.3 $\mu\text{g/kg}$, about 0.35 $\mu\text{g/kg}$, about 0.4 $\mu\text{g/kg}$, about 0.45 $\mu\text{g/kg}$, about 0.5 $\mu\text{g/kg}$, about 0.6 $\mu\text{g/kg}$, about 0.7 $\mu\text{g/kg}$, about 0.8 $\mu\text{g/kg}$, about 0.9 $\mu\text{g/kg}$, about 1 $\mu\text{g/kg}$, about 1.2 $\mu\text{g/kg}$, about 1.25 $\mu\text{g/kg}$, about 1.3 $\mu\text{g/kg}$, about 1.4 $\mu\text{g/kg}$, about 1.5 $\mu\text{g/kg}$, about 1.75 $\mu\text{g/kg}$, about 2 $\mu\text{g/kg}$, about 2.5 $\mu\text{g/kg}$, about 3 $\mu\text{g/kg}$, about 4 $\mu\text{g/kg}$, or about 5 $\mu\text{g/kg}$.

[00372] In some embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth in a subject with advanced RCC comprises the heterodimeric Fc-fused protein at an amount of about 0- about 2 mg, about 0.2- about 1.8 mg, about 0.3- about 1.7 mg, about 0.4- about 1.6 mg, about 0.5- about 1.5 mg, about 0.6- about 1.4 mg, about 0.7- about 1.3 mg, about 0.8- about 1.2 mg, or about 0.9- about 1.1 mg. In certain embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth in a subject with advanced RCC comprises the heterodimeric Fc-fused protein at an amount selected from the group consisting of about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, or about 10 mg.

[00373] In some embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth in a subject with advanced urothelial carcinoma is administered at a dose of about 0.01- about 3 $\mu\text{g/kg}$, about 0.01- about 0.02 $\mu\text{g/kg}$, about 0.01- about 0.05 $\mu\text{g/kg}$, about 0.05- about 0.1 $\mu\text{g/kg}$, about 0.05- about 0.5 $\mu\text{g/kg}$, about 0.05- about 0.75 $\mu\text{g/kg}$, about 0.05- about 1 $\mu\text{g/kg}$, about 0.05- about 1.5 $\mu\text{g/kg}$, about 0.05- about 2 $\mu\text{g/kg}$, about 0.05- about 2.5 $\mu\text{g/kg}$, about 0.05- about 3 $\mu\text{g/kg}$, about 0.1- about 3 $\mu\text{g/kg}$, about 0.1- about 1 $\mu\text{g/kg}$, about 0.5- about 1 $\mu\text{g/kg}$, about 0.1- about 2 $\mu\text{g/kg}$, about 0.5- about 2 $\mu\text{g/kg}$, about 0.1- about 0.5 $\mu\text{g/kg}$, about 0.1- about 0.25 $\mu\text{g/kg}$, about 0.2- about 1 $\mu\text{g/kg}$, about 0.2- about 2 $\mu\text{g/kg}$, about 1- about 1.2 $\mu\text{g/kg}$, about 1- about 1.5 $\mu\text{g/kg}$, about 1- about 2 $\mu\text{g/kg}$, about 1- about 2.5 $\mu\text{g/kg}$, about 0.5- about 2.5 $\mu\text{g/kg}$, about 1- about 3 $\mu\text{g/kg}$, or about 0.5- about 3 $\mu\text{g/kg}$. In certain embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth in a subject with advanced urothelial carcinoma is administered a dose selected from the group consisting of about 0.01 $\mu\text{g/kg}$, about 0.02 $\mu\text{g/kg}$, about 0.03 $\mu\text{g/kg}$, about 0.04 $\mu\text{g/kg}$, about 0.05 $\mu\text{g/kg}$, about 0.1 $\mu\text{g/kg}$, about 0.15 $\mu\text{g/kg}$, about 0.2 $\mu\text{g/kg}$, about 0.25 $\mu\text{g/kg}$, about 0.3 $\mu\text{g/kg}$, about 0.35 $\mu\text{g/kg}$, about 0.4 $\mu\text{g/kg}$, about 0.45 $\mu\text{g/kg}$, about 0.5 $\mu\text{g/kg}$, about 0.6 $\mu\text{g/kg}$, about 0.7

μg/kg, about 0.8 μg/kg, about 0.9 μg/kg, about 1 μg/kg, about 1.2 μg/kg, about 1.25 μg/kg, about 1.3 μg/kg, about 1.4 μg/kg, about 1.5 μg/kg, about 1.75 μg/kg, about 2 μg/kg, about 2.5 μg/kg, about 3 μg/kg, about 4 μg/kg, or about 5 μg/kg.

[00374] In some embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth in a subject with advanced urothelial carcinoma comprises the heterodimeric Fc-fused protein at an amount of about 0- about 2 mg, about 0.2- about 1.8 mg, about 0.3- about 1.7 mg, about 0.4- about 1.6 mg, about 0.5- about 1.5 mg, about 0.6- about 1.4 mg, about 0.7- about 1.3 mg, about 0.8- about 1.2 mg, or about 0.9- about 1.1 mg. In certain embodiments, the drug delivery formulation for use in a method of treating cancer or inhibiting tumor growth in a subject with advanced urothelial carcinoma comprises the heterodimeric Fc-fused protein at an amount selected from the group consisting of about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, or about 10 mg.

[00375] In certain embodiments, the subject treated in accordance with the methods disclosed herein has not received prior therapy for treating the cancer. In certain embodiments, the subject treated in accordance with the methods disclosed herein has not received prior chemotherapy or immunotherapy for treating the cancer. In certain embodiments, the subject treated in accordance with the methods disclosed herein has received a prior therapy (*e.g.*, a chemotherapy or immunotherapy) but continues to experience cancer progression despite the prior therapy. In certain embodiments, the subject treated in accordance with the methods disclosed herein has experienced cancer regression after receiving a prior therapy (*e.g.*, a chemotherapy or immunotherapy), but later experienced cancer relapse. In certain embodiments, the subject treated in accordance with the methods disclosed herein is intolerant to a prior therapy (*e.g.*, a chemotherapy or immunotherapy).

[00376] In certain embodiments, the subject treated in accordance with the methods disclosed herein meets all the inclusion criteria of a clinical trial cohort (*e.g.*, the dose escalation cohort, the dose expansion cohorts, the melanoma cohort, the renal cell carcinoma cohort, the urothelial carcinoma cohort, or the combination therapy with pembrolizumab or nivolumab cohorts) described in Examples 26 and 29. In certain embodiments, the subject treated in accordance with the methods disclosed herein does not meet any of the exclusion criteria described in Examples 26 and 29.

[00377] The heterodimeric Fc-fused protein disclosed herein can be used as a monotherapy or in combination with one or more therapies. In certain embodiments, the heterodimeric Fc-fused

protein is used as a monotherapy in accordance with the dosage regimen disclosed herein. In other embodiments, the heterodimeric Fc-fused protein is used in combination with one or more therapies, wherein the heterodimeric Fc-fused protein is administered in accordance with a dosage regimen disclosed herein and the one or more therapies are administered in accordance with a dosage regimen known to be suitable for treating the particular subject with the particular cancer. In certain embodiments, the method of treatment disclosed herein is used as an adjunct to surgical removal of the primary lesion. In certain embodiments, a surgical intervention of the primary lesion comprises lysing cancer cells, removing a tumor, or debulking a tumor in the subject.

[00378] Exemplary therapeutic agents that may be used in combination with the heterodimeric Fc-fused protein include, for example, radiation, mitomycin, tretinoin, ribomustin, gemcitabine, vincristine, etoposide, cladribine, mitobronitol, methotrexate, doxorubicin, carboquone, pentostatin, nitracrine, zinostatin, cetorelix, letrozole, raltitrexed, daunorubicin, fadrozole, fotemustine, thymalfasin, sobuzoxane, nedaplatin, cytarabine, bicalutamide, vinorelbine, vesnarinone, aminoglutethimide, amsacrine, proglumide, elliptinium acetate, ketanserin, doxifluridine, etretinate, isotretinoin, streptozocin, nimustine, vindesine, flutamide, drogenil, butocin, carmofur, razoxane, sizofilan, carboplatin, mitolactol, tegafur, ifosfamide, prednimustine, picibanil, levamisole, teniposide, improsulfan, encitabine, lisuride, oxymetholone, tamoxifen, progesterone, mepitiostane, epitio stanol, formestane, interferon-alpha, interferon-2 alpha, interferon-beta, interferon-gamma (IFN- γ), colony stimulating factor-1, colony stimulating factor-2, denileukin diftitox, interleukin-2, luteinizing hormone releasing factor and variations of the aforementioned agents that may exhibit differential binding to their cognate receptors, or increased or decreased serum half-life.

[00379] An additional class of agents that may be used as part of a combination therapy in treating cancer is immune checkpoint inhibitors. Exemplary immune checkpoint inhibitors include agents that inhibit one or more of (i) cytotoxic T lymphocyte-associated antigen 4 (CTLA4), (ii) programmed cell death protein 1 (PD1), (iii) PDL1, (iv) LAG3, (v) B7-H3, (vi) B7-H4, and (vii) TIM3. The CTLA4 inhibitor ipilimumab has been approved by the United States Food and Drug Administration for treating melanoma.

[00380] Yet other agents that may be used as part of a combination therapy in treating cancer are monoclonal antibody agents that target non-checkpoint targets (*e.g.*, herceptin) and non-cytotoxic agents (*e.g.*, tyrosine-kinase inhibitors).

[00381] Yet other categories of anti-cancer agents include, for example: (i) an inhibitor selected from an ALK Inhibitor, an ATR Inhibitor, an A2A Antagonist, a Base Excision Repair Inhibitor,

a Bcr-Abl Tyrosine Kinase Inhibitor, a Bruton's Tyrosine Kinase Inhibitor, a CDC7 Inhibitor, a CHK1 Inhibitor, a Cyclin-Dependent Kinase Inhibitor, a DNA-PK Inhibitor, an Inhibitor of both DNA-PK and mTOR, a DNMT1 Inhibitor, a DNMT1 Inhibitor plus 2-chloro-deoxyadenosine, an HDAC Inhibitor, a Hedgehog Signaling Pathway Inhibitor, an IDO Inhibitor, a JAK Inhibitor, a mTOR Inhibitor, a MEK Inhibitor, a MELK Inhibitor, a MTH1 Inhibitor, a PARP Inhibitor, a Phosphoinositide 3-Kinase Inhibitor, an Inhibitor of both PARP1 and DHODH, a Proteasome Inhibitor, a Topoisomerase-II Inhibitor, a Tyrosine Kinase Inhibitor, a VEGFR Inhibitor, and a WEE1 Inhibitor; (ii) an agonist of OX40, CD137, CD40, GITR, CD27, HVEM, TNFRSF25, or ICOS; and (iii) a cytokine selected from IL-12, IL-15, GM-CSF, and G-CSF.

[00382] In certain embodiments, the cancer treated with a single dose or more of a heterodimeric IL-12-Fc-fused protein (*e.g.*, comprising a first polypeptide comprising the amino acid sequence of SEQ ID NO:290 and a second polypeptide comprising the amino acid sequence of SEQ ID NO:291) is a metastatic cancer. In certain embodiments, the metastatic cancer is a local, regional, or distant metastatic cancer. In certain embodiments, a single or multiple dose of a heterodimeric IL-12-Fc-fused protein (*e.g.*, comprising a first polypeptide comprising the amino acid sequence of SEQ ID NO:290 and a second polypeptide comprising the amino acid sequence of SEQ ID NO:291) treats a distant cancer, which is not the primary cancer of the source organ or tissue and/or the direct target of a treatment regimen, by an abscopal effect. In certain embodiments the abscopal effect of a heterodimeric IL-12-Fc-fused protein is enhanced during and/or after a treatment plan including radiation and/or chemotherapy. In certain embodiments, a single or multiple dose of a heterodimeric IL-12-Fc-fused protein (*e.g.*, comprising a first polypeptide comprising the amino acid sequence of SEQ ID NO:290 and a second polypeptide comprising the amino acid sequence of SEQ ID NO:291) treats cancer in a patient by inducing a systemic anti-tumor response, determined, for example, by increased expression of IFN γ , CXCL9, and/or CXCL10 in the serum and/or the tumor of the patient.

Combination Therapy

[00383] Another aspect of the invention provides for combination therapy. A pharmaceutical formulation comprising a heterodimeric Fc fused protein described herein can be used in combination with additional therapeutic agents to treat the cancer.

[00384] In certain embodiments, the heterodimeric Fc-fused protein of the present invention (*e.g.*, a heterodimeric Fc-fused protein comprising IL-12 subunits) is administered as a combination therapy to treat a subject diagnosed with cancer. In certain embodiments, the cancer

is bladder cancer, breast cancer, cervical cancer, colorectal cancer, oesophageal cancer, gastric cancer, head and neck cancer, hepatocellular carcinoma, leukemia, lung cancer, lymphoma, mesothelioma, melanoma, myeloma, ovarian cancer, endometrial carcinoma, prostate cancer, pancreatic cancer, renal cell carcinoma (RCC), non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), brain cancer, sarcoma, neuroblastoma, classical Hodgkin lymphoma, primary mediastinal large B-Cell lymphoma, urothelial carcinoma, micro-satellite instability high cancer, Merkel cell carcinoma, endometrial carcinoma, cutaneous T cell lymphoma, triple negative breast cancer, or head and neck squamous cell carcinoma (HNSCC). In certain embodiments, the cancer is colon cancer. In certain embodiments, the heterodimeric Fc-fused protein is administered as a combination therapy to a subject diagnosed with colon cancer. In certain embodiments, the cancer is melanoma. In certain embodiments, the heterodimeric Fc-fused protein is administered as a combination therapy to a subject diagnosed with melanoma. In certain embodiments, the cancer is breast cancer. In certain embodiments, the heterodimeric Fc-fused protein is administered as a combination therapy to a subject diagnosed with breast cancer.

[00385] In some embodiments, a heterodimeric Fc-fused protein of the present invention (*e.g.*, a heterodimeric Fc-fused protein comprising IL-12 subunits) is used in treating an advanced malignancy in combination with another therapeutic agent selected from: cytotoxic chemotherapy; radiotherapy; an antibody that targets a molecule involved in an anti-tumor immune response, such as CTLA-4, PD-1, PD-L1, or TGF- β ; an antibody that acts by ADCC on a tumor-associated antigen; a multispecific antibody binding NKG2D, CD16, and a tumor-associated antigen, optionally administered in combination with an antibody that targets PD-1 or PD-L1; a personalized cancer vaccine; an oncolytic cancer vaccine; and a personalized vaccine administered in combination with an antibody that targets PD-1 or PD-L1.

[00386] In some embodiments, a heterodimeric Fc-fused protein of the present invention (*e.g.*, a heterodimeric Fc-fused protein comprising IL-12 subunits) is used in treating malignancy (*e.g.*, an advanced malignancy) in combination with another therapy including, but not limited to, an NK-targeting therapy (*e.g.*, CAR-NK therapy), an antibody therapy, a checkpoint inhibitor therapy, an additional cytokine therapy, an innate immune system agonist therapy, a chemotherapy, a target agent therapy, a radiotherapy, an adoptive NK therapy, a stem cell transplant (SCT) therapy, an agonistic antibody, a chimeric antigen receptor (CAR) T cell therapy, a T-cell receptor (TCR) engineered therapy, a multi-specific binding protein (TriNKET), an agent that induces cellular senescence, and a vaccine and/or oncolytic virus therapy. In some embodiments, a heterodimeric Fc-fused protein of the present invention is used in treating malignancy (*e.g.*, an advanced

malignancy) in combination with two or more additional therapies selected from an NK-targeting therapy (*e.g.*, CAR-NK therapy), an antibody therapy, a checkpoint inhibitor therapy, an additional cytokine therapy, an innate immune system agonist therapy, a chemotherapy, a target agent therapy, a radiotherapy, an adoptive NK therapy, a stem cell transplant (SCT) therapy, an agonistic antibody, a chimeric antigen receptor (CAR) T cell therapy, a T-cell receptor (TCR) engineered therapy, a multi-specific binding protein (TriNKET), an agent that induces cellular senescence, and a vaccine and/or oncolytic virus therapy.

[00387] In some embodiments, a heterodimeric Fc-fused protein of the present invention (*e.g.*, a heterodimeric Fc-fused protein comprising IL-12) is used in treating locally advanced malignancy that can be fully resected, in combination with a cancer vaccine or an antibody that targets PD-1 or PD-L1.

[00388] Proteins of the invention can also be used as an adjunct to surgical removal of the primary lesion.

[00389] The amount of heterodimeric Fc-fused protein of the present invention (*e.g.*, a heterodimeric Fc-fused protein comprising IL-12) and additional therapeutic agent and the relative timing of administration may be selected in order to achieve a desired combined therapeutic effect. For example, when administering a combination therapy to a patient in need of such administration, the therapeutic agents in the combination, a pharmaceutical formulation or formulations comprising the therapeutic agents, or a pharmaceutical composition or compositions comprising the therapeutic agents, may be administered in any order such as, for example, sequentially, concurrently, together, simultaneously and the like. Further, for example, a heterodimeric Fc-fused protein may be administered during a time when the additional therapeutic agent(s) exerts its prophylactic or therapeutic effect, or *vice versa*.

[00390] As disclosed herein, the methods of the invention include coadministration of the combination of a heterodimeric Fc-fused protein (*e.g.*, a heterodimeric Fc-fused protein comprising IL-12 subunits) and an additional therapeutic agent. As disclosed herein, the methods of the invention include coadministration of the combination of a heterodimeric Fc-fused protein comprising IL-12 subunits and an additional therapeutic agent.

[00391] “Coadministered” encompasses methods where a heterodimeric Fc-fused protein (*e.g.*, a heterodimeric Fc-fused protein comprising IL-12 subunits) and an additional therapeutic agent are given simultaneously, where a heterodimeric Fc-fused protein and an additional therapeutic agent are given sequentially, and where either one of, or both of, a heterodimeric Fc-fused protein and an additional therapeutic agent are given intermittently or continuously, or any combination

of: simultaneously, sequentially, intermittently and/or continuously. The skilled artisan will recognize that intermittent administration is not necessarily the same as sequential because intermittent also includes a first administration of an agent and then another administration later in time of that very same agent. Moreover, the skilled artisan understands that intermittent administration also encompasses sequential administration in some embodiments because intermittent administration does include interruption of the first administration of an agent with an administration of a different agent before the first agent is administered again. Further, the skilled artisan will also know that continuous administration can be accomplished by a number of routes including intravenous drip (IV infusion) or feeding tubes, *etc.*

[00392] Furthermore, and in a more general way, the term "coadministered" encompasses any and all methods where the individual administration of a heterodimeric Fc-fused protein and the individual administration of an additional therapeutic agent to a subject overlap during any timeframe.

[00393] The frequency of administration of a heterodimeric Fc-fused protein or an additional therapeutic agent to a subject is known in the art as Q_nd or q_nd where n is the frequency in days for successive administration of that agent. For example, Q₃d would be an administration of an agent once every three (3) days. In certain embodiments, the method comprises administering either one of, or both of, or any combinations thereof, a heterodimeric Fc-fused protein and/or an additional therapeutic agent to a subject for Q₁d, Q₂d, Q₃d, Q₄d, Q₅d, Q₆d, Q₇d, Q₈d, Q₉d, Q₁₀d, Q₁₄d, Q₂₁d, Q₂₈d, Q₃₀d, Q₉₀d, Q₁₂₀d, Q₂₄₀d, or Q₃₆₅d.

[00394] In certain embodiments, either one of or both of a heterodimeric Fc-fused protein and/or an additional therapeutic agent are administered intermittently. In certain embodiments, the method includes administering either one of, or both of a heterodimeric Fc-fused protein or an additional therapeutic agent to a subject with a delay of at least 10 minutes, 15 minutes, 20 minutes, 30 minutes, 40 minutes, 60 minutes, 2 hours, 3 hours, 4 hours, 6 hours, 8 hours, 10 hours, 12 hours, 14 hours, 18 hours, 24 hours, 36 hours, 48 hours, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 3 weeks, or 4 weeks between administrations. In certain embodiments, the administration with a delay follows a pattern where one of, or both of, or any combination thereof, of a heterodimeric Fc-fused protein and/or an additional therapeutic agent are administered continuously for a given period of time from about 10 minutes to about 365 days and then is not administered for a given period of time from about 10 minutes to about 30 days.

[00395] In certain embodiments, either one of, or any combination of, a heterodimeric Fc-fused protein and/or an additional therapeutic agent are administered intermittently while the other is given continuously. In certain embodiments, the combination of the first effective amount of a heterodimeric Fc-fused protein is administered sequentially with the second effective amount of an additional therapeutic agent.

[00396] In certain embodiments, a heterodimeric Fc-fused protein and an additional therapeutic agent are administered simultaneously. In certain embodiments, the combination of the first effective amount of a heterodimeric Fc-fused protein is administered sequentially with the second effective amount of an additional therapeutic agent. In such embodiments, the combination is also said to be "coadministered" since the term includes any and all methods where the subject is exposed to both components in the combination. However, such embodiments are not limited to the combination being given just in one formulation or composition. It may be that certain concentrations of a heterodimeric Fc-fused protein and the additional therapeutic agent are more advantageous to deliver at certain intervals and as such, the first effective amount and second effective amount may change according to the formulation being administered.

[00397] In certain embodiments, a heterodimeric Fc-fused protein and the additional therapeutic agent are administered simultaneously or sequentially. In certain embodiments, the first effective amount of a heterodimeric Fc-fused protein is administered sequentially after the second effective amount of an additional therapeutic agent. In certain embodiments, the second effective amount of an additional therapeutic agent is administered sequentially after the first effective amount of a heterodimeric Fc-fused protein.

[00398] In certain embodiments, the combination of a heterodimeric Fc-fused protein (*e.g.*, a heterodimeric Fc-fused protein comprising IL-12 subunits) and an additional therapeutic agent is administered in one formulation. In certain embodiments, the combination is administered in two (2) compositions where the first effective amount of a heterodimeric Fc-fused protein is administered in a separate formulation from the formulation of the second effective amount of an additional therapeutic agent. In certain embodiments, the combination is administered in two (2) compositions where the first effective amount of the heterodimeric Fc-fused protein is administered in a separate formulation from the formulation of the second effective amount of an additional therapeutic agent. In certain embodiments, the first effective amount of a heterodimeric Fc-fused protein is administered sequentially after the second effective amount of an additional therapeutic agent. In certain embodiments, the second effective amount of an additional therapeutic agent is administered sequentially after the first effective amount of a heterodimeric Fc-fused

protein. In certain embodiments, a heterodimeric Fc-fused protein and the additional therapeutic agent are administered; and subsequently both the heterodimeric Fc-fused protein and the additional therapeutic agent are administered intermittently for at least 24 hours. In certain embodiments, the heterodimeric Fc-fused protein and the additional therapeutic agent are administered on a non-overlapping every other day schedule.

[00399] In certain embodiments, the first effective amount of a heterodimeric Fc-fused protein is administered no less than 4 hours after the second effective amount of an additional therapeutic agent. In certain embodiments, the first effective amount of a heterodimeric Fc-fused protein is administered no less than 10 minutes, no less than 15 minutes, no less than 20 minutes, no less than 30 minutes, no less than 40 minutes, no less than 60 minutes, no less than 1 hour, no less than 2 hours, no less than 4 hours, no less than 6 hours, no less than 8 hours, no less than 10 hours, no less than 12 hours, no less than 24 hours, no less than 2 days, no less than 4 days, no less than 6 days, no less than 8 days, no less than 10 days, no less than 12 days, no less than 14 days, no less than 21 days, or no less than 30 days after the second effective amount of an additional therapeutic agent. In certain embodiments, the second effective amount of an additional therapeutic agent is administered no less than 10 minutes, no less than 15 minutes, no less than 20 minutes, no less than 30 minutes, no less than 40 minutes, no less than 60 minutes, no less than 1 hour, no less than 2 hours, no less than 4 hours, no less than 6 hours, no less than 8 hours, no less than 10 hours, no less than 12 hours, no less than 24 hours, no less than 2 days, no less than 4 days, no less than 6 days, no less than 8 days, no less than 10 days, no less than 12 days, no less than 14 days, no less than 21 days, or no less than 30 days after the first effective amount of a heterodimeric Fc-fused protein.

[00400] In certain embodiments, either one of, or both of a heterodimeric Fc-fused protein and/or additional therapeutic agent are administered by a route selected from the group consisting of: intravenous, subcutaneous, cutaneous, oral, intramuscular, and intraperitoneal. In certain embodiments, either one of, or both of a heterodimeric Fc-fused protein and/or additional therapeutic agent are administered by intravenously. In certain embodiments, either one of, or both of, or any combination thereof, a heterodimeric Fc-fused protein and/or additional therapeutic agent are administered orally.

[00401] It is understood by the skilled artisan that the unit dose forms of the present disclosure may be administered in the same or different physical forms, *i.e.* orally via capsules or tablets and/or by liquid via IV infusion, and so on. Moreover, the unit dose forms for each administration may differ by the particular route of administration. Several various dosage forms may exist for

either one of, or both of, the combination of a heterodimeric Fc-fused protein and additional therapeutic agents. Because different medical conditions can warrant different routes of administration, the same components of the combination described herein may be exactly alike in composition and physical form and yet may need to be given in differing ways and perhaps at differing times to alleviate the condition. For example, a condition such as persistent nausea, especially with vomiting, can make it difficult to use an oral dosage form, and in such a case, it may be necessary to administer another unit dose form, perhaps even one identical to other dosage forms used previously or afterward, with an inhalation, buccal, sublingual, or suppository route instead or as well. The specific dosage form may be a requirement for certain combinations of a heterodimeric Fc-fused protein and additional therapeutic agents, as there may be issues with various factors like chemical stability or pharmacokinetics.

(i) NK-Targeting Therapy

[00402] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with NK targeting therapies. For example, in an embodiment, the heterodimeric Fc-fused protein is coadministered with a therapeutic agent that targets Nkp46. In certain embodiments, the therapeutic agent that targets Nkp46 also binds CD16, one or more tumor-associated antigens, or a combination thereof. Exemplary therapeutic agents that target Nkp46 are described in more detail in U.S. Application No. US20170198038A1, herein incorporated by reference for all purposes.

[00403] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with bi- and tri-specific killer engagers (BiKEs and TriKEs) therapies, including BiKE and TriKE therapies targeting NK cells. BiKEs and TriKEs are constructed from a single heavy (VH) and light (VL) chain of the variable region of each antibody of interest. VH and VL domains are joined by a short flexible polypeptide linker to prevent dissociation. BiKEs and TriKEs are described in more detail in U.S. Application Nos. US20180282386A1 and US20180258396A1, herein incorporated by reference for all purposes. BiKEs and TriKEs can contain a binding domain specific for an NK cell.

[00404] In certain embodiments, BiKE and TriKE therapies are used in combination with the heterodimeric Fc-fused protein therapy to treat subjects known or suspected of having High-risk Myelodysplastic Syndrome, Acute Myelogenous Leukemia, Systemic Mastocytosis, or Mast Cell Leukemia. In certain embodiments, BiKE and TriKE therapies are administered as a single course of 3 weekly treatment blocks. In certain embodiments, a treatment block comprises 4 consecutive 24-hour continuous infusions (approximately 96 hours) followed by a 72 hour break. In certain

embodiments, BiKE and TriKE therapies are administered at a dose of 5 µg/kg/day, 10 µg/kg/day, 25 µg/kg/day, 50 µg/kg/day, 100 µg/kg/day, or 200 µg/kg/day. In certain embodiments, BiKE and TriKE therapies are administered at a dose of at least 5 µg/kg/day, at least 10 µg/kg/day, at least 25 µg/kg/day, at least 50 µg/kg/day, at least 100 µg/kg/day, or at least 200 µg/kg/day. In certain embodiments, BiKE and TriKE therapies are administered at a dose of at least 1 µg/kg/day. In certain embodiments, BiKE and TriKE therapies are administered at a dose of at least 5 µg/kg/day. In certain embodiments, BiKE and TriKE therapies are administered at a dose of at least 200 µg/kg/day. In certain embodiments, BiKE and TriKE therapies are administered at a dose of at least 500 µg/kg/day. In certain embodiments, BiKE and TriKE therapies are administered at a dose of at least 1000 µg/kg/day. In certain embodiments, BiKE and TriKE therapies are administered at a dose of 200 µg/kg/day or less. In certain embodiments, BiKE and TriKE therapies are administered at a dose of 500 µg/kg/day or less. In certain embodiments, BiKE and TriKE therapies are administered at a dose of 1000 µg/kg/day or less. In certain embodiments, BiKE and TriKE therapies are administered at a dose of 1-200 µg/kg/day. In certain embodiments, BiKE and TriKE therapies are administered at a dose of 5-200 µg/kg/day. In certain embodiments, BiKE and TriKE therapies are administered at a dose of 1-500 µg/kg/day. In certain embodiments, BiKE and TriKE therapies are administered at a dose of 1-1000 µg/kg/day. In certain embodiments, BiKE and TriKE therapies are administered at a dose of 5-500 µg/kg/day. In certain embodiments, BiKE and TriKE therapies are administered at a dose of 5-1000 µg/kg/day. In certain embodiments, BiKE and TriKE therapies are administered at a maximum-tolerated dose. In certain embodiments, BiKE and TriKE therapies are administered at less than maximum-tolerated dose.

(ii) Multi-specific Binding Protein (“TriNKET”) Therapy

[00405] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with a therapy comprising a multi-specific binding protein, which comprises: (a) a first antigen-binding site that binds NKG2D; (b) a second antigen-binding site that binds a tumor-associated antigen; and (c) an antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16 (“TriNKET”) (for example, multi-specific binding proteins comprising various NKG2D-binders and tumor-associated antigen-binding sites described in international publication no. WO 2019/157332, whose contents relating to the multi-specific binding proteins described therein are incorporated by reference herein), to treat subjects known or suspected of having cancer. Exemplary tumor-associated antigens include, but are not limited to, HER2, CD20, CD33, B-cell maturation antigen (BCMA), EpCAM, CD2, CD19, CD25, CD30, CD38, CD40, CD52, CD70, CLL1/CLEC12A, FLT3, EGFR/ERBB1, IGF1R, HER3/ERBB3,

HER4/ERBB4, MUC1, cMET, SLAMF7, PSCA, MICA, MICB, TRAILR1, TRAILR2, MAGE-A3, B7.1, B7.2, CTLA4, HLA-E, and PD-L1.

[00406] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with a therapy comprising a dose of a multi-specific binding protein based on body weight. For example, doses of a multi-specific binding protein based on body weight are from about 0.01 μg to about 100 mg per kg of body weight, such as about 0.01 μg to about 100 mg/kg of body weight, about 0.01 μg to about 50 mg/kg of body weight, about 0.01 μg to about 10 mg/kg of body weight, about 0.01 μg to about 1 mg/kg of body weight, about 0.01 μg to about 100 μg /kg of body weight, about 0.01 μg to about 50 μg /kg of body weight, about 0.01 μg to about 10 μg /kg of body weight, about 0.01 μg to about 1 μg /kg of body weight, about 0.01 μg to about 0.1 μg /kg of body weight, about 0.1 μg to about 100 mg/kg of body weight, about 0.1 μg to about 50 mg/kg of body weight, about 0.1 μg to about 10 mg/kg of body weight, about 0.1 μg to about 1 mg/kg of body weight, about 0.1 μg to about 100 μg /kg of body weight, about 0.1 μg to about 10 μg /kg of body weight, about 0.1 μg to about 1 μg /kg of body weight, about 1 μg to about 100 mg/kg of body weight, about 1 μg to about 50 mg/kg of body weight, about 1 μg to about 10 mg/kg of body weight, about 1 μg to about 1 mg/kg of body weight, about 1 μg to about 100 μg /kg of body weight, about 1 μg to about 50 μg /kg of body weight, about 1 μg to about 10 μg /kg of body weight, about 10 μg to about 100 mg/kg of body weight, about 10 μg to about 50 mg/kg of body weight, about 10 μg to about 10 mg/kg of body weight, about 10 μg to about 1 mg/kg of body weight, about 10 μg to about 100 μg /kg of body weight, about 10 μg to about 50 μg /kg of body weight, about 50 μg to about 100 mg/kg of body weight, about 50 μg to about 50 mg/kg of body weight, about 50 μg to about 10 mg/kg of body weight, about 50 μg to about 1 mg/kg of body weight, about 50 μg to about 100 μg /kg of body weight, about 100 μg to about 100 mg/kg of body weight, about 100 μg to about 50 mg/kg of body weight, about 100 μg to about 10 mg/kg of body weight, about 100 μg to about 1 mg/kg of body weight, about 1 mg to about 100 mg/kg of body weight, about 1 mg to about 50 mg/kg of body weight, about 1 mg to about 10 mg/kg of body weight, about 10 mg to about 100 mg/kg of body weight, about 10 mg to about 50 mg/kg of body weight, about 50 mg to about 100 mg/kg of body weight.

[00407] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with a therapy comprising doses of a multi-specific binding protein given once or more times daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the targetable construct or complex in bodily fluids or tissues. Administration of

a multi-specific binding protein could be intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, intrapleural, intrathecal, intracavitary, by perfusion through a catheter or by direct intralesional injection. This may be administered once or more times daily, once or more times weekly, once or more times monthly, and once or more times annually.

(iii) Chimeric antigen receptors (CARs) Therapy

[00408] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with a CAR therapy. The term “chimeric antigen receptor” or alternatively a “CAR” refers to a recombinant polypeptide construct comprising at least an extracellular antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising a functional signaling domain derived from a stimulatory molecule (also referred to herein as a “primary signaling domain”).

[00409] Accordingly, in certain embodiments, the CAR comprises an extracellular antigen-binding site that binds tumor-associated antigen, a transmembrane domain, and an intracellular signaling domain comprising a primary signaling domain. In certain embodiments, the CAR further comprises one or more functional signaling domains derived from at least one costimulatory molecule (also referred to as a “costimulatory signaling domain”).

[00410] In one embodiment, the CAR comprises a chimeric fusion protein comprising a tumor-associated antigen-binding domain (*e.g.*, tumor-associated antigen-binding scFv domain) comprising a heavy chain variable domain and a light chain variable domain as an extracellular antigen binding domain, a transmembrane domain, and an intracellular signaling domain comprising a primary signaling domain. In one embodiment, the CAR comprises a chimeric fusion protein comprising a tumor-associated antigen-binding domain (*e.g.*, tumor-associated antigen-binding scFv domain) comprising a heavy chain variable domain and a light chain variable domain as an extracellular antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising a costimulatory signaling domain and a primary signaling domain. In certain embodiments, the CAR comprises a chimeric fusion protein comprising a tumor-associated antigen-binding domain (*e.g.*, tumor-associated antigen-binding scFv domain) comprising a heavy chain variable domain and a light chain variable domain as an extracellular antigen binding domain, a transmembrane domain, and an intracellular signaling domain comprising two costimulatory signaling domains and a primary signaling domain. In one embodiment, the CAR comprises a chimeric fusion protein comprising a tumor-associated antigen-binding domain comprising a heavy chain variable domain and a light chain variable domain as an extracellular

antigen binding domain, a transmembrane domain, and an intracellular signaling domain comprising at least two costimulatory signaling domains and a primary signaling domain.

[00411] With respect to the transmembrane domain, in various embodiments, the CAR is designed to comprise a transmembrane domain that is fused to the extracellular domain of the CAR. In one embodiment, the transmembrane domain is one that naturally is associated with one of the domains in the CAR. In some instances, the transmembrane domain can be selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex. In another embodiment, the transmembrane domain is capable of homodimerization with another CAR on the CAR T cell surface. In another embodiment, the amino acid sequence of the transmembrane domain may be modified or substituted so as to minimize interactions with the binding domains of the native binding partner present in the same CAR T cell.

[00412] The transmembrane domain may be derived from any naturally occurring membrane-bound or transmembrane protein. In one embodiment, the transmembrane region is capable of signaling to the intracellular domain(s) whenever the CAR has bound to a target. In some embodiments, the transmembrane domain comprises the transmembrane region(s) of one or more proteins selected from the group consisting of TCR α chain, TCR β chain, TCR ζ chain, CD28, CD3 ϵ , CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, and CD154. In some embodiments, the transmembrane domain comprises the transmembrane region(s) of one or more protein(s) selected from the group consisting of KIRDS2, OX40, CD2, CD27, LFA-1 (CD11a, CD18), ICOS (CD278), 4-1BB (CD137), GITR, CD40, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD160, CD19, IL2R β , IL2R γ , IL7R α , ITGA1, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, TNFR2, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, PAG/Cbp, NKG2D, and NKG2C.

[00413] The extracellular tumor-associated antigen-binding domain (*e.g.*, tumor-associated antigen-binding scFv domain) can be connected to the transmembrane domain by a hinge region. A variety of hinges can be employed, including but not limited to the human Ig hinge (*e.g.*, an IgG4 hinge, an IgD hinge), a Gly-Ser linker, a (G4S)₄ linker, a KIR2DS2 hinge, and a CD8 α hinge.

[00414] The intracellular signaling domain of the CAR is responsible for activation of at least one of the specialized functions of the immune cell (*e.g.*, cytolytic activity or helper activity, including the secretion of cytokines, of a T cell) in which the CAR has been placed in. Thus, as used herein, the term “intracellular signaling domain” refers to the portion of a protein which transduces an effector function signal and directs the cell to perform a specialized function. While usually the entire intracellular signaling domain can be employed, in many cases it is not necessary to use the entire chain. To the extent that a truncated portion of the intracellular signaling domain is used, such truncated portion may be used in place of the intact chain as long as it transduces the effector function signal. The term intracellular signaling domain is thus meant to include any truncated portion of the intracellular signaling domain sufficient to transduce the effector function signal.

[00415] The intracellular signaling domain of the CAR comprises a primary signaling domain (*i.e.* a functional signaling domain derived from a stimulatory molecule) and one or more costimulatory signaling domains (*i.e.* functional signaling domains derived from at least one costimulatory molecule).

[00416] As used herein, the term “stimulatory molecule” refers to a molecule expressed by an immune cell, *e.g.*, a T cell, an NK cell, or a B cell, that provide the cytoplasmic signaling sequence(s) that regulate activation of the immune cell in a stimulatory way for at least some aspect of the immune cell signaling pathway. In one embodiment, the signal is a primary signal that is initiated by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with a peptide, and which leads to mediation of a T cell response, including, but not limited to, proliferation, activation, differentiation, and the like.

[00417] Primary signaling domains that act in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs or ITAMs. Examples of ITAM containing cytoplasmic signaling sequences that are of particular use in the present disclosure include those derived from CD3 zeta, common FcR gamma (FCER1G), Fc gamma RIIa, FcR beta (Fc Epsilon R1b), CD3 gamma, CD3 delta, CD3 epsilon, CD79a, CD79b, DAP10, and DAP12. In one embodiment, the primary signaling domain in any one or more CARs comprises a cytoplasmic signaling sequence derived from CD3-zeta.

[00418] In some embodiments, the primary signaling domain is a functional signaling domain of TCR zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, CD66d, 4-1BB, and/or CD3-zeta. In an embodiment, the intracellular signaling domain comprises a functional signaling domain of CD3 zeta, common FcR gamma (FCER1G), Fc gamma

RIIa, FcR beta (Fc Epsilon R1b), CD3 gamma, CD3 delta, CD3 epsilon, CD79a, CD79b, DAP10, and/or DAP12. In a particular embodiment, the primary signaling domain is a functional signaling domain of the zeta chain associated with the T cell receptor complex.

[00419] As used herein, the term “costimulatory molecule” refers to a cognate binding partner on a T cell that specifically binds with a costimulatory ligand, thereby mediating a costimulatory response by the T cell, such as, but not limited to, proliferation. A costimulatory molecule is a cell surface molecule other than an antigen receptor or its ligands that is required for an efficient response of lymphocytes to an antigen. Examples of such molecules include CD27, CD28, 4-1BB (CD137), OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18), CD2, CD7, CD258 (LIGHT), NKG2C, B7-H3, and a ligand that specifically binds with CD83, and the like. Further examples of such costimulatory molecules include CD5, ICAM-1, GITR, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD160, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, NKG2C, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, and a ligand that specifically binds with CD83. In some embodiments, the costimulatory signaling domain of the CAR is a functional signaling domain of a costimulatory molecule described herein, e.g., OX40, CD27, CD28, CD30, CD40, PD-1, CD2, CD7, CD258, NKG2C, B7-H3, a ligand that binds to CD83, ICAM-1, LFA-1 (CD11a/CD18), ICOS and 4-1BB (CD137), or any combination thereof.

[00420] As used herein, the term “signaling domain” refers to the functional portion of a protein which acts by transmitting information within the cell to regulate cellular activity via defined signaling pathways by generating second messengers or functioning as effectors by responding to such messengers.

[00421] The cytoplasmic signaling sequences within the cytoplasmic signaling portion of the CAR may be linked to each other in a random or specified order. Optionally, a short oligo- or polypeptide linker, for example, between 2 and 10 amino acids in length may form the linkage.

(iv) Antibody Therapy

[00422] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with an antibody therapy to treat subjects known or suspected of having cancer.

[00423] In certain embodiments, the heterodimeric Fc-fused protein is combined with a therapy comprising an anti-HER2 binding domain, such as an anti-HER2 antibody or anti-HER2 antibody platforms (*e.g.*, a bi-specific or tri-specific antibody comprising an anti-HER2 binding domain, anti-HER2 antibody-drug conjugates, or anti-HER2 CAR). Anti-HER2 antibodies include, but are not limited to, trastuzumab (HERCEPTIN® - Roche/Genentech; Kanjinti - Amgen), pertuzumab (PERJETA® - Roche/Genentech), and MGAH22 (described in detail in U.S. Pat. No. 8,802,093, herein incorporated by reference for all purposes). Anti-HER2 antibody platforms include, but are not limited to, ertumaxomab (REXOMUN® – Creative Biolabs) and trastuzumab emtansine (ado-trastuzumab emtansine/T-DM1; KADCYLA® - Roche/Genentech). In certain embodiments, the anti-HER2 binding domain therapy is used in combination with the heterodimeric Fc-fused protein therapy to treat subjects known or suspected of having cancer. In certain embodiments, the anti-HER2 binding domain therapy is administered by IV infusion. In certain embodiments, the anti-HER2 binding domain therapy is administered at a dose of 1 mg/kg/day, 2 mg/kg/day, 3 mg/kg/day, 4 mg/kg/day, 5 mg/kg/day, 6 mg/kg/day, 7 mg/kg/day, 8 mg/kg/day, 9 mg/kg/day, 10 mg/kg/day. In certain embodiments, the anti-HER2 binding domain therapy is administered at a dose of at least 1 mg/kg/day, at least 2 mg/kg/day, at least 3 mg/kg/day, at least 4 mg/kg/day, at least 5 mg/kg/day, at least 6 mg/kg/day, at least 7 mg/kg/day, at least 8 mg/kg/day, at least 9 mg/kg/day, at least 10 mg/kg/day. In certain embodiments, the anti-HER2 binding domain therapy is administered at a dose of less than 1 mg/kg/day.

[00424] In certain embodiments, the anti-HER2 binding domain therapy is used in combination with the heterodimeric Fc-fused protein therapy to treat subjects known or suspected of having breast cancer, *e.g.*, a subject diagnosed with metastatic HER2-overexpressing breast cancer. In certain embodiments, the anti-HER2 binding domain therapy is administered at 4 mg/kg/day. In certain embodiments, the anti-HER2 binding domain therapy is administered at 4 mg/kg/day by IV infusion over 90 minutes. In certain embodiments, the anti-HER2 binding domain therapy is administered at 2 mg/kg/day. In certain embodiments, the anti-HER2 binding domain therapy is administered at 2 mg/kg/day by IV infusion over 30 minutes. In certain embodiments, the anti-HER2 binding domain therapy is administered at an initial dose of 4 mg/kg/day, then subsequently administered weekly at 2 mg/kg/day. In certain embodiments, the anti-HER2 binding domain therapy is administered at an initial dose of 4 mg/kg/day, then subsequently administered weekly at 2 mg/kg/day for 52 weeks.

[00425] In certain embodiments, the anti-HER2 binding domain therapy is used in combination with the heterodimeric Fc-fused protein therapy to treat subjects known or suspected of having

gastric cancer, *e.g.*, a subject diagnosed with metastatic HER2-overexpressing gastric cancer. In certain embodiments, the anti-HER2 binding domain therapy is administered at 8 mg/kg/day. In certain embodiments, the anti-HER2 binding domain therapy is administered at 8 mg/kg/day by IV infusion over 90 minutes. In certain embodiments, the anti-HER2 binding domain therapy is administered at 6 mg/kg/day. In certain embodiments, the anti-HER2 binding domain therapy is administered at 6 mg/kg/day by IV infusion over 30-90 minutes. In certain embodiments, the anti-HER2 binding domain therapy is administered at an initial dose of 8 mg/kg/day, then subsequently administered weekly at 6 mg/kg/day. In certain embodiments, the anti-HER2 binding domain therapy is administered at an initial dose of 8 mg/kg/day, then subsequently administered weekly at 6 mg/kg/day for 52 weeks.

[00426] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with a therapy comprising an anti-CD20 binding domain, such as an anti-CD20 antibody or anti-CD20 antibody platforms (*e.g.*, a bi-specific or tri-specific antibody comprising an anti-CD20 binding domain, anti-CD20 antibody-drug conjugates, or anti-CD20 CAR). Anti-CD20 antibodies include, but are not limited to, rituximab (RITUXAN® - Roche/Genentech), ocrelizumab (OCREVUS® - Roche/Genentech), obinutuzumab (GAZYVA® - Roche/Genentech), ofatumumab (ARZERRA® - Novartis), and velutuzumab. In certain embodiments, the anti-CD20 binding domain therapy is used in combination with the heterodimeric Fc-fused protein therapy to treat subjects known or suspected of having cancer. In certain embodiments, the anti-CD20 binding domain therapy is administered by IV infusion. In certain embodiments, the anti-CD20 binding domain therapy is administered at a dose of 100 mg/m², 200 mg/m², 300 mg/m², 400 mg/m², 500 mg/m², 600 mg/m², 700 mg/m², 800 mg/m², 900 mg/m², or 1000 mg/m². In certain embodiments, the anti-CD20 binding domain therapy is administered at a dose of 375 mg/m². In certain embodiments, the anti-CD20 binding domain therapy is administered at a dose of at least 100 mg/m², at least 200 mg/m², at least 300 mg/m², at least 400 mg/m², at least 500 mg/m², at least 600 mg/m², at least 700 mg/m², at least 800 mg/m², at least 900 mg/m², or at least 1000 mg/m². In certain embodiments, the anti-CD20 binding domain therapy is administered at a dose of less than 400 mg/m². In certain embodiments, the anti-CD20 binding domain therapy is administered at a dose of less than 375 mg/m².

[00427] In certain embodiments, the anti-CD20 binding domain therapy is used in combination with the heterodimeric Fc-fused protein therapy to treat subjects known or suspected of having Non-Hodgkin's Lymphoma (NHL). In certain embodiments, the anti-CD20 binding domain

therapy is administered at a dose of 375 mg/m² by IV-infusion. In certain embodiments, the anti-CD20 binding domain therapy is administered at a dose less than 375 mg/m² by IV-infusion.

[00428] In certain embodiments, the anti-CD20 binding domain therapy is used in combination with the heterodimeric Fc-fused protein therapy to treat subjects known or suspected of having Chronic Lymphocytic Leukemia (CLL). In certain embodiments, the anti-CD20 binding domain therapy is administered at a dose of 375 mg/m² by IV-infusion in a first cycle, and at a dose of 500 mg/m² by IV-infusion per cycle in an additional 2-6 cycles. In certain embodiments, the anti-CD20 binding domain therapy is administered at a dose less than 375 mg/m² by IV-infusion. The combined anti-CD20 binding domain and heterodimeric Fc-fused protein therapy can be used in combination with fludarabine and cyclophosphamide (FC).

[00429] In certain embodiments, the anti-CD20 binding domain therapy is used in combination with the heterodimeric Fc-fused protein therapy to treat subjects known or suspected of having Rheumatoid Arthritis (RA). In certain embodiments, the anti-CD20 binding domain therapy is administered as two doses of 1000 mg, doses separated 2 weeks, by IV-infusion. In certain embodiments, the anti-CD20 binding domain therapy is administered as two doses of 1000 mg, doses separated 2 weeks, by IV-infusion up to 24 weeks. In certain embodiments, the combined anti-CD20 binding domain and heterodimeric Fc-fused protein therapy is coadministered with methotrexate.

[00430] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with a therapy comprising an antibody therapy comprising an agonist antibody. In certain embodiments, the agonist antibody is an anti-4-1BB antibody, an anti-CD137 antibody, an anti-FAP antibody, an anti-OX40 antibody, an anti-CD40 antibody, an anti-GITR antibody, or an anti-CD27 antibody. In certain embodiments, the agonist antibody is a bispecific antibody. In certain embodiments, the agonist antibody is a multispecific antibody, *e.g.*, a bispecific antibody, comprising two or more antigen binding domains selected from an anti-4-1BB antibody, an anti-CD137 antibody, an anti-FAP antibody, an anti-OX40 antibody, an anti-CD40 antibody, an anti-GITR antibody, or an anti-CD27 antibody. An illustrative example is a bispecific agonist antibody targeting 4-1BB and CD137, such as utomilumab (Pfizer).

(v) Checkpoint Inhibitor Therapy

[00431] In certain embodiments, the heterodimeric Fc-fused protein therapy can be combined with a checkpoint inhibitor therapy. Illustrative immune checkpoint molecules that can be targeted for blocking or inhibition include, but are not limited to, CTLA-4, 4-1BB (CD137), 4-1BBL (CD137L), PDL1, PDL2, PD1, B7-H3, B7-H4, BTLA, HVEM, TIM3, GAL9, LAG3, TIM3,

B7H3, B7H4, VISTA, KIR, 2B4 (belongs to the CD2 family of molecules and is expressed on all NK, $\gamma\delta$, and memory CD8⁺ ($\alpha\beta$) T cells), CD160 (also referred to as BY55), and CGEN-15049. Immune checkpoint inhibitors include antibodies, or antigen binding fragments thereof, or other binding proteins, that bind to and block or inhibit the activity of one or more of CTLA-4, PDL1, PDL2, PD1, B7-H3, B7-H4, BTLA, HVEM, TIM3, GAL9, LAG3, TIM3, B7H3, B7H4, VISTA, KIR, 2B4, CD160, and CGEN-15049. Illustrative immune checkpoint inhibitors include nivolumab (anti-PD-1; OPDIVO® - BMS), AMP224 (anti-PD-1; NCI), pembrolizumab (anti-PD-1; MK-3475/KEYTRUDA® - Merck), pidilizumab (anti-PD-1 antibody; CT-011 - Teva/CureTech), atezolizumab (anti-PD-L1; TECENTRIQ® - Roche/Genentech), durvalumab (anti-PD-L1; MEDI4736/IMFINZI® - Medimmune/AstraZeneca), avelumab (anti-PD-L1; BAVENCIO® - Pfizer), BMS-936559 (anti-PD-L1 - BMS), ipilimumab (anti-CTLA-4; YERVOY® - BMS), tremelimumab (anti-CTLA-4; Medimmune/AstraZeneca), lirilumab (anti-KIR; BMS), monalizumab (anti-NKG2A; Innate Pharma/AstraZeneca), BY55 (anti-CD160), anti-OX40, anti-TIM3, and anti-LAG3.

[00432] In certain embodiments, the method of the present invention further comprises administering to the subject an anti-PD-1 antibody. Many anti-PD-1 antibodies have been developed for therapeutic purposes and are described in, for example, Gong *et al.*, (2018) J. ImmunoTher Cancer (2018) 6:8. In certain embodiments, the anti-PD-1 antibody is pembrolizumab. In certain embodiments pembrolizumab can be administered via various routes, *e.g.*, intravenously, subcutaneously, intramuscularly, or intraperitoneally. In certain embodiments, pembrolizumab is administered intravenously. In certain embodiments, pembrolizumab can be administered at a dose of about 100 mg, about 125 mg, about 150 mg, about 175 mg, about 200 mg, about 225 mg, about 250 mg, about 275 mg, about 300 mg, or about 400 mg. In certain embodiments, pembrolizumab is administered at a dose of about 200 mg every 3 weeks. In certain embodiments, pembrolizumab is administered at a dose of about 400 mg every 6 weeks. In certain embodiments about 200 mg of pembrolizumab is administered on Day 1 of the initial treatment cycle. In certain embodiments, if the subject receives one or more subsequent treatment cycles, 200 mg of pembrolizumab is administered once every three weeks in the subsequent treatment cycles, starting from Day 1 of the first subsequent treatment cycle. In some embodiments, administration of pembrolizumab can precede each administration of the pharmaceutical formulation, can be concurrent with each administration of the pharmaceutical formulation, or follow each administration of the pharmaceutical formulation. In certain embodiments, administration of pembrolizumab precedes each administration of the pharmaceutical formulation.

In some embodiments, the pharmaceutical formulation can be administered within about 10 minutes, about 20 minutes, about 30 minutes, about 40 minutes, about 50 minutes, about 1 hour, about 1.5 hours, about 2 hours, about 2.5 hours, about 3 hours, about 3.5 hours, about 4 hours, about 4.5 hours, or about 5 hours after completion of administration of pembrolizumab. In certain embodiments, the pharmaceutical formulation is administered within 1 hour after completion of administration of pembrolizumab.

[00433] In some embodiments, the pharmaceutical formulation administered in combination with pembrolizumab is for the treatment of a cancer selected from the group consisting of: melanoma, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), head and neck squamous cell carcinoma (HNSCC), classical Hodgkin lymphoma, primary mediastinal large B-cell lymphoma, urothelial carcinoma, microsatellite instability-high cancer, gastric cancer, oesophageal cancer, cervical cancer, hepatocellular carcinoma, Merkel cell carcinoma, renal cell carcinoma, and endometrial carcinoma.

[00434] In certain embodiments, the anti-PD-1 antibody is nivolumab. In certain embodiments nivolumab can be administered via various routes, *e.g.*, intravenously, subcutaneously, intramuscularly, or intraperitoneally. In certain embodiments, nivolumab is administered intravenously. In some embodiments, nivolumab can be administered at a dose of about 200 mg, about 220 mg, about 240 mg, about 260 mg, about 280 mg, about 300 mg, about 320 mg, about 340 mg, about 360 mg, about 380 mg, about 400 mg, about 420 mg, about 440 mg, about 460 mg, about 480 mg, about 500 mg, about 520 mg, about 540 mg, about 560 mg, about 580 mg, or about 600 mg. In certain embodiments, nivolumab is administered at a dose of about 240 mg. In certain embodiments, nivolumab is administered at a dose of about 240 mg once about every two weeks. In certain embodiments, nivolumab is administered at a dose of about 360 mg. In certain embodiments, nivolumab is administered at a dose of about 360 mg once about every three weeks. In certain embodiments, nivolumab is administered at a dose of about 480 mg. In certain embodiments, nivolumab is administered at a dose of about 480 mg once about every four weeks. In some embodiments, administration of nivolumab can precede each administration of the pharmaceutical formulation, can be concurrent with each administration of the pharmaceutical formulation, or follow each administration of the pharmaceutical formulation. In certain embodiments, administration of nivolumab precedes each administration of the pharmaceutical formulation. In some embodiments, the pharmaceutical formulation can be administered within about 10 minutes, about 20 minutes, about 30 minutes, about 40 minutes, about 50 minutes, about 1 hour, about 1.5 hours, about 2 hours, about 2.5 hours, about 3 hours, about 3.5 hours, about 4

hours, about 4.5 hours, or about 5 hours after completion of administration of nivolumab. In certain embodiments, the pharmaceutical formulation is administered within 1 hour after completion of administration of nivolumab. In some embodiments, the pharmaceutical formulation administered in combination with nivolumab is for the treatment of a cancer selected from the group consisting of: melanoma, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), renal cell carcinoma, classical Hodgkin lymphoma, head and neck squamous cell carcinoma (HNSCC), colorectal cancer, hepatocellular carcinoma, bladder cancer, and oesophageal cancer. In certain embodiments the cancer is melanoma. In certain embodiments the melanoma is unresectable or metastatic. In some embodiments the cancer is colorectal cancer. In certain embodiments, the colorectal cancer is microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer.

[00435] In certain embodiments, the checkpoint inhibitor therapy is used in combination with the heterodimeric Fc-fused protein therapy to treat subjects known or suspected of having cancer. In certain embodiments, the checkpoint inhibitor therapy is administered by IV infusion. In certain embodiments, the checkpoint inhibitor therapy is administered by IV infusion over 30 minutes. In certain embodiments, the checkpoint inhibitor therapy is administered every 3 weeks. In certain embodiments, the checkpoint inhibitor therapy is administered at a dose of about 100 mg, about 200 mg, about 300 mg, about 400 mg, about 500 mg, about 600 mg, about 700 mg, about 800 mg, about 900 mg, or about 1000 mg. In certain embodiments, the checkpoint inhibitor therapy is administered at a dose of 200 mg. In certain embodiments, the checkpoint inhibitor therapy is administered at a dose of at least 100 mg, at least 200 mg, at least 300 mg, at least 400 mg, at least 500 mg, at least 600 mg, at least 700 mg, at least 800 mg, at least 900 mg, or at least 1000 mg. In certain embodiments, the checkpoint inhibitor therapy is administered at a dose of less than 200 mg. In certain embodiments, the checkpoint inhibitor therapy is used in combination with the heterodimeric Fc-fused protein therapy to treat subjects known or suspected of having melanoma, non-small cell lung cancer (NSCLC), head and neck squamous cell carcinoma (HNSCC), classical Hodgkin lymphoma, primary mediastinal large B-Cell lymphoma, bladder cancer, urothelial carcinoma, microsatellite instability-high cancer, colorectal cancer, gastric cancer, oesophageal cancer, cervical cancer, hepatocellular carcinoma, Merkel cell carcinoma, renal cell carcinoma (RCC), endometrial carcinoma, cutaneous T cell lymphoma, and triple negative breast cancer.

(vi) Additional Cytokine Therapy

[00436] In some embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more additional cytokine therapies, one or more chemokine therapies, or combinations thereof.

In some embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more additional cytokine therapies. In some embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more chemokine therapies. In some embodiments, the cytokine therapy comprises a pro-inflammatory cytokine, a Th1 cytokine, or a Th2 cytokine. In some embodiments, the cytokine therapy comprises a recombinant human cytokine or chemokine.

[00437] In some embodiments, the cytokine therapy includes a cytokine that is an interleukin (*e.g.*, IL-1, IL-2, IL-6, IL-7, IL-8, IL-12, IL-13, IL-15, IL-16, IL-18, IL-21 and IL-22). In some embodiments, the cytokine therapy includes a cytokine that is growth factor (*e.g.*, tumor necrosis factor (TNF), LT, EMAP-II, GM-CSF, FGF and PDGF). In some embodiments, the cytokine therapy comprises an anti-inflammatory cytokine (*e.g.*, IL-4, IL-10, IL-11, IL-13 and TGF).

[00438] In some embodiments, the chemokine therapy includes a pro-inflammatory chemokine (*e.g.*, GRO- α , GRO-b, LIX, GCP-2, MIG, IP10, I-TAC, and MCP-1, RANTES, Eotaxin, SDF-1, and MIP3a). In some embodiments, the chemokine therapy includes a chemokine receptor. In some embodiments, the chemokine therapy includes a CXC chemokine receptor (*e.g.*, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, and CXCR7), a CC chemokine receptor (*e.g.*, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, and CCR11), a CX3C chemokine receptor (*e.g.*, CX3CR1), or a XC chemokine receptor (*e.g.*, XCR1). In some embodiments, the chemokine therapy comprises a G protein-linked transmembrane receptor.

[00439] In some embodiments, the cytokine therapy comprises a cytokine therapy that synergizes with the IL-12 signaling. In some embodiments, the cytokine therapy comprises an IL-2 cytokine or a derivative thereof. In some embodiments, the IL-2 therapy is aldesleukin (Proleukin – Prometheus Therapeutics). In some embodiments, the IL-2 therapy and/or aldesleukin is administered intravenously. In some embodiments, the cytokine therapy comprises an IL-15 cytokine or a derivative thereof. In some embodiments, the IL-15 therapy is ALT-803 (Altor Bioscience) or NKTR-255 (Nektar). In some embodiments, the IL-15 therapy, NKTR-255, and/or ALT-803 is administered subcutaneously. In some embodiments, the chemokine therapy comprises a CXCL9 chemokine, a CXCL10 chemokine, or derivatives thereof.

[00440] In some embodiments, the cytokine or chemokine therapy includes administering a cytokine or chemokine to a subject. In some embodiments, the cytokine or chemokine therapy includes administering a recombinant cytokine or chemokine to a subject. In some embodiments, the cytokine or chemokine therapy includes engineering a cell to produce the cytokine or chemokine. In some embodiments, the cytokine or chemokine therapy includes engineering a cell *ex vivo*, *in vitro*, or *in vivo* to produce the cytokine or chemokine.

[00441] In some embodiments, the cytokine or chemokine therapy includes engineering a cell to produce the cytokine or chemokine using a viral vector-based delivery platform such as vaccinia, fowlpox, self-replicating alphavirus, marabavirus, adenovirus (*See, e.g., Tatsis et al., Adenoviruses, Molecular Therapy* (2004) 10, 616—629), a lentivirus, including but not limited to second, third or hybrid second/third generation lentivirus and recombinant lentivirus of any generation designed to target specific cell types or receptors (*See, e.g., Hu et al., Immunization Delivered by Lentiviral Vectors for Cancer and Infectious Diseases, Immunol Rev.* (2011) 239(1): 45-61, Sakuma *et al.*, Lentiviral vectors: basic to translational, *Biochem J.* (2012) 443(3):603-18, Cooper *et al.*, Rescue of splicing-mediated intron loss maximizes expression in lentiviral vectors containing the human ubiquitin C promoter, *Nucl. Acids Res.* (2015) 43 (1): 682-690, Zufferey *et al.*, Self-Inactivating Lentivirus Vector for Safe and Efficient *In vivo* Gene Delivery, *J. Virol.* (1998) 72 (12): 9873-9880), or an adeno-associated virus (“AAV”) vector, as described in more detail in U.S. Pat. No. 5,173,414; Tratschin *et al.*, *Mol. Cell. Biol.* 5:3251-3260 (1985); Tratschin, *et al.*, *Mol. Cell. Biol.* 4:2072-2081 (1984); Hermonat *et al.*, *PNAS* 81:6466-6470 (1984); and Samulski *et al.*, *J. Virol.* 63:03822-3828 (1989)). In some embodiments, the cytokine or chemokine therapy includes engineering a cell to produce the cytokine or chemokine using a LNP, liposome, or an exosome. In some embodiments, the cytokine or chemokine therapy includes engineering a cell to produce the cytokine or chemokine using genome editing, such as using a nuclease-based genome editing systems (*e.g.*, a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family, a Transcription activator-like effector nuclease (TALEN), a zinc-finger nuclease (ZFN), and a homing endonuclease (HE) based genome editing system or a derivative thereof). In some embodiments, the cytokine or chemokine therapy includes engineering a cell to produce the cytokine or chemokine using electroporation.

(vii) Innate Immune System Agonist Therapy

[00442] In some embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more innate immune system agonists.

[00443] In some embodiments, the innate immune system agonist comprises a toll-like receptor (TLR) agonist. In some embodiments, the TLR agonist comprises a TLR1/2, TLR2/6, TLR3, TLR4, TLR7, TLR8, TLR7/8, or TLR9 agonist. In some embodiments, a TLR2/6 agonist comprises lipoproteins, such as bacterial lipoproteins or derivatives, such as Pam2CSK4. In some embodiments, a TLR1/2 agonist comprises lipoproteins. In some embodiments, a TLR3 agonist comprises a dsRNA analog, such as rintatolimod (AMPLIGEN® - Hemispherx Biopharma) or

poly IC-LC (*e.g.*, HILTONOL®). In some embodiments, a TLR4 agonist comprises lipopolysaccharide (LPS, also referred to as endotoxin) or derivatives, such as lipid A. In some embodiments, a TLR7 agonist comprises a ssRNA or derivatives or imidazoquinoline derivatives including, but not limited to, resiquimod (also referred to as R848), imiquimod (ZYCLARA®, Aldara – Medicis), and gardiquimod. In some embodiments, a TLR7 agonist is also a TLR8 agonist, such as imiquimod or Medi-9197 (AstraZeneca/MedImmune). In some embodiments, a TLR9 agonist comprises a CpG-containing oligodeoxynucleotide (CpG-ODN) or SD-101 (Dynavax).

[00444] In some embodiments, the innate immune system agonist comprises a Stimulator of interferon genes (STING) agonist. In some embodiments, the STING agonist comprises a cyclic-di-nucleotide (CDN). In some embodiments, the CDN comprises a cyclic-di-AMP, a cyclic-di-GMP, or a cyclic-GMP-AMP (cGAMP). In some embodiments, the STING agonist comprises a nucleic acid (*e.g.*, DNA or RNA) that stimulates cGAS. In some embodiments, the STING agonist is ADU-S100 (also referred to as MIW815 - Aduro/Novartis).

(viii) Chemotherapy

[00445] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more chemotherapies. In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more chemotherapies to treat a subject diagnosed with cancer. Examples of chemotherapy agents include aldesleukin, alvociclib, antineoplaston AS2-1, antineoplaston A10, anti-thymocyte globulin, amifostine trihydrate, aminocamptothecin, arsenic trioxide, beta alethine, Bcl-2 family protein inhibitor ABT-263, ABT-199, BMS-345541, bortezomib (VELCADE®), bryostatin 1, busulfan, carboplatin, campath-1H, CC-5103, carmustine, caspofungin acetate, clofarabine, cisplatin, Cladribine (LEUSTARIN®), Chlorambucil (LEUKERAN®), Curcumin, cyclosporine, Cyclophosphamide (Cyloxan, Endoxan, Endoxana, Cyclostin), cytarabine, denileukin diftitox, dexamethasone, DT PACE, docetaxel, dolastatin 10, Doxorubicin (ADRIAMYCIN®, ADRIBLASTINE®), doxorubicin hydrochloride, enzastaurin, epoetin alfa, etoposide, Everolimus (RAD001), fenretinide, filgrastim, melphalan, mesna, Flavopiridol, Fludarabine (FLUDARA®), Geldanamycin (17-AAG), ifosfamide, irinotecan hydrochloride, ixabepilone, Lenalidomide (REVLIMID®, CC-5013), lymphokine-activated killer cells, melphalan, methotrexate, mitoxantrone hydrochloride, motexafin gadolinium, mycophenolate mofetil, nelarabine, oblimersen (GENASENSE®) Obatoclax (GX15-070), oblimersen, octreotide acetate, omega-3 fatty acids, oxaliplatin, paclitaxel, PD0332991, PEGylated liposomal doxorubicin hydrochloride, pegfilgrastim, Pentostatin (NIPENT®), perfosine, Prednisolone,

Prednisone, R-roscovitine (SELICILIB®, CYC202), recombinant interferon alfa, recombinant interleukin-12, recombinant interleukin-11, recombinant flt3 ligand, recombinant human thrombopoietin, rituximab, sargramostim, sildenafil citrate, simvastatin, sirolimus, Styryl sulphones, tacrolimus, tanespimycin, Temsirolimus (CC1-779), Thalidomide, therapeutic allogeneic lymphocytes, thiotepa, tipifarnib, VELCADE® (BORTEZOMIB® or PS-341), Vincristine (ONCOVIN®), vincristine sulfate, vinorelbine ditartrate, Vorinostat (SAHA), and FR (fludarabine, rituximab), CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone), CVP (cyclophosphamide, vincristine and prednisone), FCM (fludarabine, cyclophosphamide, mitoxantrone), FCR (fludarabine, cyclophosphamide, rituximab), hyperCVAD (hyperfractionated cyclophosphamide, vincristine, doxorubicin, dexamethasone, methotrexate, cytarabine), ICE (ifosfamide, carboplatin and etoposide), MCP (mitoxantrone, chlorambucil, and prednisolone), R-CHOP (rituximab plus CHOP), R-CVP (rituximab plus CVP), R-FCM (rituximab plus FCM), R-ICE (rituximab-ICE), and R-MCP (R-MCP).

[00446] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more chemotherapies to treat a subject diagnosed with colon cancer, rectal cancer, or colorectal cancer (CRC). In certain embodiments, the chemotherapy comprises FOLFOX (5-FU, leucovorin, and oxaliplatin/Eloxatin), FOLFIRI (leucovorin, 5-FU, and irinotecan/Camptosar), FOLFOXIRI (leucovorin, 5-FU, oxaliplatin, and irinotecan), CapeOx (capecitabine and oxaliplatin), 5-FU coadministered with leucovorin, capecitabine (XELODA®) alone, or Trifluridine and tipiracil (LONSURF®). In certain embodiments, the chemotherapy comprises a VEGF targeting agent, such as bevacizumab (AVASTIN®), ziv-aflibercept (ZALTRAP®), ramucirumab (CYRAMZA®), or Regorafenib (STIVARGA®), or an EGFR targeting agent such as cetuximab (ERBITUX) or panitumumab (VECTIBIX®). In certain embodiments, the chemotherapy coadministers a chemotherapy selected from FOLFOX, FOLFIRI, FOLFOXIRI, CapeOx, 5-FU coadministered with leucovorin, capecitabine alone, and Trifluridine/tipiracil together with a VEGF targeting agent or an EGFR targeting agent.

[00447] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more chemotherapies to treat a subject diagnosed with breast cancer. In certain embodiments, the chemotherapy comprises doxorubicin (ADRIAMYCIN®), pegylated liposomal doxorubicin, epirubicin (ELLENCE®), paclitaxel (Taxol), docetaxel (TAXOTERE®), albumin-bound paclitaxel (ABRAXANE®), 5-fluorouracil (5-FU), cyclophosphamide (CYTOXAN®), carboplatin (PARAPLATIN®), cisplatin, vinorelbine (NAVELBINE®), capecitabine (XELODA), gemcitabine (GEMZAR®), ixabepilone (IXEMPRA®), or eribulin (HALAVEN). In

certain embodiments, the chemotherapy comprises a combination of two or more chemotherapies selected from doxorubicin (ADRIAMYCIN®), pegylated liposomal doxorubicin, epirubicin (ELLENCE®), paclitaxel (Taxol), docetaxel (TAXOTERE®), albumin-bound paclitaxel (ABRAXANE®), 5-fluorouracil (5-FU), cyclophosphamide (CYTOXAN®), carboplatin (PARAPLATIN®), cisplatin, vinorelbine (NAVELBINE®), capecitabine (XELODA®), gemcitabine (GEMZAR®), ixabepilone (IXEMPRA®), and eribulin (HALAVEN®).

[00448] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more chemotherapies to treat a subject diagnosed with melanoma/skin-cancer. In certain embodiments, the chemotherapy comprises dacarbazine (also called DTIC), temozolomide, nab-paclitaxel, paclitaxel, cisplatin, carboplatin, or vinblastine.

(ix) Targeted Agent Therapy

[00449] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more targeted agents. In general, targeted agents act on specific molecular targets, such as targets associated with cancer. Targeted agents are differentiated from standard chemotherapies in that standard chemotherapies act on all rapidly dividing normal and cancerous cells. Targeted agents include, but are not limited to, a hormone therapy, a signal transduction inhibitor, a gene expression modulator, an apoptosis inducer, an angiogenesis inhibitor, an immunotherapy, a toxin delivery molecule (*e.g.*, an antibody drug-conjugate), and a kinase inhibitor. In certain embodiments, a targeted agent comprises a receptor agonist or ligand.

[00450] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more targeted agents to treat a subject diagnosed with colon cancer, rectal cancer, or colorectal cancer (CRC). In certain embodiments, the targeted agent comprises cetuximab (ERBITUX®), panitumumab (VECTIBIX®), bevacizumab (AVASTIN®), ziv-aflibercept (ZALTRAP®), regorafenib (STIVARGA®), ramucirumab (CYRAMZA®), nivolumab (OPDIVO®), or ipilimumab (YERVOY®).

[00451] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more targeted agents to treat a subject diagnosed with breast cancer. In certain embodiments, the targeted agent comprises everolimus (AFINITOR®), tamoxifen (NOLVADEX®), toremifene (FARESTON®), trastuzumab (HERCEPTIN®), fulvestrant (FASLODEX®), anastrozole (ARIMIDEX®), exemestane (AROMASIN®), lapatinib (TYKERB®), letrozole (FEMARA®), pertuzumab (PERJETA®), ado-trastuzumab emtansine (KADCYLA®), palbociclib (IBRANCE®), ribociclib (KISQALI®), neratinib maleate (NERLYNX™), abemaciclib

(VERZENIO™), olaparib (LYNPARZA™), atezolizumab (TECENTRIQ®), or alpelisib (PIQRAY®).

[00452] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more targeted agents to treat a subject diagnosed with melanoma/skin-cancer. In certain embodiments, the targeted agent comprises Vismodegib (ERIVEDGE®), sonidegib (ODOMZO®), ipilimumab (YERVOY®), vemurafenib (ZELBORAF®), trametinib (MEKINIST®), dabrafenib (TAFINLAR®), pembrolizumab (KEYTRUDA®), nivolumab (OPDIVO®), cobimetinib (COTELLIC™), alitretinoin (PANRETIN®), avelumab (BAVENCIO®), encorafenib (BRAFTOVI™), binimetinib (MEKTOVI®), or cemiplimab-rwlc (LIBTAYO®).

[00453] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with a receptor agonist or ligand therapy. In certain embodiments, the receptor agonist or ligand therapy comprises an agonist antibody. In certain embodiments, the receptor agonist or ligand therapy comprises a receptor ligand, such as 4-1BBL or CD40L.

(x) Radiotherapy

[00454] In some embodiments, the heterodimeric Fc-fused protein therapy is combined with radiotherapy. In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with a radioisotope particle, such as indium In-111, yttrium Y-90, or iodine I-131. Examples of combination therapies include, but are not limited to, Iodine-131 tositumomab (BEXXAR®), Yttrium-90 ibritumomab tiuxetan (ZEVALIN®), and BEXXAR® with CHOP. In certain embodiments, the radiotherapy comprises external-beam radiation therapy (EBRT), internal radiation therapy (brachytherapy), endocavitary radiation therapy, interstitial brachytherapy, radioembolization, hypofractionated radiation therapy, intraoperative radiation therapy (IORT), 3D-conformal radiotherapy, stereotactic radiosurgery (SRS), or stereotactic body radiation therapy (SBRT).

[00455] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more radiotherapies to treat a subject diagnosed with colon cancer, rectal cancer, or colorectal cancer (CRC). In certain embodiments, the radiotherapy comprises external-beam radiation therapy (EBRT), internal radiation therapy (brachytherapy), endocavitary radiation therapy, interstitial brachytherapy, or radioembolization.

[00456] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more radiotherapies to treat a subject diagnosed with breast cancer. In certain embodiments,

the radiotherapy comprises external-beam radiation therapy, hypofractionated radiation therapy, intraoperative radiation therapy (IORT), or 3D-conformal radiotherapy.

[00457] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more radiotherapies to treat a subject diagnosed with melanoma/skin-cancer. In certain embodiments, the radiotherapy comprises stereotactic radiosurgery (SRS; *e.g.*, using a Gamma Knife or linear accelerator) or stereotactic body radiation therapy (SBRT).

(xi) Vaccine and Oncolytic Viruses Therapy

[00458] In some embodiments, the heterodimeric Fc-fused protein therapy is combined with one or more immunogenic compositions, *e.g.*, a vaccine composition or an oncolytic virus, capable of raising a specific immune response, *e.g.*, a tumor-specific immune response.

[00459] In some embodiments, the heterodimeric Fc-fused protein therapy is combined with a vaccine composition. Vaccine compositions typically comprise a plurality of antigens and or neoantigens specific for the tumor to be targeted. Vaccine compositions can also be referred to as vaccines.

[00460] In some embodiments, a vaccine composition further comprises an adjuvant and/or a carrier. In some embodiments, a vaccine composition associates with a carrier such as a protein or an antigen-presenting cell such as a dendritic cell (DC) capable of presenting the peptide to a T-cell. In some embodiments, carriers are scaffold structures, for example a polypeptide or a polysaccharide, to which an antigen or neoantigen, is capable of being associated.

[00461] In general, adjuvants are any substance whose admixture into a vaccine composition increases or otherwise modifies the immune response to an antigen or neoantigen. Optionally, adjuvants are conjugated covalently or non-covalently. The ability of an adjuvant to increase an immune response to an antigen is typically manifested by a significant or substantial increase in an immune-mediated reaction, or reduction in disease symptoms. For example, an increase in humoral immunity is typically manifested by a significant increase in the titer of antibodies raised to the antigen, and an increase in T-cell activity is typically manifested in increased cell proliferation, or cellular cytotoxicity, or cytokine secretion. An adjuvant may also alter an immune response, for example, by changing a primarily humoral or Th response into a primarily cellular, or Th response. Suitable adjuvants include, but are not limited to 1018 ISS, alum, aluminum salts, Amplivax, AS15, BCG, CP-870,893, CpG7909, CyaA, dSLIM, GM-CSF, IC30, IC31, Imiquimod, ImuFact IMP321, IS Patch, ISS, ISCOMATRIX, JuvImmune, LipoVac, MF59, monophosphoryl lipid A, Montanide IMS 1312, Montanide ISA 206, Montanide ISA 50V, Montanide ISA-51, OK-432, OM-174, OM-197-MP-EC, ONTAK, PepTel vector system, PLG microparticles, resiquimod,

SRL172, Virosomes and other Virus-like particles, YF-17D, VEGF trap, R848, beta-glucan, Pam3Cys, Aquila's QS21 stimulon (Aquila Biotech, Worcester, Mass., USA) which is derived from saponin, mycobacterial extracts and synthetic bacterial cell wall mimics, and other proprietary adjuvants such as Ribi's Detox. Quil or Superfos. Adjuvants such as incomplete Freund's or GM-CSF are useful. Several immunological adjuvants (e.g., MF59) specific for dendritic cells and their preparation have been described previously (Dupuis M, et al., Cell Immunol. 1998; 186(1):18-27; Allison A C; Dev Biol Stand. 1998; 92:3-11). Cytokines can also be used. Several cytokines have been directly linked to influencing dendritic cell migration to lymphoid tissues (e.g., TNF-alpha), accelerating the maturation of dendritic cells into efficient antigen-presenting cells for T-lymphocytes (e.g., GM-CSF, IL-1 and IL-4) (U.S. Pat. No. 5,849,589, specifically incorporated herein by reference in its entirety) and acting as immunoadjuvants (e.g., IL-12) (Gabrilovich D I, et al., J Immunother Emphasis Tumor Immunol. 1996 (6):414-418). In some embodiments, an adjuvant comprises a CpG immunostimulatory oligonucleotide. In some embodiments, an adjuvant comprises a TLR agonist.

[00462] Other examples of useful adjuvants include, but are not limited to, chemically modified CpGs (e.g. CpR, Idera), Poly(I:C)(e.g. polyi:CI2U), non-CpG bacterial DNA or RNA as well as immunoactive small molecules and antibodies such as cyclophosphamide, sunitinib, bevacizumab, celecoxib, NCX-4016, sildenafil, tadalafil, vardenafil, sorafenib, XL-999, CP-547632, pazopanib, ZD2171, AZD2171, ipilimumab, tremelimumab, and SC58175, which may act therapeutically and/or as an adjuvant. The amounts and concentrations of adjuvants and additives can readily be determined by the skilled artisan without undue experimentation. Additional adjuvants include colony-stimulating factors, such as Granulocyte Macrophage Colony Stimulating Factor (GM-CSF, sargramostim).

[00463] In some embodiments, a vaccine composition comprises more than one different adjuvant. In some embodiments, a vaccine composition comprises any adjuvant substance including any of the above or combinations thereof. It is also contemplated that a vaccine and an adjuvant can be administered together or separately in any appropriate sequence.

[00464] In some embodiments, a carrier (or excipient) is present independently of an adjuvant. In some embodiments, the function of a carrier is to increase the molecular weight, increase activity or immunogenicity, to confer stability, to increase the biological activity, or to increase serum half-life. In some embodiments, a carrier aids presenting peptides to T-cells. In some embodiments, a carrier comprises any suitable carrier known to the person skilled in the art, for example a protein or an antigen presenting cell. Examples of carrier proteins include, but are not limited to, keyhole

limpet hemocyanin, serum proteins such as transferrin, bovine serum albumin, human serum albumin, thyroglobulin or ovalbumin, immunoglobulins, or hormones, such as insulin or palmitic acid. For immunization of humans, the carrier is generally a physiologically acceptable carrier acceptable to humans and safe. However, tetanus toxoid and/or diphtheria toxoid are suitable carriers. Alternatively, the carrier can be a dextran, for example Sepharose.

[00465] In some embodiments, a vaccine comprises a viral vector-based vaccine platform, such as vaccinia, fowlpox, self-replicating alphavirus, maraba virus, adenovirus (*See, e.g.,* Tatsis et al., *Adenoviruses, Molecular Therapy* (2004) 10, 616—629), or lentivirus, including but not limited to second, third or hybrid second/third generation lentivirus and recombinant lentivirus of any generation designed to target specific cell types or receptors (*See, e.g.,* Hu et al., *Immunization Delivered by Lentiviral Vectors for Cancer and Infectious Diseases, Immunol Rev.* (2011) 239(1): 45-61, Sakuma et al., *Lentiviral vectors: basic to translational, Biochem J.* (2012) 443(3):603-18, Cooper et al., *Rescue of splicing-mediated intron loss maximizes expression in lentiviral vectors containing the human ubiquitin C promoter, Nucl. Acids Res.* (2015) 43 (1): 682-690, Zufferey et al., *Self-Inactivating Lentivirus Vector for Safe and Efficient In vivo Gene Delivery, J. Virol.* (1998) 72 (12): 9873-9880). In general, upon introduction into a host, infected cells express the antigen or neoantigen and thereby elicits a host immune (e.g., CTL) response against the peptide(s).

[00466] Dependent on the packaging capacity of the above mentioned viral vector-based vaccine platforms, in some embodiments, the vaccine composition comprises one or more viral-vectors. In some embodiments, viral-vectors comprise sequences flanked by non-mutated sequences, separated by linkers, or preceded with one or more sequences targeting a subcellular compartment (*See, e.g.,* Gros et al., *Prospective identification of neoantigen-specific lymphocytes in the peripheral blood of melanoma patients, Nat Med.* (2016) 22 (4):433-8, Stronen et al., *Targeting of cancer neoantigens with donor-derived T cell receptor repertoires, Science.* (2016) 352 (6291):1337-41, Lu et al., *Efficient identification of mutated cancer antigens recognized by T cells associated with durable tumor regressions, Clin Cancer Res.* (2014) 20(13):3401-10). Vaccinia vectors and methods useful in immunization protocols are described in, e.g., U.S. Pat. No. 4,722,848. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover et al. (*Nature* 351:456-460 (1991)). A wide variety of other vaccine vectors useful for therapeutic administration or immunization of neoantigens, e.g., *Salmonella typhi* vectors, and the like will be apparent to those skilled in the art from the description herein.

[00467] In some embodiments, the heterodimeric Fc-fused protein therapy is combined with an oncolytic virus therapy. In general, an oncolytic virus is a virus engineered to infect and kill mainly

cancer cells. In some embodiments, in addition to an oncolytic virus killing a cancer cell, the oncolytic virus induces an immune response to the cancer cell.

[00468] In certain embodiments, the heterodimeric Fc-fused protein therapy is combined with oncolytic virus therapy to treat a subject diagnosed with melanoma/skin-cancer. In certain embodiments, the oncolytic virus comprises talimogene laherparepvec (IMLYGIC®), also referred to as T-VEC. In some embodiments, a heterodimeric Fc-fused protein comprising subunits of IL-12 is used for treating cancer (*e.g.*, an advanced malignancy) in combination with an oncolytic virus (for example, Talimogene Laherparepvec (IMLYGIC®) or T-VEC).

(xii) Surgical Interventions

[00469] In some embodiments, the heterodimeric Fc-fused protein therapy is combined with surgical interventions, where abnormal tissue (*e.g.*, a tumor) is surgically removed. In some embodiments, the tumor is cut from the subject's body using scalpels or other sharp tools to cut the tumor and/or surrounding tissue. In some embodiments, lasers can be used to cut abnormal tissue (*e.g.*, a tumor). In some embodiments, surgical interventions can include the use of hyperthermia treatment, which exposes abnormal tissue (*e.g.*, a tumor) to kill the cells of the abnormal tissue or make them more sensitive to radiation and certain chemotherapy drugs. In some embodiments, surgical interventions can include the use of photodynamic therapy, where certain drugs are activated by light to kill cancer cells. The surgical intervention can involve open surgery or minimally invasive surgery. In some embodiments, the surgical intervention can be used to remove the entire tumor, to debulk a tumor, or to ease cancer symptoms.

[00470] In some embodiments, the surgical intervention can be performed in a subject prior to administering the heterodimeric Fc-fused protein therapy. In other embodiments, the surgical intervention can be performed in a subject concurrently with the heterodimeric Fc-fused protein therapy. In other embodiments, the surgical intervention can be performed in a subject after the heterodimeric Fc-fused protein therapy.

(xiii) Cryotherapy

[00471] In some embodiments, the heterodimeric Fc-fused protein therapy is combined with cryotherapy (also called cryoablation or cryosurgery). In some embodiments, the cryotherapy is administered to a patient by applying liquid nitrogen or argon gas to destroy abnormal tissue (*e.g.*, a tumor). In some embodiments, for tumors inside a subject's body, cryotherapy can be administered using a cryoprobe, and imaging procedures such as ultrasound or MRI can be used to guide a cryoprobe and/or to monitor freezing of target tissue.

[00472] In some embodiments, the cryotherapy can be administered to the patient prior to the heterodimeric Fc-fused protein therapy. In other embodiments, the cryotherapy can be administered to the patient concurrently with the heterodimeric Fc-fused protein therapy. In other embodiments, the cryotherapy can be administered to the subject after the heterodimeric Fc-fused protein therapy.

[00473] In certain embodiments, the method of treatment disclosed herein results in a disease response or improved survival of the subject or patient. For example, in certain embodiments, the disease response is a complete response, a partial response, or a stable disease. In certain embodiments, the improved survival is improved progression-free survival (PFS) or overall survival. Improvement (*e.g.*, in PFS) can be determined relative to a period prior to initiation of the treatment of the present disclosure. Methods of determining disease response (*e.g.*, complete response, partial response, or stable disease) and patient survival (*e.g.*, PFS, overall survival) for BTC (*e.g.*, advanced BTC, metastatic BTC), or biliary tract tumor therapy, are routine in the art and are contemplated herein. In some embodiments, disease response is evaluated according to RECIST 1.1 after subjecting the treated patient to contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI) of the affected area (*e.g.*, chest/abdomen and pelvis covering the area from the superior extent of the thoracic inlet to the symphysis pubis).

[00474] In some embodiments, biomarkers of immune activation are measured in order to assess biological activity. In certain embodiments, cellular parameters are assessed, *e.g.* peripheral blood mononuclear cell (PBMCs) for immunophenotyping (IPT) by flow cytometry. In certain embodiments, soluble factors are assessed, *e.g.*, cytokines and chemokines in serum samples. In certain embodiments, serum levels of c-reactive protein (CRP) are assessed to determine toxicity. In certain embodiments, if the CRP concentration in the subject's blood is higher than a threshold CRP concentration, then the subject is identified as being at risk for developing an adverse drug reaction. In certain embodiments, if the CRP concentration in the subject's blood is about the same or lower than the threshold C-reactive protein concentration, the subject is not identified as being at risk for developing an adverse drug reaction. In specific embodiments, if the CRP concentration in the subject's blood is higher than the threshold CRP concentration, the administration of the pharmaceutical formulation is paused; the heterodimeric Fc-fused protein is administered at a lower dose; or a remedial action is taken to reduce or alleviate the formulation's toxicity effects in the subject.

[00475] In certain embodiments, an *ex vivo* IL12 response assay is used to assess activity, wherein PBMCs are collected for *ex vivo* stimulation followed by analysis of IFN γ production.

In certain embodiments, circulating tumor (ct) deoxyribonucleic acid (DNA) may be assessed. In certain embodiments, tissue derived biomarkers are evaluated on pre-treatment and post-treatment biopsies, *e.g.*, to investigate a possible correlation between clinical efficacy and analyzed markers. In certain embodiments, levels of PD-L1 expression are determined, *e.g.*, using a commercially available kit (*e.g.*, Dako PD-L1 IHC 22C3 pharmDx). In certain embodiments, CD3 positivity as an assay for T cell infiltration is determined by immunohistochemistry (IHC). In certain embodiments, frequency and/or localization of tumor-infiltrating leukocytes (*e.g.*, CD8 T-cells, CD4 T-cells, T_{reg}, NK cells, macrophages [M1/2 profile]) is determined by IHC or immunofluorescence microscopy (IF). In some embodiments, a gene expression profile is performed. In some embodiments, pharmacogenomics is performed.

(m) Kits

[00476] The formulation of DF hIL12-Fc si is prepared as a lyophilized formulation or a liquid formulation. For preparing the lyophilized formulation, freeze-dried DF hIL12-Fc si is sterilized and stored in single-use glass vials. Several such glass vials are then packaged in a kit for delivering a dose to a subject diagnosed with a cancer or a tumor.

[00477] In one aspect, the present disclosure provides a kit including one or more vessels collectively including a formulation of about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 0.6 mg, about 0.7 mg, about 0.8 mg, about 0.9 mg, about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, about 6 mg, about 7 mg, about 8 mg, about 9 mg, or about 10 mg of a heterodimeric Fc-fused protein. In certain embodiments, the present disclosure provides a kit including one or more vessels collectively including a formulation of about 1 mg of a heterodimeric Fc-fused protein. In certain embodiments, the present disclosure provides a kit including one or more vessels collectively including a formulation of about 1 mg of the heterodimeric Fc-fused protein comprising a first polypeptide comprising the amino acid sequence of SEQ ID NO:290 and a second polypeptide comprising the amino acid sequence of SEQ ID NO:291

[00478] In certain embodiments, the formulation is prepared and packaged as a liquid formulation and stored as about as about 0.5 mg/vial to about 1.5 mg/vial (*e.g.*, about 0.5 mg/vial to about 1.5 mg/vial, about 0.6 mg/vial to about 1.4 mg/vial, about 0.7 mg/vial to about 1.3 mg/vial, about 0.8 mg/vial to about 1.2 mg/vial, about 0.5 mg/vial to about 1.1 mg/vial, about 0.5 mg/vial to about 1.4 mg/vial, about 0.5 mg/vial to about 1.3 mg/vial, about 0.5 mg/vial to about 1.2 mg/vial, about 0.5 mg/vial to about 1.1 mg/vial, about 0.5 mg/vial to about 1.1 mg/vial, about 0.6 mg/vial to about 1.5 mg/vial, about 0.6 mg/vial to about 1.4 mg/vial, about 0.6 mg/vial to about 1.3 mg/vial,

about 0.6 mg/vial to about 1.2 mg/vial, about 0.6 mg/vial to about 1.1 mg/vial, about 0.7 mg/vial to about 1.5 mg/vial, about 0.7 mg/vial to about 1.4 mg/vial, about 0.7 mg/vial to about 1.3 mg/vial, about 0.7 mg/vial to about 1.2 mg/vial, about 0.7 mg/vial to about 1.1 mg/vial, about 0.8 mg/vial to about 1.5 mg/vial, about 0.8 mg/vial to about 1.4 mg/vial, about 0.8 mg/vial to about 1.3 mg/vial, about 0.8 mg/vial to about 1.2 mg/vial, about 0.8 mg/vial to about 1.1 mg/vial, about 0.9 mg/vial to about 1.5 mg/vial, about 0.9 mg/vial to about 1.4 mg/vial, about 0.9 mg/vial to about 1.3 mg/vial, about 0.9 mg/vial to about 1.2 mg/vial, about 0.9 mg/vial to about 1.1 mg/vial). In certain embodiments, the formulation is a liquid formulation and stored as about 1 mg/vial.

[00479] In certain embodiments, the formulation is prepared and packaged as a lyophilized formulation and stored as about 0.5 mg/vial to about 1.5 mg/vial (*e.g.*, about 0.5 mg/vial to about 1.5 mg/vial, about 0.6 mg/vial to about 1.4 mg/vial, about 0.7 mg/vial to about 1.3 mg/vial, about 0.8 mg/vial to about 1.2 mg/vial, about 0.5 mg/vial to about 1.1 mg/vial, about 0.5 mg/vial to about 1.4 mg/vial, about 0.5 mg/vial to about 1.3 mg/vial, about 0.5 mg/vial to about 1.2 mg/vial, about 0.5 mg/vial to about 1.1 mg/vial, about 0.5 mg/vial to about 1.1 mg/vial, about 0.6 mg/vial to about 1.5 mg/vial, about 0.6 mg/vial to about 1.4 mg/vial, about 0.6 mg/vial to about 1.3 mg/vial, about 0.6 mg/vial to about 1.2 mg/vial, about 0.6 mg/vial to about 1.1 mg/vial, about 0.7 mg/vial to about 1.5 mg/vial, about 0.7 mg/vial to about 1.4 mg/vial, about 0.7 mg/vial to about 1.3 mg/vial, about 0.7 mg/vial to about 1.2 mg/vial, about 0.7 mg/vial to about 1.1 mg/vial, about 0.8 mg/vial to about 1.5 mg/vial, about 0.8 mg/vial to about 1.4 mg/vial, about 0.8 mg/vial to about 1.3 mg/vial, about 0.8 mg/vial to about 1.2 mg/vial, about 0.8 mg/vial to about 1.1 mg/vial, about 0.9 mg/vial to about 1.5 mg/vial, about 0.9 mg/vial to about 1.4 mg/vial, about 0.9 mg/vial to about 1.3 mg/vial, about 0.9 mg/vial to about 1.2 mg/vial, about 0.9 mg/vial to about 1.1 mg/vial). In certain embodiments, the formulation is a lyophilized formulation and stored as about 1 mg/vial.

[00480] In certain embodiments, the vessels collectively may include about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 0.6 mg, about 0.7 mg, about 0.8 mg, about 0.9 mg, about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, about 6 mg, about 7 mg, about 8 mg, about 9 mg, about 10 mg, about 12 mg, about 15 mg, about 20 mg, about 21 mg, about 24 mg, about 25 mg, about 27 mg, about 30 mg, about 35 mg, about 36 mg, about 40 mg, about 45 mg, about 50 mg, about 55 mg, about 60 mg, about 65 mg, about 70 mg, about 75 mg, about 80 mg, about 85 mg, about 90 mg, about 95 mg, or about 100 mg of the heterodimeric Fc-fused protein of the present disclosure (*e.g.*, DF hIL12-Fc si). In certain embodiments, the vessels include about 1 mg of the heterodimeric Fc-fused protein of the present disclosure (*e.g.*, DF hIL12-Fc si). In certain embodiments, the vessels include about 1 mg of the heterodimeric Fc-fused protein

comprising a first polypeptide comprising the amino acid sequence of SEQ ID NO: 290 and a second polypeptide comprising the amino acid sequence of SEQ ID NO: 291

[00481] In certain embodiments, the formulation in the vessels may be a lyophilized formulation. In certain embodiments, the formulation in the vessels may be a liquid formulation.

[00482] In certain embodiments, the formulation may be packed in kits containing a suitable number of vials. The information on the medication may be included, which are in accordance with approved submission documents. The kit may be shipped in transport cool containers (2° C to 8°C) that are monitored with temperature control devices.

[00483] The formulation may be stored at 2° C to 8°C until use. The vials of the formulations may be sterile and nonpyrogenic, and may not contain bacteriostatic preservatives.

[00484] The description above describes multiple aspects and embodiments of the invention. The patent application specifically contemplates all combinations and permutations of the aspects and embodiments.

EXAMPLES

[00485] The invention now being generally described, will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention and are not intended to limit the invention.

Example 1 – Method of Preparation

[00486] The proteins of the present invention are typically made using recombinant DNA technology. In one exemplary embodiment, a first nucleic acid sequence encoding the first polypeptide comprising a first subunit of a multisubunit protein (p40 subunit of human IL-12) fused to a first antibody Fc domain polypeptide was cloned into a first expression vector (pET-pSURE-Puro); a second nucleic acid sequence encoding a second polypeptide comprising a second, different subunit of a multisubunit protein (p35 subunit of human IL-12) fused to a second antibody Fc domain polypeptide was cloned into a second expression vector (pET-pSURE-Puro); and the first and the second expression vectors were stably transfected together into host cells (*e.g.*, Chinese Hamster Ovary cells) to produce the heterodimeric Fc-fused proteins.

[00487] Exemplary amino acid sequence encoded by the first expression vector is shown in SEQ ID NO:292. The first expression vector encoded a first polypeptide comprising a p40 subunit of human IL-12 fused to a human IgG1 Fc sequence comprising a Y349C mutation. The first

polypeptide also included K360E and K409W mutations that promote heterodimerization, and LALAPA (L234A, L235A, and P329A) mutations that reduce effector functions. In SEQ ID NO: 292, leader sequence is shown in italics, the p40 subunit sequence of human IL-12 is underlined, and the mutations are shown in bold.

*MDMRVPAQLLGLLLLWLP**GARC**IWELKKDVYVVELDWYPDAPGEMVVLT*
*CDTPEEDGITWTL**DQSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGEVL**SH*
*SLLLLHKKEDGIWSTDILKDQKEPKNKTF**LRCEAKNYSGRFTCW**WLT**TIS*
*TDLTFSVKSSRGSSDPQGVTCGAATLSAERVRGDNKEYEYSVE**CQEDSAC*
*PAAEESLPIEVMVDAVHKLKYENYTSSFFIRDI**KPDPPKNLQLKPLKNSRQ*
VEVSWEYPDTWSTPHSYFSLTFCVQVOGKSKREKKDRVFTDKTSATVICR
KNASISVRAQDRYYSSSWSEWASVPCSPKSSDKTHTCPPCPAPEAAGGPS
VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK
TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALAAPIEKTISK
*AKGQPREPQVCTLP**PSRDEL**TENQVSLTCLVKGFYPSDIAVEWESNGQPE*
*NNYKTTTPVLDS**SGSFFLYSWLTVDKSRWQQGNVFSCSVMHEALHNHY*
TQKSLSLSPG (SEQ ID NO:292)

[00488] Exemplary amino acid sequence encoded from the second expression vector is shown in SEQ ID NO: 293. The second expression vector encoded a second polypeptide comprising a p35 subunit of human IL-12 fused to a human IgG1 Fc sequence comprising a S354C mutation. The second polypeptide also included Q347R, D399V, and F405T mutations that promote heterodimerization, and LALAPA (L234A, L235A, and P329A) mutations that reduce effector functions. In SEQ ID NO: 293, leader sequence is shown in italics, the p35 subunit sequence of human IL-12 is underlined, and mutations are shown in bold.

*MDMRVPAQLLGLLLLWLP**GARC**RNL**PVATPD**PGMF**PCLHHSQNLLRAVSN*
*MLQKARQTLEFYPCTSEEIDHEDITKDKTSTVEACLP**LELT**KNESCLNSRE*
TSFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVEFKTMNAKLLMDPKR
QIFLDQNMLAVIDELMQALNFNSETVPQKSSLEEDFYKTKIKLCILLHAF
RIRAVTIDRVMSYLNASGGGGSGGGGSGGGGSEPKSSDKTHTCPPCPAPE
AAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE
VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALAAAP
*IEKTISKAKGQPREPRVYTLPP**CRDEL**TKNQVSLTCLVKGFYPSDIAVEWE*
SNGQPENNYKTTTPVLVSDGSFTLYSKLTVDKSRWQQGNVFSCSVMHEA
LHNHYTQKSLSLSPG (SEQ ID NO:293)

[00489] To achieve the highest yield of the protein, different ratios of the first and second expression vectors are explored to determine the optimal ratio for transfection into the host cells. After transfection, single clones are isolated for cell bank generation using methods known in the art, such as limited dilution, ELISA, FACS, microscopy, or Clonepix.

[00490] Clones are cultured under conditions suitable for bio-reactor scale-up and maintained expression of the proteins. The proteins are isolated and purified using methods known in the art including centrifugation, depth filtration, cell lysis, homogenization, freeze-thawing, affinity purification, gel filtration, ion exchange chromatography, hydrophobic interaction exchange chromatography, and mixed-mode chromatography.

Example 2 – Tumor Suppression by IL-12 Fused with a Silent Fc domain polypeptide in a CT26 Tumor Model

[00491] This example describes relative abilities of two IL-12-Fc fusion constructs of recombinant murine IL-12 (rmIL-12) to control tumor progression in a mouse colon cancer model. The two IL-12-Fc fusion variants used in this example were mIL-12-Fc wildtype (DF-mIL-12-Fc wt), which includes wild-type murine IL-12 p40 and p35 subunits fused to the N-termini of wild-type murine IgG2a Fc domain polypeptides, and mIL-12-Fc silent (DF-mIL-12-Fc si), which includes wild-type murine IL-12 p40 and p35 subunits fused to the N-termini of murine IgG2a Fc domain polypeptides with mutations L234A, L235A, and P329G. The amino acid sequences of the proteins are shown below:

mIL-12-p40-mIgG2A-EW (first chain of DF-mIL-12-Fc wt)

MWELEKDVYVVEVDWTPDAPGETVNLTCDTPEEDDITWTSDQRHGVIGS
GKTLTITVKEFLDAGQYTCHKGGETLSHSHLLHKKENGIWSTEILKNFKN
KTLKCEAPNYSGRFTCSWLVRNMDLKFNKSSSSSPDSRAVTCGMASL
SAEKVTLDRDYEKYSVSCQEDVTCPTAEETLPIELALEARQQNKYENYS
TSFFIRDIKPDPPKNLQMKPLKNSQVEVSWEYPDSWSTPHSYFSLKFFVRI
QRKKEKMKETEEGCNQKGAFLVEKTSTEVQCKGGNVCVQAQDRYYNSS
CSKWACVPCRVRSPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKIKDVLMS
LSPIVTCVVVDVSEDDPDVQISWVFNNEVHTAQTQTHREDYNSTLRVVS
ALPIQHQQDWMSGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLP
EEMTEKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDGS
YFMYSWLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPG (SEQ ID
NO:286)

mIL-12-p35-mIgG2A-RVT (second chain of DF-mIL-12-Fc wt)

RVIPVSGPARCLSQSRNLLKTDDMVKTAREKLKHYSCTAEDIDHEDITR
DQTSTLKTCLPLELHKNESCLATRETSSTTRGSCLPPQKTSMMTLCLGSI
YEDLKMYQTEFQAINAALQNHNHQQIILDKGMLVAIDELMQSLNHNGET
LRQKPPVGEADPYRVKMKLCILLHAFSTRVVTINRVMGYLSSAGGGGSG
GGGSGGGGSPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKIKDVLMSLSPIV
TCVVVDVSEDDPDVQISWVFNNEVHTAQTQTHREDYNSTLRVVSALPI
QHQQDWMSGKEFKCKVNNKDLPAPIERTISKPKGSVRAPRVYVLPPEEEM
TKKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDGSYTM
YSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPG (SEQ ID
NO:287)

mIL-12-p40-mIgG2A-EW-LALAPG (first chain of DF-mIL-12-Fc si)

MWELEKDVYVVEVDWTPDAPGETVNLTCDTPEEDDITWTSDQRHGVIGS
 GKTLTITVKEFLDAGQYTCHKGGETLSHSHLLHKKENGIWSTEILKNFKN
 KTFKCEAPNYSGRFTCSWLVRNMDLKFNIKSSSSSPDSRAVTCGMASL
 SAEKVTLQDRDYEKYSVSCQEDVTCPTAEETLPIELALEARQQNKYENYS
 TSFFIRDIKPDPPKNLQMKPLKNSQVEVSWEYPDSWSTPHSYFSLKFFVRI
 QRKKEKMKETEEGCNQKGAFLVEKTSTEVQCKGGNVCVQAQDRYYNSS
 CSKWACVPCRVRSPRGPTIKPCPPCKCPAPNAAGGPSVFIFPPKIKDVLMS
 LSPIVTCVVVDVSEDDPDVQISWVFNNEVHTAQTQTHREDYNSTLRVVS
 ALPIQHQQDWMSGKEFKCKVNNKDLGAPIERTISKPKGSVRAPQVYVLP
 EEEMTEKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSGDS
 YFMYSWLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPG (SEQ ID
 NO:288)

mIL-12-p35-mIgG2A-RVT-LALAPG (second chain of DF-mIL-12-Fc si)

RVIPVSGPARCLSQSRNLLKTDDMVKTAREKLKHYSCTAEDIDHEDITR
 DQTSTLKTCLPLELHKNESCLATRETSSTTRGSCLPPQKTSMMTLCLGSI
 YEDLKMYQTEFQAINAALQNHNHQQIILDKGMLVAIDELMQSLNHNGET
 LRQKPPVGEADPYRVKMKLCILLHAFSTRVVTINRVMGYLSSAGGGGSG
 GGGSGGGGSPRGPTIKPCPPCKCPAPNAAGGPSVFIFPPKIKDVLMSLPIV
 TCVVVDVSEDDPDVQISWVFNNEVHTAQTQTHREDYNSTLRVVSALPI
 QHQDWMSGKEFKCKVNNKDLGAPIERTISKPKGSVRAPRVYVLPPEEE
 MTKKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLVSDGSYT
 MYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPG (SEQ ID
 NO:289)

[00492] Briefly, 10^6 CT26-Tyrp1 colon carcinoma cells were injected subcutaneously into the flank of Balb/c mice. On Day 14 after tumor inoculation, when tumor volume reached 270 mm^3 , the mice were randomized into different treatment groups ($n = 10$ per group) and treated intraperitoneally with $1 \mu\text{g}$ of rmIL-12, DF-mIL-12-Fc wt at a molar dose equivalent to $1 \mu\text{g}$ rmIL-12, DF-mIL-12-Fc si at a molar dose equivalent to $1 \mu\text{g}$ rmIL-12, or $1 \mu\text{g}$ of mIgG2a isotype control once a week. Tumor growth was assessed for 60 days.

[00493] As shown in **FIGs. 2A-2C**, although IL-12 (**FIG. 2A**) and DF-mIL-12-Fc wt (**FIG. 2B**) were efficient in controlling tumor progression in some mice, only DF-mIL-12-Fc si induced robust tumor regression and yielded 100% complete tumor regression (**FIG. 2C**). Moreover, overall survival was significantly extended by the treatment of DF-mIL-12-Fc si therapy – 100% of treated mice were still alive at day 60, whereas median survival times of the mice treated with isotype control, DF-mIL-12-Fc wt, and IL-12 were 27 days, 33 days, and 46 days, respectively (**FIG. 3**).

[00494] Next, different doses of DF-mIL-12-Fc wt and DF-mIL-12-Fc si in controlling tumor progression were compared. Briefly, 10^6 CT26-Tyrp1 colon carcinoma cells were injected

subcutaneously into the flank of Balb/c mice. On Day 14 after tumor inoculation, when tumor volume reached 300 mm³, the mice were randomized into different treatment groups (n = 10 per group) and treated intraperitoneally with DF-mIL-12-Fc wt at molar doses equivalent to 1 µg or 0.1 µg rmIL-12, or DF-mIL-12-Fc si at molar doses equivalent to 1 µg or 0.1 µg IL-12 once a week. Tumor growth was assessed for 55 days.

[00495] As shown in **FIGs. 4A-4D**, treatment with DF-mIL-12-Fc wt led to reduced tumor progression in some mice and complete regression in two mice at the 1 µg rmIL-12 molar equivalents dose (**FIG. 4A**), but no tumor suppression was observed at the 0.1 µg IL-12 molar equivalents dose (**FIG. 4C**). By contrast, the DF-mIL-12-Fc si treatment at the 1 µg IL-12 molar equivalents dose yielded 100% complete tumor regression (**FIG. 4B**) and induced a robust delay in tumor growth at the lower dose of 0.1 µg IL-12 molar equivalents (**FIG. 4D**). The median survival of the mice treated with 1 µg IL-12 molar equivalents of DF-mIL-12-Fc wt was 32 days, similar to the 34 days of median survival of the mice treated with 0.1 µg IL-12 molar equivalents of DF-mIL-12-Fc si, suggesting that DF-mIL-12-Fc si was 10-fold more potent than its wildtype variant (**FIG. 5**). DF-mIL-12-Fc wt was not efficient at the dose of 0.1 µg IL-12 molar equivalents, and showed the same median survival of 24 days as the isotype treated group.

[00496] Next, *in vivo* efficacy for different routes of administering DF-mIL-12-Fc si were compared. Briefly, 10⁶ CT26-Tyrp1 colon carcinoma cells were injected subcutaneously into the flank of Balb/c mice. On Day 14 after tumor inoculation, when tumor volume reached 270 mm³, the mice were randomized into different treatment groups (n = 10 per group) and treated either intraperitoneally or subcutaneously with DF-mIL-12-Fc si at a molar dose equivalent to 1 µg IL-12 or molar equivalent of mIgG2a isotype control once a week. Tumor growth was assessed for over 60 days.

[00497] As shown in **FIGs. 19A – 19B**, both intraperitoneal (**FIG. 19A**) and subcutaneous (**FIG. 19B**) administration of DF-mIL-12-Fc si induced robust tumor regression and yielded 100% complete tumor regression. Thus, DF-mIL-12-Fc si treatment demonstrated efficacy using various routes of administration.

Example 3 – Tumor Suppression by IL-12 Fused with a Silent Fc domain polypeptide in a B16F10 Tumor Model

[00498] This example describes relative abilities of DF-mIL-12-Fc wt and DF-mIL-12-Fc si in controlling tumor progression in a mouse melanoma model. Briefly, 10⁶ B16F10 melanoma cells were injected subcutaneously into C57BL/6 mice. On Day 8 after tumor inoculation, when tumor volume reached 250 mm³, the mice were randomized into different treatment groups (n = 10) and

treated with 0.5 µg of IL-12, DF-mIL-12-Fc wt at a molar dose equivalent to 0.5 µg IL-12, DF-mIL-12-Fc si at a molar dose equivalent to 0.5 µg IL-12, or 0.5 µg of mIgG2a isotype control once a week. Tumor growth was assessed for 32 days.

[00499] As shown in **FIGs. 6A-6C**, although each of the IL-12-Fc constructs tested delayed tumor progression, DF-mIL-12-Fc si was the most efficient in controlling tumor growth. Median survival time of the mice treated with DF-mIL-12-Fc si was 29 days, which was longer than the median survival times of the mice treated with isotype control, DF-mIL-12-Fc wt, and IL-12, which were 16 days, 26 days, and 22 days, respectively (**FIG. 7**).

[00500] Next, different doses of DF-mIL-12-Fc wt and DF-mIL-12-Fc si in controlling tumor progression were compared. Briefly, 10⁶ B16F10 melanoma cells were injected subcutaneously into the flank of C57BL/6 mice. On Day 8 after tumor inoculation, the mice were randomized into different treatment groups (n = 10 per group) and treated intraperitoneally with 0.5 µg or 0.1 µg IL-12 molar equivalents of DF-mIL-12-Fc wt, or 0.5 µg or 0.1 µg IL-12 molar equivalents of DF-mIL-12-Fc si once a week. Tumor growth was assessed for 30 days.

[00501] As shown in **FIGs. 8A-8D**, DF-mIL-12-Fc si was superior to DF-mIL-12-Fc wt in suppression of tumor growth at both doses. Moreover, at each dose, the median survival of the mice treated with DF-mIL-12-Fc wt was 20 days. By contrast, the median survival of the mice treated with 0.1 µg IL-12 molar equivalents of DF-mIL-12-Fc si was 21 days, and the median survival of the mice treated with 0.5 µg IL-12 molar equivalents of DF-mIL-12-Fc si was 28 days (**FIG. 9**). These results demonstrated that a high dose (0.5 µg IL-12 molar equivalents) of DF-mIL-12-Fc si significantly increased the survival of mice compared to its wildtype counterpart or isotype control.

[00502] Next, single dose administrations of the DF-mIL-12-Fc si treatment was compared to the weekly treatments previously described. Briefly, 10⁶ B16F10 melanoma cells were injected subcutaneously into C57BL/6 mice. On Day 8 after tumor inoculation, when tumor volume reached 200 mm³, the mice were randomized into different treatment groups (n = 10) and treated with DF-mIL-12-Fc si at a molar dose equivalent to 0.5 µg IL-12 or molar equivalent of mIgG2a isotype control once a week. Tumor growth was assessed for 39 days.

[00503] As shown in **FIG. 20**, a single administration of DF-mIL-12-Fc si resulted in reduced tumor outgrowth in 100% of mice, although tumor outgrowth occurred sooner when compared to weekly administrations (**FIG. 6C**). Additionally, mice demonstrated transient weight loss, but after the first dose only (data not shown). Accordingly, a single administration of DF-mIL-12-Fc si

demonstrated initial efficacy in a hard-to-treat tumor model, although subsequent weekly administrations are better at delaying tumor outgrowth in this model.

[00504] Next, *in vivo* efficacy for different routes of administering DF-mIL-12-Fc si were compared. Briefly, 10^6 B16F10 melanoma cells were injected subcutaneously into C57BL/6 mice. On Day 7 after tumor inoculation, when tumor volume reached 260 mm^3 , the mice were randomized into different treatment groups ($n = 10$) and treated either intraperitoneally or subcutaneously with DF-mIL-12-Fc si at a molar dose equivalent to $1 \mu\text{g}$ IL-12 or molar equivalent of mIgG2a isotype control once a week. Tumor growth was assessed for 40 days.

[00505] As shown in **FIGs. 21A – 21B**, both intraperitoneal (**FIG. 21A**) and subcutaneous (**FIG. 21B**) administration of DF-mIL-12-Fc si induced tumor regression in 100% of mice. Thus, DF-mIL-12-Fc si treatment demonstrated efficacy using various routes of administration.

Example 4 – *In vitro* potency of DF-hIL-12-Fc wt and rhIL-12

[00506] The potency of DF-hIL-12-Fc si in comparison to rhIL-12 was assessed using *in vitro* bioassays.

[00507] IL-12 potency was assessed using a HEK-Blue IL-12 reporter assay. IL-12R+ HEK-Blue reporter cells (InvivoGen) were harvested from culture and adjusted to 1×10^6 cells/mL in culture media. DF-hIL-12-Fc si (DF IL-12-Fc) and recombinant human IL-12 (rhIL-12; PeproTech) were diluted in media. $100 \mu\text{L}$ of PBMC suspension was mixed with $100 \mu\text{L}$ of diluted test article and incubated for 48 hours. The supernatant was harvested and engagement of IL-12 receptor and signaling components stably expressed by the reporter cells was detected by measurement of secreted embryonic alkaline phosphatase from the cells following manufacturer instructions. Briefly, $25 \mu\text{L}$ of sample supernatant was mixed with $200 \mu\text{L}$ of QUANTI-Blue reagent and incubated in the dark at RT for 10 minutes. The plate was then read with a SpectraMax i3x plate-reader at 620 nM and optical density reported to represent relative IL-12 activity.

[00508] As shown in **FIG. 10A**, production of SEAP by IL-12R+ HEK reporter cells increased with increasing concentrations of DF-hIL-12-Fc si or rhIL-12. The measured IL-12 responses in the HEK-Blue reporter assay were comparable between DF-hIL-12-Fc si and rhIL-12 at the concentrations examined.

[00509] Next, IL-12 potency was assessed by quantifying IFN γ production from human PBMCs. PBMCs were isolated from human peripheral blood buffy coats using density gradient centrifugation and adjusted to 1×10^6 cells/mL in culture media. DF-hIL-12-Fc si and recombinant human IL-12 (rhIL-12) were diluted in media. $100 \mu\text{L}$ of PBMC suspension was mixed with $100 \mu\text{L}$ of diluted test article and incubated for 48hrs. The supernatant was harvested and IFN γ was

quantified using a Human IFN- γ ELISA MAX kit (BioLegend). After development of the IFN γ ELISA plates, they were read using a SpectraMax i3x instrument at 450 nm with a background subtraction at 540 nm. IFN γ content in sample wells was approximated by interpolating sample readings from the assay standard curve.

[00510] As shown in **FIG. 10B**, IFN γ production increased when human PBMCs were cultured with DF-hIL-12-Fc si or rhIL-12, with concurrent treatment with 5 μ g/ml of PHA to amplify the magnitude of IFN γ responses. IFN- γ production following IL-12 stimulation was comparable between DF-hIL-12-Fc si and rhIL-12 at the concentrations examined.

[00511] Accordingly, although the EC50 values with the two cell types and stimulation conditions differed by over an order of magnitude, comparable activity of DF-hIL-12-Fc si and rhIL-12 was demonstrated in both assays suggesting the potency of the DF-hIL-12-Fc si construct exhibits similar potency to that of native recombinant human IL-12.

Example 5 – IL-12, DF-hIL-12-Fc si and IFN γ concentrations in monkey plasma following IV infusion of DF-hIL-12-Fc si or rhIL-12

[00512] The pharmacodynamics (PD) and pharmacokinetics (PK) were assessed in cynomolgus monkeys following IV infusion of DF-hIL-12-Fc si or rhIL-12.

[00513] Cynomolgus monkeys were administered DF-hIL-12-Fc si and recombinant human IL-12 at 10 μ g/kg by IV-infusion.

[00514] An immunoassay was used to detect DF-hIL-12-Fc si and Human IL-12 based on a Quantikine ELISA Human IL-12 p70 Immunoassay kit: This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human IL-12 p70 was used as a solid phase capture and detection was accomplished using an antibody HRP-tagged reporter. Standards and QCs spiked with rhIL-12 or DF-hIL-12-Fc si reference standard, along with test samples were pipetted into the wells of microtiter plate and any IL-12 p70 present in the samples were bound by the immobilized antibody, on the solid phase. Unbound substances were washed away and the enzyme-linked polyclonal antibody specific for human IL-12 p70 was added to the wells. Unbound antibody-enzyme reagents were washed away and TMB substrate was added to each well. The resulting enzyme reaction yields a blue product that turns yellow when an acid stop solution is added. The intensity of the color measured in each well is directly proportional to the amount of rhIL-12 or DF-hIL-12-Fc si is bound in the initial step. Plates were read at 450 nm with a reference of 540 nm on a SpectraMax microplate reader with data collection software, SoftMax Pro Enterprise version 4.6. Data was converted into a text file and imported/processed in

Watson LIMS v.7.2.0.02. Regression was performed using a Logistic (Auto Estimate) curve fitting with a weighting factor of 1.

[00515] An immunoassay (meso scale discovery (MSD) – an ELISA like immunoassay) was also used to detect DF-hIL-12-Fc si that involved coating an untreated MSD microtiter plate with monkey-adsorbed goat anti-human IgG and incubating at room temperature. The plate was washed, blocked, washed, and incubated with standard curve and quality control samples spiked with DF-hIL-12-Fc si reference standard, along with test samples. After this incubation, the plate was washed and biotin anti-human IL-12/IL-23 p40 was added to the plate as the primary detection antibody. After another wash step, streptavidin-conjugated Sulfo-Tag was added as the secondary detection antibody. The plate was washed a final time, MSD Read Buffer T was added to the plate, and the plate was read using a MSD Sector Imager S600. Raw MSD data was exported into a text file, which was then converted into a Watson LIMS compatible file using a programmed Excel spreadsheet, which was custom designed at Envigo. Data was imported and regressed in Watson LIMS Software v.7.2.0.02.

[00516] A meso scale discovery method was performed for the relative quantitative measurement of NHP proinflammatory biomarkers in cynomolgus monkey plasma. The method used a sandwich immunoassay procedure for the relative quantitative measurement of Pro-inflammatory Panel 1 Biomarkers: IFN γ , IL-1 β , IL-2, IL-6 IL-8, and IL-10 in cynomolgus monkey K2 EDTA plasma (referred to as monkey plasma). The method is based on MSD non-human primate (NHP) kits for V-PLEX and V-PLEX Plus, Catalog No. K15056D-1, K15056D-2, K15056D-4, K15056D-6, K15056G-1, K15056G-2, K15056G-4, K15056G-6. The method employs human capture and detection antibodies that react with cynomolgus monkeys. The kit provides plates pre-coated with capture antibodies on independent well-defined spots in each well of a 96-well multi-spot plate. The plate was incubated with monkey plasma samples, washed and then incubated with detection antibodies (specific for each analyte) that are conjugated with electrochemiluminescent (ECL) labels (MSD SULFO-TAG). Analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the detection antibodies by the bound analytes completes the sandwich. The plate was washed and an MSD Read Buffer was added to create the appropriate chemical environment for electrochemiluminescence (ECL). The plate was loaded into an MSD Sector Imager 600 (SI600) instrument where a voltage was applied to the plate electrodes causing the captured labels to emit light. The instrument measures the intensity of emitted light in terms of Relative Light Units (RLU) to provide a relative quantitative measure of analytes in the sample. Raw RLU data was exported into a text file, which

then was converted into a Watson LIMS compatible file using a programmed Excel spread sheet, which was custom designed at Envigo. Data was subsequently imported and regressed in Watson LIMS Software v.7.2.0.02.

[00517] **FIG. 11** shows the relative plasma concentrations of DF-hIL-12-Fc si and recombinant human IL-12 over time following IV-administration. The data indicate that concentrations of DF-hIL-12-Fc si and rhIL-12 decreased over time, as expected. However, DF-hIL-12-Fc si demonstrated a prolonged half-life and overall greater exposure compared to rhIL-12 over the time course.

[00518] **FIG. 11** also shows the relative concentrations of IFN γ (PD) in monkey plasma following IV-administration. The data indicate that the pharmacodynamics of DF-hIL-12-Fc si and rhIL-12, as assessed by IFN γ production, both demonstrated activity following IV-administration. However, DF-hIL-12-Fc si demonstrated a higher peak activity and a longer duration compared to rhIL-12.

Example 6 – Pharmacological characterization of the mouse surrogate DF-mIL-12-Fc si

[00519] The serum half-life and *in vivo* pharmacodynamics of a half-life prolonged murine IL-12 variant, designated DF-mIL-12-Fc si, was examined.

[00520] An equivalent molar amount of DF-mIL-12-Fc si, corresponding to 1 μ g IL-12, was intravenously injected in non-tumor bearing Balb/c mice and PK/PD characteristics were compared to IL-12. Naïve Balb/c (n=6) were injected intravenously with 1 μ g DF-mIL-12-Fc si and IL-12 (equivalent molar to 1 μ g IL-12). Blood was sampled at 0.017, 0.5, 3, 6, 24, 48, 72, 96, 144 and 219 hours post-injection. IL-12 and IFN γ levels in serum were analyzed by ELISA, as previously described.

[00521] As shown in **FIGs. 12A** and **12B** and quantified in Table 15, DF-mIL-12-Fc si showed protracted serum half-life of approximately 30 hours (**FIG. 12B**, DF-mIL-12-Fc si $T_{1/2}$ = 29.85 hours), which was 5 times longer than that of IL-12 (**FIG. 12A**; IL-12 $T_{1/2}$ = 6.05 hours). In addition to an extended half-life, DF-mIL-12-Fc si-mediated IFN γ production (AUC = 916654) was also prolonged compared to IL-12 (AUC = 20304).

[00522] Next, the PK/PD properties for different routes of administering DF-mIL-12-Fc si were compared. An equivalent molar amount of DF-mIL-12-Fc si, corresponding to 1 μ g IL-12, was injected as a single dose in non-tumor bearing Balb/c mice by intravenous, intraperitoneal, or subcutaneous administration and PK/PD characteristics were assessed, as described.

[00523] As shown in **FIGs. 12C-12E** and quantified in Table 16, intravenous (**FIG. 12C**), intraperitoneal (**FIG. 12D**), or subcutaneous (**FIG. 12E**) administration all resulted in DF-mIL-12-

Fc si-mediated IFN γ production comparable across the different routes of administration. Notably, subcutaneous administration resulted in a lower IL-12 C_{max}. Accordingly, the pharmacokinetic properties (*e.g.*, IL-12 concentration) of DF-mIL-12-Fc si administration varied depending on the route of administration, while the pharmacodynamic properties (IFN γ production) remained protracted and relatively comparable across the different routes.

Table 15: Pharmacological characteristics of DF-mIL-12-Fc si and rmIL-12

	IL-12					IFN γ
	T _{1/2}	Span	T _{max}	C _{max}	AUC	AUC
rmIL-12	6.05	9.15	0.13	358.65	999.81	20304
DF-mIL-12-Fc	29.85	6.92	0.19	258.20	5636.49	916654

Table 16: Pharmacological characteristics of DF-mIL-12-Fc si via IV, IP, and SC

	T _{1/2}	Span	T _{max}	C _{max}	AUC
Intravenous	31.5	6.4	0.2	257.2	5985.6
Intraperitoneal	34.70	5.19	N/A	75.09	3964.21
Subcutaneous	37.5	4.1	36.0	23.1	1938.1

Example 7 – Combination of DF-mIL-12-Fc si and PD-1 blockade in B16F10 mouse model

[00524] Combination therapy of DF-mIL-12-Fc si and PD-1 blockade was performed to analyze whether anti-tumor immune response can be amplified in established B16F10 tumors.

[00525] C57BL/6 mice were injected with 10⁶ B16F10 melanoma cells subcutaneously into the flank of mice. On Day 8 after tumor inoculation, mice were randomized (n=10 per group). When average tumor volume reached ~245 mm³, mice were treated intraperitoneally with 0.5 μ g isotype control, 0.5 μ g DF-mIL-12-Fc si, 200 μ g anti-PD-1 clone RMP1-14, or combined DF-mIL-12-Fc si/anti-PD-1. Animals were injected once a week with DF-mIL-12-Fc si and twice weekly with anti-PD-1. Tumor growth was assessed for 60 days, and survival and body weight was monitored.

[00526] As shown in **FIGs. 13A – 13C**, while administration of DF-mIL-12-Fc si alone delayed tumor regression (**FIG. 13A**) and PD-1 alone had a minimal effect on tumor growth (**FIG. 13B**), the combination of DF-mIL-12-Fc si with PD-1 blockade further delayed tumor growth (**FIG. 13C**), suggesting anti-PD-1 treatment further amplified anti-tumor responses to DF-mIL-12-Fc si treatment.

[00527] As shown in **FIGs. 14A and 14B**, overall survival with DF-mIL-12-Fc si therapy and in combination with PD-1 blockade was extended showing a median survival of 29 days (DF-mIL-12-Fc si monotherapy) and 36 days (combination) compared to 15 days of isotype and 17.5 days of 200 μ g anti-PD-1 treated mice (**FIG. 14A**). Notably, despite the high response rate, the regimen

of DF-mIL-12-Fc si and combination therapy appeared to be well tolerated by B16F10 tumor-bearing mice (**FIG. 14B**).

[00528] Accordingly, a combination therapy of DF-mIL-12-Fc si and PD-1 blockade demonstrated improved efficacy compared to either treatment alone.

Example 8 – Combination of DF-mIL-12-Fc si and with mcFAE-C26.99 TriNKETs in B16F10 mouse model

[00529] Combination therapy of DF-mIL-12-Fc si and mcFAE-C26.99 TriNKETs was performed to analyze whether anti-tumor immune response can be amplified in established B16F10 tumors.

[00530] C57BL/6 mice were injected with 10^6 B16F10 melanoma cells subcutaneously into the flank of the mice. On Day 7 after tumor inoculation mice were randomized (n=10 per group). When tumor average reached 200 mm^3 , mice were treated intraperitoneally with 150 μg isotype control, or 0.5 μg DF-mIL-12-Fc si, 150 μg TriNKET, or the combination DF-mIL-12-Fc si/TriNKET. Tumor growth was assessed for 60 days, and survival and body weight was monitored.

[00531] As shown in **FIG. 15A**, monotherapy with DF-mIL-12-Fc si led to reduced tumor growth. Treatment with mcFAE-C26.99 TriNKET as single agent at a starting tumor volume of 200 mm^3 did not result in delayed tumor progression (**FIG. 15B**). In contrast, the combination of DF-mIL-12-Fc si with mcFAE-C26.99 further enhanced anti-tumor responses in comparison to DF-mIL-12-Fc si alone (**FIG. 15C**) and resulted in 30% complete responders (CR) (n=3), suggesting TriNKET treatment further amplified anti-tumor responses to DF-mIL-12-Fc si treatment.

[00532] As shown in **FIG. 16A**, overall survival with DF-mIL-12-Fc si therapy and in combination with mcFAE-C26.99 TriNKET was extended showing a median survival of 29 days (DF-mIL-12-Fc si monotherapy) and 60 days (TriNKET combination) compared to 16 days of isotype and 17 days of TriNKET treated mice. Notably, despite the high response rate, the regimen of DF-mIL-12-Fc si and combination therapy appeared to be well tolerated by B16F10 tumor-bearing mice (**FIG. 16B**).

[00533] The three complete responders (the CRs from the experiment described above and the data presented in **FIG. 15C**) were re-challenged with 2×10^6 B16F10 melanoma cells 72 days after first tumor inoculation. Age-matched naïve C57BL/6 mice were used as control group. 1 out of 3 mice from the initial DF-mIL-12-Fc si /TriNKET combo treated group remained tumor-free, another mouse showed tumor formation starting at day 95 and the tumor progression of the third

mouse was similar to the age-matched control group (**FIG. 17**), suggesting the formation of immunological memory with combination therapy.

[00534] Accordingly, a combination therapy of DF-mIL-12-Fc si and TriNKETs demonstrated improved efficacy compared to either treatment alone, including demonstrating a complete, durable response in a population of mice.

Example 9 – Treatment with DF-mIL-12-Fc si promotes complete recovery in CT26 tumor model

[00535] This example shows that treatment with DF-mIL-12-Fc si promotes recovery in mice bearing CT26 tumors.

[00536] Briefly, 10^6 CT26-Tyrp1 colon carcinoma cells were injected subcutaneously into the flank of Balb/c mice. On Day 14 after tumor inoculation, when tumor volume reached 270 mm^3 , the mice were randomized into different treatment groups and intraperitoneally injected with $1 \mu\text{g}$ of DF-mIL-12-Fc si at a molar dose equivalent to $1 \mu\text{g}$ mIL-12 or $1 \mu\text{g}$ of mIgG2a isotype control once a week. Tumor growth was assessed for 60 days. The complete responders were re-challenged with 10^6 CT26 cells 72 days after first tumor inoculation. Age-matched naïve Balb/C mice were used as control group.

[00537] **FIG. 18A** is a graph showing tumor growth curves of individual mice inoculated with CT26 tumor cells and administered a single dose of $1 \mu\text{g}$ of DF-mIL-12-Fc si or mIgG2a isotype. **FIG. 18B** is a graph showing body weights of individual mice inoculated with CT26 tumor cells and administered a weekly dose of $1 \mu\text{g}$ of DF-mIL-12-Fc si or mIgG2a isotype. **FIG. 18C** is a graph showing tumor growth curves of individual mice re-challenged with inoculation of CT26 tumor cells.

[00538] As shown in **FIGs. 18A-B**, administration of DF-mIL-12-Fc si resulted in robust tumor regression in comparison to mIgG2a isotype with no observable toxicity affecting the body weight of treatment animals. As shown in **FIG. 18C**, the initial DF-mIL-12-Fc si treated mice remained tumor-free suggesting the formation of immunological memory with DF-mIL-12-Fc si treatment.

Example 10 –DF mIL-12-Fc si delivered intraperitoneally or subcutaneously is effective to reduce tumor volume in CT26 tumor model

[00539] This example shows that intraperitoneal or subcutaneous administration of DF-mIL-12-Fc si ensures 100% complete recovery in mice bearing CT26 tumors.

[00540] Briefly, 10^6 CT26-Tyrp1 colon carcinoma cells were injected subcutaneously into the flank of Balb/c mice. On Day 14 after tumor inoculation, when tumor volume reached 270 mm^3 , the mice were randomized into different treatment groups and intraperitoneally injected with $1 \mu\text{g}$

of DF-mIL-12-Fc si at a molar dose equivalent to 1 μ g IL-12 or 1 μ g of mIgG2a isotype control once a week, or subcutaneously injected with 1 μ g of DF-mIL-12-Fc si at a molar dose equivalent to 1 μ g IL-12 or 1 μ g of mIgG2a isotype control once a week. Tumor growth was assessed for more than 60 days.

[00541] **FIG. 19A** is a graph showing tumor growth curve of individual mice inoculated with CT26 tumor cells and administered a weekly dose of 1 μ g of DF-mIL-12-Fc si or mIgG2a isotype delivered intraperitoneally. **FIG. 19B** is a graph showing tumor growth curve of individual mice inoculated with CT26 tumor cells and administered a weekly dose of 1 μ g of DF-mIL-12-Fc si or mIgG2a isotype delivered subcutaneously.

[00542] As shown in **FIGs. 19A-B**, either intraperitoneal or subcutaneous delivery of DF-mIL-12-Fc si was effective at reducing CT26 tumor volume.

Example 11 – Single dose administration of DF-mIL-12-Fc si is effective to reduce tumor volume in B16F10 mouse model

[00543] This example shows that a single dose of DF-mIL-12-Fc si is effective at reducing tumor volume in mice bearing B16F10 melanoma tumors.

[00544] In brief, C57BL/6 mice were injected with 10^6 B16F10 melanoma cells subcutaneously into the flank of the mice. On Day 7 after tumor inoculation mice were randomized. When tumor average reached 200 mm³, mice were treated intraperitoneally with a single dose of isotype control, or 1 μ g of DF-mIL-12-Fc si. Tumor growth was assessed for 50 days.

[00545] As shown in **FIG. 20**, a single administration of μ g of DF-mIL-12-Fc si is effective to reduce tumor volume in B16F10 tumor-bearing mice.

Example 12 – DF-mIL-12-Fc si delivered intraperitoneally or subcutaneously is effective to reduce tumor volume in B16F10 mouse model

[00546] This example shows that intraperitoneal or subcutaneous administration of DF-mIL-12-Fc si led to 100% complete recovery in mice bearing B16F10 melanoma tumors.

[00547] Briefly, 10^6 B16F10 melanoma cells were injected subcutaneously into the flank of C57BL/6 mice. On Day 7 after tumor inoculation, mice were randomized. When tumor average reached 200 mm³, mice were intraperitoneally injected with 1 μ g of DF-mIL-12-Fc si at a molar dose equivalent to 1 μ g IL-12 or 1 μ g of mIgG2a isotype control once a week, or subcutaneously injected with 1 μ g of DF-mIL-12-Fc si at a molar dose equivalent to 1 μ g IL-12 or 1 μ g of mIgG2a isotype control once a week. Tumor growth was assessed for 40 days.

[00548] As shown in **FIGs. 21A-B**, either intraperitoneal or subcutaneous delivery of DF-mIL-12-Fc si was effective at reducing B16F10 tumor volume as compared to isotype control.

Example 13 – DF-mIL-12-Fc si is efficacious as a single dose

[00549] This example shows that DF-mIL-12-Fc si is effective at reducing CT26 tumor volume when administered as a single dose, and when administered via repeat dosing, is even more effective.

[00550] Briefly, 10^6 CT26-Tyrp1 colon carcinoma cells were injected subcutaneously into the flank of Balb/c mice. On Day 14 after tumor inoculation, when tumor volume reached 270 mm^3 , the mice were randomized into different treatment groups ($n = 10$ per group) and intraperitoneally injected with a single dose of $1 \mu\text{g}$ of DF-mIL-12-Fc si at a molar dose equivalent to $0.1 \mu\text{g}$ IL-12 or $1 \mu\text{g}$ of mIgG2a isotype control once a week. Alternatively, mice were intraperitoneally injected with $1 \mu\text{g}$ of DF-mIL-12-Fc si at a molar dose equivalent to $1 \mu\text{g}$ IL-12 or $1 \mu\text{g}$ of mIgG2a isotype control once a week. Tumor growth was assessed for more than 60 days.

[00551] **FIG. 22A** is a graph showing tumor growth curve of individual mice inoculated with CT26 tumor cells and administered a single dose of $1 \mu\text{g}$ of DF-mIL-12-Fc si or mIgG2a isotype. **FIG. 22B** is a graph showing tumor growth curve of individual mice inoculated with CT26 tumor cells and administered a weekly dose of $1 \mu\text{g}$ of DF-mIL-12-Fc si or mIgG2a isotype.

[00552] As shown in **FIG. 18A** and **FIG. 22A**, a single administration of $1 \mu\text{g}$ of DF-mIL-12-Fc si resulted in robust 70% complete recovery of tumor-bearing mice as compared to mIgG2a isotype. However, as shown in **FIG. 2C** and **FIG. 22B**, repeat weekly dosing of $1 \mu\text{g}$ of DF-mIL-12-Fc si ensured 100% complete recovery of tumor-bearing mice as compared to mIgG2a isotype. As shown in **FIG. 18B**, even repeat administration of DF-mIL-12-Fc si was well-tolerated with no toxicities observed, as assessed by body weight.

[00553] Additionally, complete responders (CR) were re-challenged with 5×10^5 CT26-Tyrp1 colon carcinoma cells at the opposite flank and tumor progression was compared to naïve mice challenged at the same tumor dose. As shown in **FIG. 18C**, while tumors grew in naïve mice, 100% of complete responders remained tumor-free following re-challenge. Accordingly, a single administration of DF-mIL-12-Fc si demonstrated a complete, durable response in a population of mice.

Example 14 – Pharmacokinetics in cynomolgus monkeys treated with a single subcutaneous dose of DF-hIL-12-Fc si

[00554] Pharmacokinetics were determined following a subcutaneous injection of DF-hIL-12-Fc si at $1 \mu\text{g}/\text{kg}$ (**FIG. 25A**), $2 \mu\text{g}/\text{kg}$ (**FIG. 25B**), or $4 \mu\text{g}/\text{kg}$ (**FIG. 25C**) in cynomolgus monkeys utilizing an ELISA like immunoassay-Meso Scale Discovery (MSD) immunoassay method. Briefly, an untreated MSD microtiter plate was coated with monkey-adsorbed goat anti-human IgG

and incubated at room temperature. Following coating and incubation, the plate was washed, blocked, washed, and incubated with standard curve and quality control samples spiked with a DF-hIL-12-Fc si reference standard, along with test samples. Following incubation, the plate was washed and biotin anti-human IL-12/IL-23 p40 was added to the plate as the primary detection antibody. Following another wash step, streptavidin-conjugated Sulfo-Tag was added as the secondary detection antibody. The plate was washed a final time before adding MSD read buffer T to the plate. The plate was read using an MSD Sector Imager S6000.

[00555] FIGs. 25A-25C are line graphs showing pharmacokinetics in cynomolgus monkeys treated with a single subcutaneous dose of 1 µg/kg (FIG. 25A), 2 µg/kg (FIG. 25B), or 4 µg/kg (FIG. 25C) of DF-hIL-12-Fc si.

[00556] The data indicate that concentrations of DF-hIL-12-Fc si and rhIL-12 decreased over time, as expected, with similar pharmacokinetic profiles at all doses tested.

Example 15 – Cytokine release in cynomolgus monkeys treated with a single subcutaneous dose of DF-hIL-12-Fc si

[00557] Quantitative measurements of cytokines following a subcutaneous injection of DF-hIL-12-Fc si at 1 µg/kg (FIGs. 26A and 26B), 2 µg/kg (FIGs. 26C and 26D), or 4 µg/kg (FIGs. 26E and 26F) in cynomolgus monkeys were determined using MSD immunoassay kits. The method used sandwich immunoassay kits (Pro-inflammatory Panel 1 Biomarkers and V-PLEX Plus Chemokine Panel 1 NHP Kit) for the relative quantitative measurement of Pro-inflammatory Panel 1 Biomarkers: IFN γ , IL-1 β , IL-2, IL-6 IL-8, and IL-10 in cynomolgus monkey K2 EDTA plasma (referred to as monkey plasma). The method is based on MSD non-human primate (NHP) kits for V-PLEX and V-PLEX Plus, Catalog No. K15056D-1, K15056D-2, K15056D-4, K15056D-6, K15056G-1, K15056G-2, K15056G-4, K15056G-6. The method employs human capture and detection antibodies that react with cynomolgus monkeys. The kit provides plates pre-coated with capture antibodies on independent, well-defined spots within each well of a 96-well multi-spot plate. The plate was incubated with monkey plasma samples, washed and then incubated with detection antibodies (specific for each analyte) that are conjugated with electrochemiluminescent (ECL) labels (MSD SULFO-TAG). Analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the detection antibodies by the bound analytes completes the sandwich. The plate was washed and an MSD Read Buffer was added to create the appropriate chemical environment for electrochemiluminescence (ECL). The plate was loaded into an MSD Sector Imager 600 (SI600) instrument where a voltage was applied to the plate electrodes causing the captured labels to emit light. The instrument measures the intensity of emitted light in

terms of Relative Light Units (RLU) to provide a relative quantitative measure of analytes in the sample. Raw RLU data was exported into a text file, which then was converted into a Watson LIMS compatible file using a programmed Excel spread sheet, which was custom designed at Envigo. Data was subsequently imported and regressed in Watson LIMS Software v.7.2.0.02.

[00558] **FIGs. 26A-26F** are line graphs showing concentrations of IFN γ (**FIGs. 26A, 26C, and 26E**) and IP10/CXCL10 (**FIGs. 26B, 26D, and 26F**) in cynomolgus monkeys treated with a single subcutaneous dose of 1 μ g/kg (**FIGs. 29A and 29B**), 2 μ g/kg (**FIGs. 26C and 26D**), or 4 μ g/kg (**FIG. 26E and 26F**) of DF-hIL-12-Fc si.

[00559] As shown in **FIG. 26A**, a single subcutaneous dose of DF-hIL-12-Fc si at 1 μ g/kg did not result in detectable levels of IFN γ . Subcutaneous doses of DF-hIL-12-Fc si at 2 μ g/kg and 4 μ g/kg, resulted in an increase in IFN γ levels in some animals that peaked at day 4 post-dosing (**FIGs. 26C and 26E**). Subcutaneous doses of DF-hIL-12-Fc si at 1 μ g/kg, 2 μ g/kg, and 4 μ g/kg all resulted in elevated IP10/CXCL10 levels that peaked at day 4 post-dosing (**FIGs. 26B, 26D, and 26F**).

Example 16 – DF-mIL-12-Fc si combination therapy using radiation or chemotherapy in 4T1 orthotopic mouse model

[00560] In order to show whether anti-tumor activity elicited by administration of DF-mIL-12-Fc si can be amplified, combination studies using radiation or chemotherapy were performed. Briefly, Balb/c mice were injected orthotopically into the mammary fat pad with 5×10^5 4T1-luc tumor cells. On Day 14 after tumor inoculation, mice were randomized (n = 10 per group). Mice were treated subcutaneously with either isotype, DF-mIL-12-Fc si (both equimolar to 1 μ g IL-12), 5mg/kg Doxil® (chemotherapy) intravenously, or irradiated with 10 Gy as monotherapy, or DF-mIL-12-Fc si in combination with Doxil® or radiation. Tumor growth was assessed over time. **FIG. 27** is a graph showing tumor growth curves of individual mice inoculated with breast cancer cells and administered a weekly dose of isotype control, DF-mIL-12-Fc si, Doxil (chemotherapy), or irradiated with 10 Gy as monotherapy or DF-mIL-12-Fc si in combination with Doxil® or radiation. Graph shows group averages of tumor growth $\pm$ standard error mean.

[00561] As seen in **FIG. 27**, although monotherapy with DF-mIL-12-Fc si was effective by itself in 4T1 tumor-bearing mice, combination therapy amplified anti-tumor immune responses leading to full tumor regression in 10-30% of mice.

Example 17 – DF-mIL-12-Fc si mediated anti-tumor efficacy against large, PD-1 blockade-resistant CT26 colon carcinoma tumors

[00562] This example analyzes whether DF-mIL-12-Fc si elicited potent, anti-tumor responses against PD-1 blockade-resistant CT26-Tyrp1 tumors. Briefly, Balb/c mice were injected with 0.5×10^6 CT26-Tyrp1 tumor cells. Following inoculation when average tumor volume reached $\sim 120 \text{ mm}^3$, mice were randomized on Day 9. Mice were either treated with 200 μg isotype or anti-PD-1 antibody (twice weekly). **FIG. 28A** is a graph showing tumor growth curve of Balb/c mice inoculated with CT26-Tyrp1 tumor cells and treated (bi-weekly) either with isotype control or anti-PD-1 antibody. On day 17, the group previously treated with anti-PD-1 (with an average tumor volume $\sim 800 \text{ mm}^3$) was subdivided into two treatment groups. Group 1 continued to receive PD-1 blockade treatment twice weekly, Group 2 received PD-1 blockade (twice weekly) along with DF-mIL-12-Fc si (1 μg weekly). **FIG. 28B** is a graph showing tumor growth curve of the previously anti-PD-1 antibody-treated Balb/c mice treated with anti-PD-1 antibody (bi-weekly) along with weekly treatment with 1 μg of DF-mIL-12-Fc si.

[00563] As shown in **FIG. 28A**, anti-PD-1 monotherapy failed to control tumor progression. However, as shown in **FIG. 28B**, the addition of DF-mIL-12-Fc si resulted in effective tumor regression.

Example 18 – Local treatment of DF-mIL-12-Fc si against large CT26 colon carcinoma tumors induces abscopal anti-tumor responses

[00564] This example shows whether DF-mIL-12-Fc si treatment can induce abscopal therapeutic effects. Briefly, Balb/c were implanted subcutaneously with CT26-Tyrp1 colon carcinoma cells on both the left (0.8×10^6 tumor cells) and right (0.4×10^6 tumor cells) flank. On Day 13 after tumor inoculation, left tumors were injected either with 0.1 μg isotype control or 0.1 μg DF-mIL-12-Fc si once weekly for 2-3 weeks. **FIG. 29A** is a graph showing tumor growth curve of the treated (Tr) tumor in Balb/c mice inoculated with CT26-Tyrp1 tumor cells and treated once (weekly) with either isotype control or DF-mIL-12-Fc si. Right tumors were left untreated (NT).

[00565] **FIG. 29B** is a graph showing tumor growth curves of the untreated (NT) tumors in Balb/c mice inoculated with CT26-Tyrp1 tumor cells.

[00566] As shown in **FIGs. 29A-29B**, control isotype-treated tumors grew progressively at both right and left sites. As shown in **FIGs. 29A-29B**, DF-mIL-12-Fc si caused effective anti-tumor responses at the local injected site (**FIG. 29A**) and the distant non-treated tumor (**FIG. 29B**) indicating abscopal therapeutic effects.

Example 19 – DF-mIL-12-Fc si mediated anti-tumor efficacy against large CT26 colon carcinoma tumors

[00567] This example shows that DF-mIL-12-Fc si which includes wild-type murine IL-12 p40 and p35 subunits fused to the N-termini of murine IgG2a Fc domain polypeptides with mutations L234A, L235A, and P329G (discussed in Example 2) is efficacious against larger tumor volumes.

[00568] DF-mIL-12-Fc si-mediated anti-tumor efficacy against large CT26 colon carcinoma tumors was tested. Briefly, Balb/c mice were injected subcutaneously with 10^6 CT26-Tyrp1 colon carcinoma cells. On Day 18 after tumor inoculation, when tumor volume reached 800 mm^3 , the mice were randomized into different treatment groups ($n=10$ per group) and treated intraperitoneally with DF-mIL-12-Fc si at a molar dose equivalent to $1 \mu\text{g}$ or $2 \mu\text{g}$ IL-12, or molar equivalent of mIgG2a isotype once or once weekly.

[00569] Tumor growth was assessed for 65 days. **FIG. 23A** is a graph showing tumor growth curves of Balb/c mice inoculated with CT26-Tyrp1 tumor cells and treated once (weekly) with either $2 \mu\text{g}$ mIgG2a isotype control or $1 \mu\text{g}$ DF-mIL-12-Fc si. **FIG. 23B** is a graph showing tumor growth curves of Balb/c mice inoculated with CT26-Tyrp1 tumor cells and treated once (weekly) with either $2 \mu\text{g}$ mIgG2a isotype control or $2 \mu\text{g}$ DF-mIL-12-Fc si. **FIG. 30A** is a graph showing tumor growth curves of Balb/c mice inoculated with CT26-Tyrp1 tumor cells and treated once with either $2 \mu\text{g}$ mIgG2a isotype control or $2 \mu\text{g}$ DF-mIL-12-Fc si. **FIG. 30B** is a graph showing average tumor growth curves of Balb/c mice inoculated with CT26-Tyrp1 tumor cells and treated with $2 \mu\text{g}$ mIgG2a isotype control, $1 \mu\text{g}$ DF-mIL-12-Fc si (weekly administration), $2 \mu\text{g}$ DF-mIL-12-Fc si (weekly administration), or $2 \mu\text{g}$ DF-mIL-12-Fc si (once). **FIGs. 23A, 23B, and 30A** show tumor growth curves of individual mice. **FIG. 30B** shows tumor average $\pm$ standard error mean.

[00570] As shown in **FIGs. 23A, 23B, and 30B**, weekly doses ($1 \mu\text{g}$ or $2 \mu\text{g}$) of DF-mIL-12-Fc si were efficient in controlling tumor progression and 100% of mice responded to DF-mIL-12-Fc si treatment. Additionally, as shown in **FIG. 30A**, a single treatment with $2 \mu\text{g}$ DF-mIL-12-Fc si showed tumor regression yielding a 100% response rate. The data and figures described in this example show that DF-mIL-12-Fc si is not only effective at reducing larger CT26 tumor volume but also effective at reducing CT26 tumor volume when administered as a single dose.

Example 20 – DF-mIL-12-Fc si treatment against B16F10 melanomas induces production of cytokines and chemokines in serum and in tumors

[00571] This example shows that DF-mIL-12-Fc si treatment results in elevated levels of IFN γ , CXCL9, and CXCL10 in blood and tumors of C57BL/6 mice bearing B16F10 tumors. Briefly, C57BL/6 mice were injected subcutaneously with 10^6 B16F10 melanoma cells. On Day 7 after

tumor inoculation (when average tumor volume reached 150 mm³), mice were randomized (n = 8 per group). Mice were treated intraperitoneally with isotype control, IL-12, or DF-mIL-12-Fc equimolar to 1 µg IL-12.

[00572] After 72 hours post-treatment, serum and tumor lysates were prepared and analyzed for IFN γ (**FIG. 24A**), CXCL9 (**FIG. 24B**), and CXCL10 (**FIG. 24C**) expression using multiplex technology. **FIGs. 31A-C** show average cytokine/chemokine levels in mice.

[00573] As shown in **FIGs. 24A-24C**, a single administration of 0.5 µg of DF-mIL-12-Fc si resulted in increased expression of IFN γ (**FIG. 24A**), CXCL9 (**FIG. 24B**), and CXCL10 (**FIG. 24C**) in serum (left panel) and within tumors (right panel), whereas IL-12 treatment had little or no effect.

Example 21 – DF hIL-12-Fc si having LALAPA and LALAPG mutations have similar IFN γ -stimulating activity and abrogated Fc γ R binding

[00574] This example shows the IFN γ -stimulating and Fc γ R-binding activities of DF hIL-12-Fc si with IgG1 Fc having LALAPA (L234A, L235A, and P329A) mutations, or LALAPG (L234A, L235A, and P329G) mutations. In brief, human PBMCs were cultured for 2 days with both 5 µg/ml phytohemagglutinin (PHA) and a dose-titration of DF hIL-12-Fc-si, having LALAPA or LALAPG mutations. After 2-day stimulation, supernatants were harvested and IFN γ content measured by ELISA. For determining Fc γ R-binding activities, fluorophore-conjugated hIgG1 isotype antibody (83 nM) bound to THP-1 cells that express high affinity Fc γ Rs CD32 and CD64 was detected by flow cytometry.

[00575] As shown in **FIG. 31A**, hIL-12-Fc-LALAPA and hIL-12-Fc-LALAPG have similar abilities to stimulate IFN γ production from PBMCs concurrently with PHA, well above the amount produced with PHA alone.

[00576] As shown in **FIG. 31B**, Simultaneous inclusion of 16-fold molar excess of hIL-12-Fc-wt (1.3 µM) in a mixture with the labeled hIgG1 isotype antibody resulted in substantial reduction of binding signal, likely due to competition for IgG1 binding to CD32 and CD64. In contrast, at the same concentration, neither incubation with hIL-12-Fc-LALAPA nor hIL-12-Fc-LALAPG resulted in detectable IgG1 isotype binding, suggesting abrogated Fc γ R engagement for both proteins attributable to the LALAPA and LALAPG mutations.

Example 22: Manufacturing Process and Process Controls for DF hIL12-Fc si

[00577] DF hIL12-Fc si is expressed in Chinese Hamster Ovary (CHO) cells in a suspension culture. Cells from the Master Cell Bank (MCB) are used to inoculate shake flasks containing chemically defined medium free of animal components. The cells are then used to inoculate

progressively larger volume cultures to expand the cell number to enable inoculation of the production bioreactor.

[00578] The production bioreactor is operated in fed-batch mode to increase expression of the DF hIL12-Fc si protein. After approximately 14 days, the culture is harvested by depth filtration to remove cells and cell debris prior to initial purification. DF hIL12-Fc si is purified from the CHO harvest medium using a series of chromatography and filtration steps, including Protein A capture chromatography, Mixed Mode chromatography and Cation exchange chromatography (CEX).

[00579] Two dedicated, orthogonal viral inactivation and removal steps are included – low pH inactivation and nanofiltration. The viral inactivation step included addition of acetate to the filtered DF hIL12-Fc si solution to adjust the pH to about 3.65, and incubating for at least 60 minutes.

[00580] Finally, DF hIL12-Fc si is concentrated and formulated in a final composition of 20 mM Citrate, 6% Sucrose, 1% Mannitol and 0.01% (w/v) polysorbate 80. The formulated drug substance is then filtered through a 0.2 μm membrane into polycarbonate bottles prior to storage at $\leq -65^{\circ}\text{C}$. A schematic of the entire DF hIL12-Fc si drug substance manufacturing process is provided in **FIG. 32**.

Batch Scale and Definition

[00581] A single vial of DF hIL12-Fc si MCB is expanded to one production bioreactor and each harvest is purified into one lot of drug substance.

Cell Culture and Upstream Manufacturing Process

[00582] The upstream drug substance manufacturing process for DF hIL12-Fc si is shown in **FIG. 33** and additional details for each unit operation are provided.

Shake Flask Passages

[00583] A vial of the master cell bank is thawed in a 37°C water bath and the contents are slowly mixed by pipette and then added to a 125 mL shake flask containing pre-equilibrated growth medium (BalanCD CHO Growth Medium A, Irvine Scientific) supplemented with 6 mM L-glutamine. A cell count is taken after inoculation, and if necessary, the cell density is diluted to a target of 0.30×10^6 to 0.50×10^6 viable cells/mL. The flask is then placed on an orbital shaker in an incubator with temperature and %CO₂ (g) control. Cell density and viability are checked on day 3 prior to Passage 2.

[00584] For Passage 2, a 500 mL shake flask is pre-equilibrated with growth medium supplemented with 6 mM L-glutamine. The flask is then inoculated with cells from Passage 1 and

placed on an orbital shaker in an incubator with temperature and %CO₂ (g) control. Cell density and viability are measured, and the cells are used to inoculate Passage 3 once the forward processing criteria are met.

[00585] For Passage 3, three 1000 mL shake flasks are pre-equilibrated with growth medium supplemented with 6 mM L-glutamine. The flasks are then inoculated with cells from Passage 2 and then placed on an orbital shaker in an incubator with temperature and %CO₂ (g) control. Cell density and viability are measured, and the cells are used to inoculate Passage 4 once the forward processing criteria are met.

[00586] For Passage 4, four 5000 mL shake flasks are pre-equilibrated with growth medium supplemented with 6 mM L-glutamine. The flasks are then inoculated with cells from Passage 2 and then placed on an orbital shaker in an incubator with temperature and %CO₂ (g) control. Cell density and viability are measured, and the cells are used to inoculate the 50L Wave bioreactor once the forward processing criteria are met. Process parameter ranges and in-process tests are summarized in Table 17.

Table 17: Shake Flask Passages – Process Parameters and In-Process Tests

Unit Operation	Parameter Setpoints/Ranges	In-Process Tests
Passage #1: 125 mL shake flask	30 mL working volume; 36.5°C 5% CO ₂ (g); 120 rpm	Viable cell density Viability
Passage #2: 500 mL shake flask	150 mL working volume; 36.5°C 5% CO ₂ (g); 120 rpm	Viable cell density Viability
Passage #3: 3 x 1000 mL shake flasks	400 mL working volume; 36.5°C 5% CO ₂ (g); 120 rpm	Viable cell density Viability
Passage #4: 4 x 5000 mL shake flasks	1500 mL working volume; 36.5°C 5% CO ₂ (g); 120 rpm	Viable cell density Viability

Wave Bioreactor

[00587] A 50L Wave Bioreactor™ platform (GE Healthcare LifeSciences) is setup and inoculated with growth medium (BalanCD CHO Growth Medium A, Irvine Scientific) supplemented with 6 mM L-glutamine. The media is pre-conditioned at 36.5°C and 5% CO₂ (g) and then inoculated with culture from Passage 4. The bioreactor is sampled daily for cell density and viability, and the culture is used to inoculate the 200 L production bioreactor once the transfer cell density criteria is achieved. Metabolite concentrations (*e.g.*, glucose and lactate) and pH are also monitored on a daily basis for information. Process parameter ranges and in-process tests are summarized in Table 18.

Table 18: Wave Bioreactor – Process Parameters and In-Process Tests

Unit Operation	Parameter Setpoints/Ranges	In-Process Tests
Wave Bioreactor	20 L working volume; 36.5°C; 5% CO ₂ (g)	Viable cell density Viability

Production Bioreactor

[00588] A 200 L disposable bioreactor is setup and inoculated with growth medium supplemented with 6 mM L-glutamine. The media is pre-equilibrated at 37°C and then inoculated with culture from the 50 L Wave Bioreactor. Initial inoculation volume is approximately 130 L and final culture volume is approximately 180 L. Dissolved oxygen is controlled with air and oxygen supplementation and pH is controlled with addition of carbon dioxide gas and/or sodium carbonate base. The production bioreactor is sampled daily for cell density and viability and once the viable cell density is $\geq 14 \times 10^6$ viable cells/mL, the temperature setpoint is shifted from 37°C to 33°C and maintained at 33°C until harvest criteria is met. The culture is harvested when the viability is $\leq 85\%$ viability or day 14 of culture, whichever comes first. Metabolite concentrations (*e.g.*, glucose and lactate) and DF hIL12-Fc si titer (starting on day 8) are monitored during the culture period.

[00589] Starting on day 3 of culture, concentrated nutrient feeds are added on a daily basis until day 13. In addition, a concentrated glucose solution is added as needed to maintain a minimum concentration of glucose in the bioreactor after feeding. Beginning on day 3, antifoam is added to the bioreactor each day to minimize foam build-up. On the day of harvest, samples of the bioreactor culture are taken for adventitious agent testing. Process parameter ranges and in- process tests are summarized in Table 19.

Table 19: Production Bioreactor – Process Parameters and In-Process Tests

Unit Operation	Parameter Setpoints/Ranges	In-Process Tests
Production Bioreaction	130 L initial working volume 36.5 $\pm$ 0.5°C (prior to temp shift) 6.75 – 7.05 pH (prior to temp shift) 33.0 $\pm$ 0.5°C (after temp shift) 6.80 – 7.20 pH (after temp shift) 40% dissolved oxygen	Viable cell density Viability Mycoplasma Non-host contamination Mouse minute virus by qPCR <i>In vitro</i> adventitious agents Transmission electron microscopy DF hIL12-Fc si titer

Harvest Clarification

[00590] The bioreactor is clarified by depth filtration to remove cells and cell debris in preparation for further purification steps. A two-stage single-use depth filtration system consisting of DOHC and XOHC filters is used for clarification. Prior to the start of filtration, the bioreactor temperature is adjusted to 18°C and the dissolved oxygen setpoint is increased to 70% of saturation.

[00591] The harvest filters are rinsed with water for injection (WFI) and then equilibrated with buffer. The cell suspension is passed through the harvest filters using a peristaltic pump and the filters are flushed to collect the product. Pressure is monitored and maintained at ≤ 15 psig. The filtrate is then filtered through a 0.45/0.2 μm membrane into a sterile bag. Process parameter ranges and in-process tests are summarized in Table 20.

[00592] Clarified harvest is stored at 2-8°C prior to the capture chromatography step unless processed immediately.

Table 20: Harvest Clarification – Process Parameters and In-Process Tests

Unit Operation	Parameter Setpoints/Ranges	In-Process Tests
Clarification	≤ 25 psig	DF hIL12-Fc si concentration by Protein A HPLC Bioburden; Endotoxin

Downstream Purification Manufacturing Process

[00593] The downstream drug substance manufacturing process for DF hIL12-Fc si is shown in **FIG. 32** and additional details for each unit operation are provided in the text below. Downstream purification consists of three chromatography steps and two dedicated virus clearance steps, low pH inactivation and nanofiltration. For each process intermediate, hold-time studies have been performed to established allowed hold times and temperatures.

Protein A Capture Chromatography

[00594] The clarified harvest is captured with Amsphere 3 Protein A (JSR Life Sciences) resin to remove process-related impurities (*e.g.*, DNA and host cell proteins), media additives and serves as a volume reduction step prior to subsequent purification. Multiple cycles are performed for each lot as needed. Prior to each load, the resin is first equilibrated with 20 mM Tris, 150 mM NaCl, pH 7.5. Following loading, the column is washed with equilibration buffer to remove unbound or loosely bound impurities, and then a second wash with 50 mM acetate, pH 5.4 is performed to lower the pH and prepare the column for elution. DF hIL12-Fc si is eluted with 50 mM acetate, 100 mM arginine, pH 3.7 and collected by 280 nm UV wavelength starting at 1.25 AU/cm ascending and then ending at 1.25 AU/cm descending. The eluate is collected in one pool and each column cycle is individually processed by low pH virus inactivation. Process parameter ranges and in- process tests are summarized in Table 21.

Table 21: Protein A Capture Chromatography – Process Parameters and In-Process Tests

Unit Operation	Parameter Targets/Ranges	In-Process Tests
Protein A Capture Chromatography	20 - 33 g/L resin; 15-25°C; Equilibration pH: 7.5; Elution pH: 3.7; Load/Wash/Elution flow rate: 180 cm/hr	Bioburden Endotoxin

Low pH Virus Inactivation

[00595] The protein A eluate is incubated at low pH to inactivate potentially present viruses. The pH of the capture eluate is adjusted with 0.5 M acetic acid as necessary and incubated for a minimum of 60 minutes. After the end of the incubation period, the inactivated pools are neutralized with 2 M Tris base and the material is passed through a 0.2 µm filtration assembly. Process parameter ranges and in-process tests are summarized in Table 22.

Table 22: Low pH Virus Inactivation – Process Parameters and In-Process Tests

Unit Operation	Parameter Targets/Ranges	In-Process Tests
Low pH viral inactivation	Acidification pH: 3.65 ± 0.05 pH 60-75 minutes	Bioburden Endotoxin
	Neutralization pH: 5.2 ± 0.1 ; 15-25°C	

X0SP Depth Filtration

[00596] The Virus Inactivated Neutralized (VIN) pool is processed through the X0SP intermediate depth filter to remove process related impurities (*e.g.*, host cell proteins (HCP), host cell DNA). The system is flushed with WFI prior to loading DF hIL12-Fc si within the range of 500 – 1000 g/m². Following loading, the system is chased with 50 mM Acetate, pH 5.2 to complete product hold-up recovery. The X0SP pool conductivity is subsequently adjusted to ≤ 6.0 mS/cm with 50 mM Acetate pH 5.2 prior to loading onto the first chromatography step. Process parameter ranges and in-process tests are summarized in Table 23.

Table 23: X0SP Depth Filtration

Unit Operation	Parameter Targets/Ranges	In-Process Tests
X0SP Depth Filtration	500 – 1000 g/m ² ; ≤ 30 psig; 15-25°C Conductivity Adjustment: ≤ 6.0 mS/cm	Bioburden Endotoxin

Mixed Mode Chromatography

[00597] Mixed Mode chromatography by CaptoAdhere ImpRes (GE Healthcare) is performed in bind- elute mode to remove high molecular weight (HMW) species. The X0SP filtrate

conductivity is adjusted to ≤ 6.0 mS/cm with 50 mM Acetate pH 5.2 as described above and split into multiple load cycles as needed. Prior to loading, the column is equilibrated with 50 mM Acetate pH 5.2 and loaded. After loading, the column is washed with 50 mM Acetate pH 5.2 and then eluted with 50 mM Acetate 250 mM NaCl pH 5.2. Collection is initiated by 280 nm UV detection at 0.625 AU/cm ascending and ended at 1.50 AU/cm descending. Following collection, each cycle is passed through a filter train containing a terminal 0.2 μ m filter. Process parameter ranges and in-process tests are summarized in Table 24.

Table 24: Mixed Mode Chromatography – Process Parameters and In-Process Tests

Unit Operation	Parameter Targets/Ranges	In-Process Tests
Mixed Mode Chromatography	Load conductivity: 3.0 – 6.0 mS/cm; Load pH: 5.1 – 5.3; Flow rate: 150 cm/hr 15-23.5 g/L resin load 15-25°C	Bioburden Endotoxin

Cation Exchange Chromatography

[00598] Cation exchange chromatography with Eshmuno CPX resin (EMD Millipore) is performed to remove product-related impurities (e.g., high molecular weight species, low molecular weight species), as well as additional process related impurity clearance. Multiple cycles are performed for each lot as needed. Prior to loading, the CaptoAdhere ImpRes cycles are pooled and dilute with 50mM Tris, pH 7.4 buffer and pH adjusted to 7.50 ± 0.20 with 2M Tris Base. The column is equilibrated with 50 mM Tris, pH 7.4 prior to loading the diluted and pH adjusted CaptoAdhere ImpRes pool. The column is then washed with 50 mM Tris, pH 7.4, and then eluted with a gradient of 50 mM Tris, pH 7.4 (Buffer A) and 50mM Tris, 0.5 M NaCl, pH 7.4 (Buffer B). **[00599]** Product collection is initiated by 280 nm UV detection starting at 2.5 AU/cm ascending and ending at 4.5 AU/cm descending. Following collection, each cycle is passed through a filter train containing a terminal 0.2 μ m filter. Process parameter ranges and in-process tests are summarized in Table 25.

Table 25: Eshmuno CPX – Process Parameters and In-Process Tests

Unit Operation	Parameter Targets/Ranges	In-Process Tests
Cation Exchange Chromatography	10-14 g/L resin; Load pH: 7.50 ± 0.20 ; Load/Elution flow rate: 200 cm/h 15-25°C	Bioburden Endotoxin

Nanofiltration

[00600] Nanofiltration is performed to remove any potentially present viruses based on size. The Eshmuno CPX eluate is passed through a prefilter (Viresolve Prefilter Pod, EMD Millipore) and

then through a 20 nm nominal filter (Viresolve Pro Modus, EMD Millipore). Prior to loading, the system is flushed with WFI and equilibrated with 50 mM Tris, 265 mM NaCl, pH 7.4. After loading, the system is rinsed with equilibration buffer to recover the system hold-up. The filtrate is then passed through a 0.2 µm membrane prior to the next step. Process parameter ranges and in-process tests are summarized in Table 26.

Table 26: Nanofiltration – Process Parameters and In-Process Tests

Unit Operation	Parameter Targets/Ranges	In-Process Tests
Nanofiltration	$\leq 500 \text{ L/m}^2 \text{ load}$ $\leq 30 \text{ psig}$ $15\text{-}25^\circ\text{C}$	Bioburden Endotoxin Filter integrity test

Ultrafiltration and Diafiltration (UF/DF)

[00601] Ultrafiltration and diafiltration are performed using Pellicon Ultracel D Screen regenerated cellulose 30 kDa molecular weight cut-off membranes. This step concentrates and exchange the DF hIL12-Fc si into the final formulation buffer at the intended concentration prior to final filtration and bottling. The system is first equilibrated with 50 mM Tris, 265 mM NaCl, pH 7.4 and then the viral filtrate pool is concentrated to a target of 5.0 g/L. Buffer exchange is then performed against a minimum of 7 diavolumes of 20 mM Citrate, pH 6.5. Following diafiltration, a second concentration is performed targeting 11.0 g/L and then the product is diluted to a final retentate target concentration of 7.5 g/L with diafiltration buffer.

[00602] A 20 mM Citrate, 18 % (w/v) Sucrose, 3 % (w/v) Mannitol, 0.03% (w/v) polysorbate-80, pH 6.5 stock solution is spiked into the UF/DF pool to target a final concentration of 20 mM Citrate, 6 % (w/v) Sucrose, 1 % (w/v) Mannitol, 0.01 % (w/v) polysorbate-80 in the drug substance. Process parameter ranges and in-process tests are summarized in Table 27.

Table 27: UF/DF – Process Parameters and In-Process Tests

Unit Operation	Parameter Targets/Ranges	In-Process Tests
Ultrafiltration and Diafiltration	$\leq 250 \text{ g/m}^2 \text{ load};$ 12.0-18.0 psig transmembrane pressure 7-9 diavolumes; $15\text{-}25^\circ\text{C}$	Bioburden Endotoxin DF hIL12-Fc si Concentration

Filtration, Bottling, and Storage of Bulk Drug Substance

[00603] The formulated UF/DF retentate is filtered through a 0.2 µm membrane into the final drug substance storage containers, 2 L polycarbonate bottles with a polypropylene closures (Nalgene Biotainer). Filtration is performed in an ISO 5/Grade A area. Following filtration, each

bottle is aseptically sampled, labeled and frozen at $\leq -65^{\circ}\text{C}$. Process parameter ranges and in-process tests are summarized in Table 28.

Table 28: Filtration, Bottling, and BDS Storage – Process Parameters and In-Process Tests

Unit Operation	Parameter Targets/Ranges	In-Process Tests
Filtration, Bottling, and BDS Storage	1.0 L fill volume Storage at $\leq -65^{\circ}\text{C}$	Filter integrity testing

Example 23: Formulation, Packaging, and Storage of DF hIL12-Fc si

[00604] The DF hIL12-Fc si drug product manufacturing process flow diagram, indicating manufacturing steps and in-process controls (IPCs), is shown in **FIG. 32**. Filtration and filling are performed following aseptic procedures that meet applicable standards described in ICH guidelines and current Good Manufacturing Practices.

Thawing Bulk Drug Substance

[00605] The DF hIL12-Fc si Drug Substance (DS) is thawed for ≤ 96 hours at $2-8^{\circ}\text{C}$ in the dark. Complete thawing of DS is confirmed by visual examination of the bottle(s).

Dilution to 80% of Target Batch Volume

[00606] A buffer consisting of 20 mM citrate, 6% (w/v) sucrose, 1% (w/v) mannitol, 0.01% polysorbate 80 (w/v), pH 6.0 is prepared in a 10 L glass carboy. Solid sodium citrate dihydrate, citric acid monohydrate, sucrose, and mannitol are weighed, added to WFI, and mixed to dissolution. A polysorbate 80 stock solution is prepared in WFI and added to the buffer. The pH of the buffer is tested (acceptance criteria 6.5 ± 0.4). The buffer is diluted with WFI to the target volume, mixed, tested to confirm the pH (6.5 ± 0.4) and osmolality, and filtered through a $0.2 \mu\text{m}$ membrane.

[00607] The weight of drug substance is used to calculate a target batch volume. The drug substance is added to buffer in a clean, 10 L glass carboy to approximately 80% of the calculated batch volume and mixed. The 80% drug product solution is tested for pH (acceptance criteria 6.5 ± 0.3) and protein concentration by absorbance at 280 nm using an Extinction coefficient of $1.44 \text{ L}/(\text{g}\cdot\text{cm})$.

[00608] The buffer components are designed to yield a pH of 6.5. If the pH does not meet acceptance criteria at either the buffer or 80% bulk drug product steps, a titration with 1N sodium hydroxide or 1N hydrochloric acid may be performed to bring the pH within the acceptance criteria.

Dilution of DF hIL12-Fc si to 1 mg/mL

[00609] The protein concentration result from the previous step is used to calculate the required amount of buffer to reach a DF HIL12-FC SI concentration of 1 mg/mL. The concentration is

verified by absorbance at 280 nm (acceptance criteria 1.0 ± 0.2 mg/mL), and samples are taken to confirm the pH (acceptance criteria 6.5 ± 0.3) and osmolality.

[00610] The compounded bulk drug product solution is passed through a sterile 0.2 μ m filter into a clean, 10 L glass carboy for bioburden reduction, and held until sterile filtration and filling. Samples for pre-filtration bioburden are removed from the 10 L glass carboy.

Sterile Filtration

[00611] The bulk drug product is filtered through two filter capsules in series, each filter capsule consisting of a 0.45 μ m polyethersulfone (PES) pre-filter membrane and a 0.2 μ m PES sterilizing membrane. The drug product is filtered into a sterile, disposable fill bag inside a controlled Grade B area of the filling suite. Both sterilizing filter capsules are tested for integrity by bubble point after filtration (acceptance criteria ≥ 3200 mbar, using WFI).

Filling into Vials

[00612] The bulk drug product solution is filled from the disposable bag residing immediately outside of the restricted access barrier system (RABS). The product is filled into ready-to-use 2R borosilicate type I vials inside the controlled, Grade A RABS area of the filling suite.

[00613] The vials are stoppered with sterilized, 13 mm serum stoppers and capped with 13 mm aluminum overseals. Fill volume of the vials is verified by weight checks of 100% of the batch during filling operations (acceptance criteria $1.3 \text{ mL} \pm 5\%$). After filling, vials are moved to 2-8°C storage.

Visual Inspection, Packaging and Storage

[00614] The filled vials undergo 100% manual visual inspection for container, closure, and product defects, followed by an Acceptance Quality Limit inspection (AQL). The inspected vials are bulk packaged and stored at 2-8°C prior to shipment.

Example 24: Formulation Analysis

Buffer Analysis

[00615] The formulations listed in Table 29 were evaluated to assess the effects of various buffer and pH conditions on the stability of DF hIL12-Fc si. DF hIL12-Fc si was buffer exchanged using centrifugal ultrafiltration devices (Amicon Ultra-4 30k MWCO) into the buffers listed in Table 29 to a target protein concentration of 1 mg/mL. Following the final buffer-exchange, the protein concentration was measured using UV-Visible spectroscopy with the sponsor-provided extinction coefficient (1.43 mL/cm*mg). The samples were then split into three equal sized aliquots. One aliquot was stored at 2 – 8°C and the other two were stored at 50°C. The aliquot stored at a 2 – 8°C

and one of the aliquots at 50°C were both removed at 1 week for testing as listed in Table 30. The other vial at 50°C was removed after 2 weeks and stored at -75°C.

Table 29: Buffers screened for DF hIL12-Fc si preformulation

Buffer	pH
Succinate (20 mM) pKa = 5.64	5.5
	6.5
Citrate (20 mM) pKa = 6.4	5.5
	6.0
	7.0
Histidine (20 mM) pKa = 6.04	6.5
	7.0
Phosphate (20 mM) pKa = 7.2	6.5
	7.0
	7.5
Tris (20 mM) pKa = 8.1	7.5
	8.0

Table 30: Assay panel

Test Panel	Assay Volume (μL)
Visual Appearance	In Vial
DSF	100
A ₂₈₀	100
DLS	100
SEC-HPLC ATM-2606	100
CE (reduced) ATM-2747	100

Results

[00616] Samples of DF hIL12-Fc si were buffer exchanged into 12 buffer/pH conditions. The pH and concentration of the samples were immediately assessed. After which, the samples were aliquoted and stored at 2 – 8°C and 50°C. After a one-week incubation, the samples were then assessed per the assay panel in Table 30. The results are shown below, and in **FIGs. 34A-42B**.

Table 31: Concentration of DF hIL12-Fc si (buffer exchange)

Buffer	Target pH	Concentration (mg/mL)
Succinate (20 mM) pKa = 5.64	5.5	1.08
	6.5	1.07

Citrate (20 mM) pKa = 6.4	5.5	1.09
	6.0	1.09
	7.0	1.09
Histidine (20 mM) pKa = 6.04	6.5	1.08
	7.0	1.11
Phosphate (20 mM) pKa = 7.2	6.5	1.05
	7.0	1.09
	7.5	1.07
Tris (20 mM) pKa = 8.1	7.5	1.12
	8.0	1.15

Please note: concentration = $((A_{280}-A_{320})/1.43)*1.0$ (cm path length) *

Dilution Factor (if applicable)

Table 32: pH values (buffer exchange)

Buffer	Target pH	Buffer pH	Sample pH
Succinate (20 mM) pKa = 5.64	5.5	5.4	5.4
	6.5	6.4	6.5
Citrate (20 mM) pKa = 6.4	5.5	5.5	5.5
	6.0	5.9	5.9
	7.0	6.9	7.0
Histidine (20 mM) pKa = 6.04	6.5	6.4	6.4
	7.0	6.9	6.8
Phosphate (20 mM) pKa = 7.2	6.5	6.5	6.5
	7.0	6.9	7.0
	7.5	7.5	7.4
Tris (20 mM) pKa = 8.1	7.5	7.4	7.4
	8.0	7.9	7.9

Visual Appearance (1-week incubation)

[00617] Visual appearances of all samples were assessed 1 week after incubating the samples at 5°C or at 50°C. All samples were colorless, clear liquids, free of visible particulates (see **FIGs. 35A-35B**).

Differential Scanning Fluorimetry (DSF) (1-week incubation)

[00618] The DSF experiments were performed using an Unchained Labs UNcle. Samples were evaluated for their thermal stability. DF hIL12-Fc si thermal unfolding (T_m) and onset of aggregation (T_{agg}) were monitored by evaluating changes in intrinsic protein fluorescence and static light scattering (SLS at 266 nm), respectively, as a function of temperature. Samples were evaluated in triplicate, and the triplicate results were averaged. Samples were analyzed over a

temperature ramp of 25°C – 95°C at a constant linear ramp rate of 0.5°C/min. See Tables 33-36, below, and **FIGs. 36A-37D**.

Table 33: Determined DSF T_m Temperatures (1-week incubation at 5°C)

Buffer	pH	Average T_{m1} (°C)	Average T_{m2} (°C)	Average T_{m3} (°C)
Succinate (20 mM) pKa = 5.64	5.5	61.0	76.7	85.8
	6.5	64.4	75.9	N/A
Citrate (20 mM) pKa = 6.4	5.5	62.0	78.6	N/A
	6.0	64.6	75.7	N/A
	7.0	65.8	74.7	N/A
Histidine (20 mM) pKa = 6.04	6.5	61.3	76.5	N/A
	7.0	61.3	78.8	N/A
Phosphate (20 mM) pKa = 7.2	6.5	64.0 ¹	76.8 ¹	N/A ¹
	7.0	65.1	76.0	N/A
	7.5	64.8	75.4	N/A
Tris (20 mM) pKa = 8.1	7.5	62.9	75.0	80.6
	8.0	62.2	76.8	N/A

¹One of the triplicate wells was excluded from analysis. A low intensity reading occurred around T_{m1} , negatively affecting the differential and preventing proper analysis of T_{m1} .

Table 34: Determined DSF T_m Temperatures (1-week incubation at 50°C)

Buffer	pH	Average T_{m1} (°C)	Average T_{m2} (°C)	Average T_{m3} (°C)
Succinate (20 mM) pKa = 5.64	5.5	63.0	83.3	N/A
	6.5	65.1	76.7	N/A
Citrate (20 mM) pKa = 6.4	5.5	63.0	79.1	N/A
	6.0	65.0 ¹	76.5 ¹	N/A ¹
	7.0	66.4	75.5	N/A
Histidine (20 mM) pKa = 6.04	6.5	62.8	77.3	N/A
	7.0	62.4	80.5 ²	N/A ¹
Phosphate (20 mM) pKa = 7.2	6.5	64.8	77.5	N/A
	7.0	65.5	76.4	N/A
	7.5	64.9	75.1	N/A
Tris (20 mM) pKa = 8.1	7.5	63.9	76.0	83.4
	8.0	62.4	76.8	N/A

¹One of the triplicate wells was excluded from analysis. Data acquired for this well was indicative of either an air bubble or misread, preventing proper analysis.

²An additional T_m (67.4°C) was identified between T_{m1} and T_{m2} . The T_m was not reported as T_{m2} given its low temperature as well as poor differential and separation from T_{m1} .

Table 35: Determined DSF T_{agg} 266 Temperatures (1-week incubation at 5°C)

Buffer	pH	T_{agg} 266(°C) Average
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Succinate (20 mM) pKa = 5.64	5.5	70.7
	6.5	75.3
Citrate (20 mM) pKa = 6.4	5.5	72.9
	6.0	76.1
	7.0	63.9
Histidine (20 mM) pKa = 6.04	6.5	65.4
	7.0	70.1
Phosphate (20 mM) pKa = 7.2	6.5	75.8
	7.0	63.7
	7.5	63.2
Tris (20 mM) pKa = 8.1	7.5	74.8
	8.0	63.4

Table 36: Determined DSF T_{agg} 266 Temperatures (1-week incubation at 50°C)

Buffer	pH	T _{agg} 266(°C) Average
Succinate (20 mM) pKa = 5.64	5.5	64.7
	6.5	74.9
Citrate (20 mM) pKa = 6.4	5.5	70.1
	6.0	76.7
	7.0	63.3
Histidine (20 mM) pKa = 6.04	6.5	62.2
	7.0	71.5
Phosphate (20 mM) pKa = 7.2	6.5	76.0
	7.0	63.4
	7.5	61.8
Tris (20 mM) pKa = 8.1	7.5	75.8
	8.0	63.1

Concentrations of DF hIL12-Fc si and pH were assessed in the various buffer formulations after 1 week of incubation at 5°C and 50°C. See Tables 37-40 and **FIGs. 38A-39B**.

Table 37: Concentration of DF hIL12-Fc si (1-week incubation at 5°C)

Buffer	Target pH	Concentration (mg/mL)
Succinate (20 mM) pKa = 5.64	5.5	0.80
	6.5	0.83
Citrate (20 mM) pKa = 6.4	5.5	0.92
	6.0	0.92
	7.0	0.89
Histidine (20 mM) pKa = 6.04	6.5	0.89
	7.0	0.97
Phosphate (20 mM)	6.5	0.91
	7.0	0.89

pKa = 7.2	7.5	0.96
Tris (20 mM)	7.5	0.96
pKa = 8.1	8.0	1.02

Please note: concentration = $((A_{280}-A_{320})/1.43)*1.0$ (cm path length) *

Dilution Factor (if applicable)

Table 38: Concentration of DF hIL12-Fc si (1-week incubation at 50°C)

Buffer	Target pH	Concentration (mg/mL)
Succinate (20 mM) pKa = 5.64	5.5	0.91
	6.5	0.93
Citrate (20 mM) pKa = 6.4	5.5	0.97
	6.0	0.98
	7.0	0.96
Histidine (20 mM) pKa = 6.04	6.5	0.90
	7.0	1.01
Phosphate (20 mM) pKa = 7.2	6.5	0.91
	7.0	0.98
	7.5	1.01
Tris (20 mM) pKa = 8.1	7.5	1.00
	8.0	1.06

Please note: concentration = $((A_{280}-A_{320})/1.43)*1.0$ (cm path length) *

Dilution Factor (if applicable)

Table 39: pH values (1-week incubation at 5°C)

Buffer	Target pH	Buffer pH	Sample pH
Succinate (20 mM) pKa = 5.64	5.5	5.4	5.4
	6.5	6.4	6.5
Citrate (20 mM) pKa = 6.4	5.5	5.5	5.6
	6.0	5.9	6.0
	7.0	6.9	7.1
Histidine (20 mM) pKa = 6.04	6.5	6.4	6.5
	7.0	6.9	6.9
Phosphate (20 mM) pKa = 7.2	6.5	6.5	6.5
	7.0	6.9	7.0
	7.5	7.5	7.5
Tris (20 mM) pKa = 8.1	7.5	7.4	7.4
	8.0	7.9	8.0

Table 40: pH values (1-week incubation at 50°C)

Buffer	Target pH	Buffer pH	Sample pH
Succinate (20 mM) pKa = 5.64	5.5	5.4	5.5
	6.5	6.4	6.6

Citrate (20 mM) pKa = 6.4	5.5	5.5	5.6
	6.0	5.9	6.0
	7.0	6.9	7.1
Histidine (20 mM) pKa = 6.04	6.5	6.4	6.5
	7.0	6.9	7.0
Phosphate (20 mM) pKa = 7.2	6.5	6.5	6.5
	7.0	6.9	7.0
	7.5	7.5	7.5
Tris (20 mM) pKa = 8.1	7.5	7.4	7.5
	8.0	7.9	8.0

Dynamic Light Scattering (DLS) (1-week incubation)

[00619] The DLS experiments were performed using a Malvern Zeta sizer. For each sample measurement, five consecutive scans were acquired at 25°C. The Z-average hydrodynamic diameter and polydispersity index (PDI) were determined from the cumulants analysis and the Stokes Einstein equation. Polydispersity is a measurement of non-uniformity in a sample. If particles are not uniform in size, a higher polydispersity will be measured. A low polydispersity index (PDI) (≤ 0.200) indicates greater uniformity in the size of the particle. See Tables 41-42 and FIGs. 40A-40L.

Table 41: Determined DLS Sizes (1-week incubation at 5°C)

Buffer	pH	Average Size (d.nm)	PdI	Monomer Size (d.nm)	Monomer %Pd
Succinate (20 mM) pKa = 5.64	5.5	13.71	0.16	15.33	37.70
	6.5	13.55	0.20	15.35	42.60
Citrate (20 mM) pKa = 6.4	5.5	18.94	0.20	22.52	47.10
	6.0	11.22	0.07	12.17	28.80
	7.0	11.62	0.17	12.93	35.20
Histidine (20 mM) pKa = 6.04	6.5	15.98	0.10	17.75	33.00
	7.0	14.88	0.06	15.92	26.90
Phosphate (20 mM) pKa = 7.2	6.5	13.38	0.17	14.81	36.50
	7.0	14.66	0.25	16.00	43.50
	7.5	21.24	0.36	18.42	29.40
Tris (20 mM) pKa = 8.1	7.5	85.19	0.15	20.42	27.10
	8.0	13.41	0.14	15.01	36.00

Table 42: Determined DLS Sizes (1-week incubation at 50°C)

Buffer	pH	Average Size (d.nm)	PdI	Monomer Size (d.nm)	Monomer %Pd
Succinate (20 mM) pKa = 5.64	5.5	63.12	0.21	80.95	50.20
	6.5	14.46	0.12	15.76	30.10
Citrate (20 mM)	5.5	44.20	0.28	76.39	49.30
	6.0	16.46	0.25	15.99	33.00

pKa = 6.4	7.0	14.31	0.25	14.13	28.90
Histidine (20 mM) pKa = 6.04	6.5	98.61	0.44	185.60	46.10
	7.0	22.52	0.13	24.23	30.30
Phosphate (20 mM) pKa = 7.2	6.5	27.91	0.33	26.08	38.40
	7.0	14.69	0.20	15.46	32.00
	7.5	12.06	0.11	13.39	33.50
Tris (20 mM) pKa = 8.1	7.5	16.22	0.16	17.20	31.00
	8.0	17.42	0.25	17.25	32.50

Size Exclusion Chromatography High-performance Liquid Chromatography (SEC-HPLC)

[00620] The SEC experiments were performed using a TOSOH G3000SWxl (7.8 x 300 mm). For each sample, 90 µL was injected to achieve a column load of 90 µg (target column load of 100 µg was not achievable given the low concentration of 1 mg/mL). Given the lack of historic data, the largest peak was defined as the main peak. Peaks prior to the main peak were defined as high molecular weight (HMW) species and peaks after as low molecular weight (LMW) species. See Tables 43-44 and **FIG. 41**. The purity of the main peak was greater than 93% for all of the conditions tested for the 5°C one-week incubation.

Table 43: SEC-HPLC Purity (1-week incubation at 5°C)

Buffer	pH	% HMW	% Main	% LMW
Succinate (20 mM) pKa = 5.64	5.5	1.0	96.6	2.4
	6.5	1.9	96.0	2.2
Citrate (20 mM) pKa = 6.4	5.5	1.1	97.0	1.9
	6.0	0.4	96.7	2.8
	7.0	1.1	97.4	1.4
Histidine (20 mM) pKa = 6.04	6.5	3.9	93.7	2.4
	7.0	2.0	96.5	1.5
Phosphate (20 mM) pKa = 7.2	6.5	1.4	97.4	1.2
	7.0	1.4	95.2	3.5
	7.5	1.3	95.2	3.5
Tris (20 mM) pKa = 8.1	7.5	2.2	96.8	1.0
	8.0	2.0	95.5	2.5

Table 44: SEC-HPLC Purity (1-week incubation at 50°C)

Buffer	pH	% HMW	% Main	% LMW
Succinate (20 mM) pKa = 5.64	5.5	46.9	51.0	2.1
	6.5	11.4	86.9	1.7
Citrate (20 mM) pKa = 6.4	5.5	26.7	71.9	1.4
	6.0	6.4	92.0	1.5
	7.0	4.2	94.3	1.5
Histidine (20 mM)	6.5	44.7	53.7	1.7

pKa = 6.04	7.0	41.2	57.8	1.0
Phosphate (20 mM) pKa = 7.2	6.5	17.2	81.0	1.8
	7.0	8.8	89.7	1.5
	7.5	8.8	89.4	1.8
Tris (20 mM) pKa = 8.1	7.5	21.2	77.4	1.4
	8.0	22.0	76.6	1.4

Capillary Electrophoresis sodium dodecyl sulfate (CE-SDS) (Reduced)

[00621] The CE-SDS experiments were performed using a Beckman Coulter PA 800 plus Capillary Electrophoresis System. Given the lack of historic data, the largest peak was defined as the main peak (peak 7). Peaks prior to the main peak were defined as LMW species and peaks after as HMW species. See Tables 45-46 and **FIG. 42**. The purity of the main peak was greater than 49% for all of the conditions tested for the 5°C one-week incubation.

Table 45: CE-SDS Purity (1-week incubation at 5°C)

Buffer	pH	% Purity/Impurity							
		Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8
Succinate (20 mM) pKa = 5.64	5.5	0.2	6.9	8.0	1.3	5.6	24.8	52.8	0.4
	6.5	0.3	6.9	7.9	1.2	5.7	25.0	52.5	0.4
Citrate (20 mM) pKa = 6.4	5.5	0.2	6.9	7.9	1.2	5.8	25.0	52.5	0.4
	6.0	0.2	6.8	7.9	1.2	5.8	24.9	52.8	0.4
	7.0	0.2	6.8	7.8	1.2	5.7	24.8	53.1	0.4
Histidine (20 mM) pKa = 6.04	6.5	0.2	7.0	7.7	1.2	5.4	25.2	52.8	0.4
	7.0	0.3	7.1	7.8	1.1	5.4	25.1	52.8	0.4
Phosphate (20 mM) pKa = 7.2	6.5	2.2	8.5	10.1	1.0	5.2	22.9	49.1	0.4
	7.0	0.3	7.0	7.7	1.1	5.5	25.3	52.7	0.4
	7.5	0.3	7.0	7.8	1.2	5.7	24.9	52.8	0.4
Tris (20 mM) pKa = 8.1	7.5	0.3	7.0	7.9	1.2	5.5	25.1	52.6	0.5
	8.0	0.3	7.0	7.9	1.3	5.5	24.6	52.0	0.9

Table 46: CE-SDS Purity (1-week incubation at 50°C)

Buffer	pH	% Purity/Impurity							
		Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8
Succinate (20 mM) pKa = 5.64	5.5	1.4	5.7	10.1	1.7	6.8	24.5	46.7	1.6
	6.5	0.6	7.4	8.6	1.4	5.6	24.4	51.3	0.7
Citrate (20 mM) pKa = 6.4	5.5	1.0	5.9	9.5	1.5	6.5	25.8	49.1	0.4
	6.0	0.5	7.0	8.4	1.5	5.8	25.1	51.1	0.5
	7.0	0.6	7.8	8.9	1.3	5.7	24.6	50.5	0.2
Histidine (20 mM) pKa = 6.04	6.5	0.6	6.2	7.9	1.4	5.7	24.9	50.4	1.7
	7.0	0.7	7.0	8.6	1.5	5.4	24.5	49.5	1.6

Phosphate (20 mM) pKa = 7.2	6.5	2.0	17.0	18.0	1.3	5.7	22.0	32.5	0.6
	7.0	0.8	8.1	9.2	1.5	5.3	24.5	49.5	0.9
	7.5	1.1	9.1	10.0	1.6	5.5	22.4	46.5	2.5
Tris (20 mM) pKa = 8.1	7.5	0.6	7.3	8.5	1.5	5.4	24	50.1	1.5
	8.0	0.6	7.5	8.8	1.5	5.0	23.7	49.2	2.4

Summary and Conclusions

[00622] All the samples were analyzed by visual appearance, pH, A₂₈₀, DLS, DSF, SEC, and CE-SDS. Evaluation by visual appearance and pH showed no significant differences between the various samples. The concentrations of all the samples were slightly decreased after the 1- week incubation (a decrease of 0.1 or 0.2 mg/mL). The concentration of the succinate samples (pH 5.5 and 6.5) after a 1-week incubation at 5°C was more decreased (from 1.1 mg/mL to 0.8 mg/mL). The CE-SDS data showed minimal variation in the main peak purity (with the exception of the phosphate pH 6.5 buffer, which had a significantly lower mean peak purity after the 50°C 1- week incubation).

[00623] The thermal stability data indicated that low pH (5.5) and histidine buffers negatively affected the molecule. Citrate buffers (with the exception of pH 5.5) and phosphate buffers displayed the highest thermal stability (for both the 5°C and the 50°C 1-week incubation samples).

[00624] The light scattering data after the 5°C 1-week incubation indicated that the citrate buffer samples (with the exception of pH 5.5) had the smallest average size with low polydispersity. The phosphate and succinate buffers similarly had a small average size with low polydispersity. After the 50°C 1-week incubation, the citrate buffers (with the exception of pH 5.5), the phosphate buffers (with the exception of pH 6.5), and the succinate pH 6.5 buffer, all had small average sizes.

[00625] The SEC data of the 5°C 1-week incubation samples showed minimal variation in the main peak purity. The SEC data of the 50°C 1-week incubation samples showed a greater degree of variation, ranging from 51.0% to 94.3%. The most desirable buffers were again the citrate buffers (with the exception of pH 5.5) and the phosphate buffers

[00626] Performance of the citrate pH 6.0 buffer or the citrate pH 7.0 buffer was more desirable than the alternative buffers tested in the assays described *supra*.

Excipient Analysis

[00627] The formulations listed in Table 47 were evaluated to assess the effects of various excipients and surfactants on the stability of DF-hIL-12-Fc si when buffered in 20 mM Citrate, pH 6.5. DF-hIL-12-Fc si was buffer exchanged using centrifugal ultrafiltration devices (Amicon Ultra-15 30k MWCO) into the buffers listed in Table 47 to a target protein concentration of either 1

mg/mL or 10 mg/mL. Following the final buffer-exchange, the protein concentration was measured using UV-Visible spectroscopy (UV-Vis) with the sponsor-provided extinction coefficient (1.43 mL/cm*mg). The samples were then sterile filtered using a 0.22 µm EMD Millipore Ultrafree – CL centrifugal filter devices with Durapore membrane (Fisher Scientific Cat. #UFC40GV0S). Following sterile filtration, each formulation was handled aseptically in a laminar flow hood. The formulated samples, as specified in Table 47, were either spiked with polysorbate 80 (PS80) to a final concentration of 0.01% or were not spiked with a surfactant. The samples were then split into six equal sized aliquots. Two aliquots were stored at 2 – 8°C, three were stored at 50°C, and the final aliquot underwent 5 freeze thaw cycles. For the freeze thaw aliquot, the samples were frozen at -75±10°C for at least an hour and thawed at room temperature, with visual confirmation of no ice. Both aliquots stored at 2 – 8°C were pulled at 2 weeks. One aliquot was used for testing and the other aliquot was frozen at -75±10°C. A single aliquot at 50°C was pulled at 2 weeks for testing. The other two aliquots at 50°C were pulled at 3 weeks and frozen at -75±10°C, shown in Table 48. The testing panel is shown in Table 49. An evaluation of particulate matter by high accuracy liquid particle count (HIAC) was also executed, to evaluate surfactant.

Table 47: Buffers Screened for DF-hIL-12-Fc si Excipient DOE Screen

Code	Buffer pH	Sugar	Sugar Conc.	Surfactant	DF-hIL-12-Fc si Conc.
A	20 mM Citrate pH 6.5	Sucrose	High (8% w/v, 233 mM)	None	1 mg/mL
B				0.01% PS80	1 mg/mL
C				0.01% PS80	10 mg/mL
D		Sucrose	Low (4% w/v, 117 mM)	None	1 mg/mL
E		Mannitol	High (6% w/v, 330 mM)	None	1 mg/mL
F				0.01% PS80	1 mg/mL
G				0.01% PS80	10 mg/mL
H		Mannitol	Low (2% w/v, 110 mM)	None	1 mg/mL
I		Sucrose Mannitol	6% w/v (175 mM) 1% w/v (55 mM)	None	1 mg/mL
J				0.01% PS80	1 mg/mL
K				0.01% PS80	10 mg/mL
L		Sucrose Mannitol	4% w/v (117 mM) 2% w/v (110 mM)	None	1 mg/mL
M				0.01% PS80	1 mg/mL
N				0.01% PS80	10 mg/mL

Table 48: Vial Distribution

Vial Number	Condition	Testing
Vial 1	Two weeks at 2 – 8 °C	Batch Tested on the 2 – 8 °C sample only
Vial 2	Two weeks 50 °C	
Vial 3	5X Freeze Thaw Cycles Freezing at -75±10°C Thawing at Room Temperature	
Vial 4	Two weeks at 2 – 8 °C Then frozen at -75±10°C	Tested as needed
Vials 5 and 6	Three weeks at 50 °C Then frozen at -75±10°C	

Table 49: Assay panel

Test Panel	Assay Volume (µL)
Visual Appearance	In Vial
A ₂₈₀	100
DLS	100
SEC-HPLC, ATM-2606	100
DSF (2-8°C Vial 1 or retain during sample prep) ¹	100 ¹
HIAC ²	100

¹DSF performed at 1 week with material remaining from sample prep²HIAC performed with 0.1 mL tare volume and 0.3 mL sample volume (single draw/read)*Results*

[00628] Samples of DF-hIL-12-Fc si were buffer exchanged into 14 formulations. The pH and concentration of the samples were immediately assessed. After which, the samples were aliquoted and stored at 2 – 8°C, 50°C, or underwent 5 freeze thaw cycles. After a two-week incubation, the samples were then assessed per the assay panel in Table 49. The untested samples were pulled and frozen as outlined previously.

[00629] Table 50 and **FIGs. 43A-43B** show UV-Vis Concentration Determination (time zero and 2 week samples).

Table 50: Concentration of DF-hIL-12-Fc si

Code	Sugar	Surfactant	Buffer Exchange Concentration (mg/mL)	2 – 8°C Concentration (mg/mL)	50°C Concentration (mg/mL)	Freeze Thaw Concentration (mg/mL)
A	8% w/v Sucrose	None	0.90	0.79	0.80	0.80
B		0.01% PS80		0.84	0.85	0.85
C		0.01% PS80	10.26	9.94	10.27	10.20
D	4% w/v Sucrose	None	1.11	0.97	1.00	0.99

E	6% w/v Mannitol	None	0.89	0.81	0.82	0.82
F		0.01% PS80		0.87	0.87	0.86
G		0.01% PS80	10.61	10.69	10.91	10.74
H	2% w/v Mannitol	None	1.04	0.93	0.94	0.95
I	6% w/v Sucrose 1% w/v Mannitol	None	1.03	0.93	0.92	0.95
J		0.01% PS80		0.98	0.98	0.98
K		0.01% PS80	9.96	9.59	10.09	9.95
L	4% w/v Sucrose 2% w/v Mannitol	None	1.05	0.92	0.93	0.94
M		0.01% PS80		0.99	0.98	0.98
N		0.01% PS80	10.14	9.41	10.19	9.48

Please note: concentration = $((A_{280}-A_{320})/1.43)*1.0$ (cm path length) *

Dilution Factor (if applicable)

[00630] Table 51 shows pH Determination at time zero and 2 week samples.

Table 51: pH values

Code	Sugar	Surfactant	Buffer Exchange pH	2 – 8°C pH	50°C pH	Freeze Thaw pH
A	8% w/v Sucrose	None	6.5	6.6	6.5	6.5
B		0.01% PS80		6.5	6.5	6.5
C		0.01% PS80	6.5	6.5	6.5	6.5
D	4% w/v Sucrose	None	6.5	6.6	6.5	6.5
E	6% w/v Mannitol	None	6.5	6.5	6.5	6.5
F		0.01% PS80		6.5	6.5	6.5
G		0.01% PS80	6.5	6.5	6.5	6.5
H	2% w/v Mannitol	None	6.5	6.5	6.5	6.4
I	6% w/v Sucrose 1% w/v Mannitol	None	6.5	6.5	6.5	6.5
J		0.01% PS80		6.5	6.5	6.5
K		0.01% PS80	6.5	6.5	6.5	6.5
L	4% w/v Sucrose 2% w/v Mannitol	None	6.5	6.5	6.5	6.5
M		0.01% PS80		6.4	6.5	6.5
N		0.01% PS80	6.5	6.5	6.5	6.4

Visual Appearance (2 week samples)

[00631] All samples were colorless and clear liquids. Particulates were observed in some samples as outlined in Table 52.

Table 52: Appearance: Visible Particulates

Code	Sugar	Surfactant	2 – 8°C Appearance	50°C Appearance	Freeze Thaw Appearance
A	8% w/v Sucrose	None	Free of visible particulates	Some small round visible particulates	Free of visible particulates
B		0.01% PS80	Free of visible particulates	Free of visible particulates	Free of visible particulates
C		0.01% PS80	Free of visible particulates	Free of visible particulates	Free of visible particulates
D	4% w/v Sucrose	None	Free of visible particulates	Free of visible particulates	Some small round visible particulates
E	6% w/v Mannitol	None	Free of visible particulates	Free of visible particulates	Free of visible particulates
F		0.01% PS80	Free of visible particulates	Free of visible particulates	Free of visible particulates
G		0.01% PS80	Free of visible particulates	Free of visible particulates	Free of visible particulates
H	2% w/v Mannitol	None	Free of visible particulates	Free of visible particulates	Numerous small round visible particulates
I	6% w/v Sucrose 1% w/v Mannitol	None	Free of visible particulates	Free of visible particulates	Some small round visible particulates
J		0.01% PS80	Free of visible particulates	Free of visible particulates	Free of visible particulates
K		0.01% PS80	Free of visible particulates	Free of visible particulates	Free of visible particulates
L	4% w/v Sucrose 2% w/v Mannitol	None	Free of visible particulates	Free of visible particulates	Some small round visible particulates
M		0.01% PS80	Free of visible particulates	Free of visible particulates	Free of visible particulates
N		0.01% PS80	Free of visible particulates	Free of visible particulates	Free of visible particulates

Differential Scanning Fluorimetry (DSF) (1 week samples)

[00632] The DSF experiments were performed using an Unchained Labs UNcle. Samples were evaluated for their thermal stability. DF-hIL-12-Fc si thermal unfolding (T_m) and onset of aggregation (T_{agg}) were monitored by evaluating changes in intrinsic protein fluorescence and static light scattering (SLS at 266 nm), respectively, as a function of temperature. Samples were evaluated in triplicate, and the triplicate results were averaged. Samples were analyzed over a

temperature ramp of 25°C – 95°C at a constant linear ramp rate of 0.5°C/min. Results are shown in Table 53 and **FIGs. 44A-46F**.

Table 53: Determined DSF T_{m1} , T_{m2} , and T_{agg} 266 Temperatures (1 week material)

Code	Sugar	Surfactant	Average T_{m1} (°C)	Average T_{m2} (°C)	Average T_{agg} 266 (°C)
A	8% w/v Sucrose	None	66.1	76.1	66.2 ¹
B		0.01% PS80	66.3	76.4	66.1
C		0.01% PS80	66.4	77.9	75.6
D	4% w/v Sucrose	None	66.5	76.2	66.1
E	6% w/v Mannitol	None	67.3	76.8	66.3
F		0.01% PS80	66.8	76.8	75.6 ¹
G		0.01% PS80	67.1	77.6	75.9
H	2% w/v Mannitol	None	66.7	76.4	66.7
I	6% w/v Sucrose 1% w/v Mannitol	None	67.3	77.3	66.5
J		0.01% PS80	67.0	77.3	67.1
K		0.01% PS80	67.1	78.3	76.0
L	4% w/v Sucrose 2% w/v Mannitol	None	66.9	77.2	66.5 ¹
M		0.01% PS80	66.8	76.9	66.7
N		0.01% PS80	67.1	78.0	75.7

¹One of the triplicates was excluded from analysis due to discrepancy in the acquired data

Dynamic Light Scattering (DLS) (2 week samples)

[00633] The DLS experiments were performed using a Malvern Zeta sizer. For each sample measurement, five consecutive scans were acquired at 25°C. The Z-average hydrodynamic diameter and polydispersity index were determined from the cumulants analysis and the Stokes Einstein equation. Polydispersity is a measurement of non-uniformity in a sample. If particles are not uniform in size, a higher polydispersity will be measured. A low polydispersity (≤ 0.200) indicates greater uniformity in the size of the particle. Results are shown in Tables 54-62 and in **FIGs. 47A-48H**.

Table 54: DLS Average Size

Code	Sugar	Surfactant	2 – 8°C	50°C	Freeze Thaw
A	8% w/v Sucrose	None	13.85	28.51	21.60
B		0.01% PS80	18.61	23.61	21.67
C		0.01% PS80	14.75	30.59	14.66

D	4% w/v Sucrose	None	17.99	17.39	19.72
E	6% w/v Mannitol	None	16.18	19.05	20.20
F		0.01% PS80	18.93	19.92	17.07
G		0.01% PS80	14.38	36.92	13.92
H	2% w/v Mannitol	None	11.83	19.68	25.23
I	6% w/v Sucrose 1% w/v Mannitol	None	13.82	16.13	19.94
J		0.01% PS80	11.59	14.40	15.32
K		0.01% PS80	14.19	40.96	13.99
L	4% w/v Sucrose 2% w/v Mannitol	None	24.18	21.08	19.50
M		0.01% PS80	16.03	17.43	17.20
N		0.01% PS80	14.83	28.27	13.65
Starting Material			12.25		

Table 55: DLS Polydispersity

Code	Sugar	Surfactant	2 – 8°C	50°C	Freeze Thaw
A	8% w/v Sucrose	None	0.42	0.45	0.48
B		0.01% PS80	0.33	0.37	0.34
C		0.01% PS80	0.15	0.26	0.14
D	4% w/v Sucrose	None	0.32	0.30	0.52
E	6% w/v Mannitol	None	0.29	0.42	0.58
F		0.01% PS80	0.29	0.20	0.46
G		0.01% PS80	0.14	0.24	0.09
H	2% w/v Mannitol	None	0.16	0.30	0.44
I	6% w/v Sucrose 1% w/v Mannitol	None	0.34	0.30	0.48
J		0.01% PS80	0.26	0.25	0.33
K		0.01% PS80	0.11	0.22	0.11
L	4% w/v Sucrose 2% w/v Mannitol	None	0.35	0.35	0.47
M		0.01% PS80	0.32	0.23	0.40
N		0.01% PS80	0.18	0.26	0.10
Starting Material			0.09		

Table 56: DLS Monomer Size

Code	Sugar	Surfactant	2 – 8°C	50°C	Freeze Thaw
A	8% w/v	None	15.79	31.20	17.77
B		0.01% PS80	22.74	53.08	24.89

C	Sucrose	0.01% PS80	16.76	44.53	16.87
D	4% w/v Sucrose	None	19.19	20.40	15.55
E	6% w/v Mannitol	None	18.69	18.72	14.68
F		0.01% PS80	20.36	23.60	14.29
G		0.01% PS80	16.32	52.95	15.35
H	2% w/v Mannitol	None	13.70	20.91	15.72
I	6% w/v Sucrose 1% w/v Mannitol	None	17.21	19.56	17.91
J		0.01% PS80	15.72	18.98	18.12
K		0.01% PS80	16.08	55.60	15.78
L	4% w/v Sucrose 2% w/v Mannitol	None	17.34	27.61	17.82
M		0.01% PS80	19.60	22.01	18.15
N		0.01% PS80	17.17	40.77	15.18
Starting Material			13.47		

Table 57: DLS Monomer %Pd

Code	Sugar	Surfactant	2 – 8°C	50°C	Freeze Thaw
A	8% w/v Sucrose	None	35.50	55.10	28.60
B		0.01% PS80	36.50	213.40	36.20
C		0.01% PS80	36.60	68.00	36.80
D	4% w/v Sucrose	None	35.70	54.30	29.10
E	6% w/v Mannitol	None	42.70	40.70	29.00
F		0.01% PS80	37.00	44.70	30.10
G		0.01% PS80	36.20	67.10	30.70
H	2% w/v Mannitol	None	38.50	47.90	28.20
I	6% w/v Sucrose 1% w/v Mannitol	None	38.40	43.40	30.40
J		0.01% PS80	39.50	43.50	37.30
K		0.01% PS80	34.50	59.50	33.60
L	4% w/v Sucrose 2% w/v Mannitol	None	34.90	66.00	32.20
M		0.01% PS80	42.80	49.10	36.10
N		0.01% PS80	41.00	65.40	32.20
Starting Material			31.20		

Table 58: DLS Species 2 Size (200 d.nm to 1500 d.nm)

Code	Sugar	Surfactant	2 – 8°C	50°C	Freeze Thaw
A	8% w/v	None	631.10	838.60	493.10
B		0.01% PS80	N/A	N/A	N/A

C	Sucrose	0.01% PS80	N/A	N/A	N/A
D	4% w/v Sucrose	None	N/A	474.50	333.50
E	6% w/v Mannitol	None	N/A	418.90	384.60
F		0.01% PS80	N/A	N/A	523.10
G		0.01% PS80	N/A	N/A	N/A
H	2% w/v Mannitol	None	N/A	347.50	380.50
I	6% w/v Sucrose 1% w/v Mannitol	None	N/A	N/A	578.00
J		0.01% PS80	N/A	N/A	N/A
K		0.01% PS80	N/A	N/A	N/A
L	4% w/v Sucrose 2% w/v Mannitol	None	971.20	496.60	600.80
M		0.01% PS80	N/A	N/A	1047.00
N		0.01% PS80	N/A	N/A	N/A
Starting Material					

Table 59: DLS Species 3 Size (> 1500 d.nm)

Code	Sugar	Surfactant	2 – 8°C	50°C	Freeze Thaw
A	8% w/v Sucrose	None	4548.00	3861.00	5120.00
B		0.01% PS80	4152.00	3025.00	3261.00
C		0.01% PS80	4335.00	N/A	N/A
D	4% w/v Sucrose	None	1771.00	3873.00	5181.00
E	6% w/v Mannitol	None	2538.00	4350.00	N/A
F		0.01% PS80	3079.00	4073.00	N/A
G		0.01% PS80	4557.00	N/A	N/A
H	2% w/v Mannitol	None	4364.00	3896.00	5307.00
I	6% w/v Sucrose 1% w/v Mannitol	None	3155.00	2512.00	4862.00
J		0.01% PS80	N/A	4256.00	2769.00
K		0.01% PS80	N/A	N/A	N/A
L	4% w/v Sucrose 2% w/v Mannitol	None	4428.00	3420.00	4906.00
M		0.01% PS80	3883.00	3969.00	4581.00
N		0.01% PS80	4356.00	N/A	N/A
Starting Material			N/A		

Table 60: 2 – 8°C Samples Species

Code	Sugar	Surfactant	Monomer	Species 2	Species 3
A		None	15.79	631.10	4548.00

B	8% w/v Sucrose	0.01% PS80	22.74	N/A	4152.00
C		0.01% PS80	16.76	N/A	4335.00
D	4% w/v Sucrose	None	19.19	N/A	1771.00
E	6% w/v Mannitol	None	18.69	N/A	2538.00
F		0.01% PS80	20.36	N/A	3079.00
G		0.01% PS80	16.32	N/A	4557.00
H	2% w/v Mannitol	None	13.70	N/A	4364.00
I	6% w/v Sucrose 1% w/v Mannitol	None	17.21	N/A	3155.00
J		0.01% PS80	15.72	N/A	N/A
K		0.01% PS80	16.08	N/A	N/A
L	4% w/v Sucrose 2% w/v Mannitol	None	17.34	971.20	4428.00
M		0.01% PS80	19.60	N/A	3883.00
N		0.01% PS80	17.17	N/A	4356.00

Table 61: 50°C Samples Species

Code	Sugar	Surfactant	Monomer	Species 2	Species 3
A	8% w/v Sucrose	None	31.20	838.60	3861.00
B		0.01% PS80	53.08	N/A	3025.00
C		0.01% PS80	44.53	N/A	N/A
D	4% w/v Sucrose	None	20.40	474.50	3873.00
E	6% w/v Mannitol	None	18.72	418.90	4350.00
F		0.01% PS80	23.60	N/A	4073.00
G		0.01% PS80	52.95	N/A	N/A
H	2% w/v Mannitol	None	20.91	347.50	3896.00
I	6% w/v Sucrose 1% w/v Mannitol	None	19.56	N/A	2512.00
J		0.01% PS80	18.98	N/A	4256.00
K		0.01% PS80	55.60	N/A	N/A
L	4% w/v Sucrose 2% w/v Mannitol	None	27.61	496.60	3420.00
M		0.01% PS80	22.01	N/A	3969.00
N		0.01% PS80	40.77	N/A	N/A

Table 62: Freeze Thaw Samples Species

Code	Sugar	Surfactant	Monomer	Species 2	Species 3
A	8% w/v	None	17.77	493.10	5120.00
B		0.01% PS80	24.89	N/A	3261.00

C	Sucrose	0.01% PS80	16.87	N/A	N/A
D	4% w/v Sucrose	None	15.55	333.50	5181.00
E	6% w/v Mannitol	None	14.68	384.60	N/A
F		0.01% PS80	14.29	523.10	N/A
G		0.01% PS80	15.35	N/A	N/A
H	2% w/v Mannitol	None	15.72	380.50	5307.00
I	6% w/v Sucrose 1% w/v Mannitol	None	17.91	578.00	4862.00
J		0.01% PS80	18.12	N/A	2769.00
K		0.01% PS80	15.78	N/A	N/A
L	4% w/v Sucrose 2% w/v Mannitol	None	17.82	600.80	4906.00
M		0.01% PS80	18.15	1047.00	4581.00
N		0.01% PS80	15.18	N/A	N/A

Size Exclusion Chromatography – High Performance Liquid Chromatography (SEC-HPLC) (2 weeks samples)

[00634] The SEC experiments were performed using a TOSOH G3000SWxl (7.8 x 300 mm). The 1 mg/mL samples were injected neat, at 90 μ L, to achieve a column load of 90 μ g (target column load of 100 μ g was not achievable given the low concentration of 1 mg/mL). The 10 mg/mL samples were injected neat, at 10 μ L, to achieve a column load of 100 μ g. The main peak was defined as the peak with the greatest peak area, and was consistent in retention time across all samples. Peaks eluting prior to the main peak were defined as high molecular weight (HMW) species and peaks eluting after as low molecular weight (LMW) species. The LOQ for the draft method was defined as 0.1% peak area of the total peak area.

Table 63: SEC-HPLC %Main

Code	Sugar	Surfactant	2 – 8°C	50°C	Freeze Thaw
A	8% w/v Sucrose	None	99.3	77.6	99.0
B		0.01% PS80	99.1	77.5	99.0
C		0.01% PS80	99.3	74.8	99.2
D	4% w/v Sucrose	None	98.9	83.8	98.9
E	6% w/v Mannitol	None	99.1	88.9	98.0
F		0.01% PS80	99.1	68.8	98.6
G		0.01% PS80	99.2	66.3	99.0
H	2% w/v Mannitol	None	99.2	76.7	98.7
I	6% w/v	None	99.1	85.0	98.7

J	Sucrose 1% w/v	0.01% PS80	99.0	85.2	98.9
K	Mannitol	0.01% PS80	99.2	57.4	99.1
L	4% w/v	None	99.1	76.6	98.5
M	Sucrose 2% w/v	0.01% PS80	99.1	78.6	99.0
N	Mannitol	0.01% PS80	99.2	76.5	99.1
Starting Material			99.4		

Table 64: SEC-HPLC %HMW

Code	Sugar	Surfactant	2 – 8°C	50°C	Freeze Thaw
A	8% w/v Sucrose	None	0.7	20.2	0.9
B		0.01% PS80	0.9	20.4	1.0
C		0.01% PS80	0.7	23.8	0.8
D	4% w/v Sucrose	None	0.7	14.6	1.1
E	6% w/v Mannitol	None	0.9	9.3	2.0
F		0.01% PS80	0.9	29.5	1.4
G		0.01% PS80	0.8	32.4	1.0
H	2% w/v Mannitol	None	0.7	21.9	1.3
I	6% w/v Sucrose 1% w/v Mannitol	None	0.9	13.5	1.3
J		0.01% PS80	1.0	13.3	1.1
K		0.01% PS80	0.8	41.3	0.9
L	4% w/v Sucrose 2% w/v Mannitol	None	0.9	22.0	1.5
M		0.01% PS80	0.9	20.0	1.0
N		0.01% PS80	0.8	22.4	0.9
Starting Material			0.6		

Table 65: SEC-HPLC %LMW

Code	Sugar	Surfactant	2 – 8°C	50°C	Freeze Thaw
A	8% w/v Sucrose	None	<LOQ of 0.1%	2.2	<LOQ of 0.1%
B		0.01% PS80	<LOQ of 0.1%	2.2	<LOQ of 0.1%
C		0.01% PS80	<LOQ of 0.1%	1.4	<LOQ of 0.1%
D	4% w/v Sucrose	None	0.4	1.6	<LOQ of 0.1%
E	6% w/v Mannitol	None	<LOQ of 0.1%	1.8	<LOQ of 0.1%
F		0.01% PS80	<LOQ of 0.1%	1.6	<LOQ of 0.1%
G		0.01% PS80	<LOQ of 0.1%	1.4	<LOQ of 0.1%
H	2% w/v Mannitol	None	<LOQ of 0.1%	1.4	<LOQ of 0.1%

I	6% w/v Sucrose 1% w/v Mannitol	None	<LOQ of 0.1%	1.4	<LOQ of 0.1%
J		0.01% PS80	<LOQ of 0.1%	1.5	<LOQ of 0.1%
K		0.01% PS80	<LOQ of 0.1%	1.3	<LOQ of 0.1%
L	4% w/v Sucrose 2% w/v Mannitol	None	<LOQ of 0.1%	1.4	<LOQ of 0.1%
M		0.01% PS80	<LOQ of 0.1%	1.5	<LOQ of 0.1%
N		0.01% PS80	<LOQ of 0.1%	1.1	<LOQ of 0.1%
Starting Material			<LOQ of 0.1%		

HIAC (2 week samples)

[00635] The particulate matter experiments were performed using a HIAC 9703+. For the analysis, the instrument was tared with 0.1 mL of sample prior to evaluation of 0.3 mL of sample volume. Between each run, the instrument was washed with water. In addition to the samples, the buffers and starting material were analyzed, and showed no significant number of particles. Results are shown in Tables 66-69.

Table 66: HIAC $\geq 2 \mu\text{m}$ Particles per mL

Code	Sugar	Surfactant	2 – 8°C	50°C	Freeze Thaw
A	8% w/v Sucrose	None	113.33	740.00	210.00
B		0.01% PS80	53.33	50.00	63.33
C		0.01% PS80	176.67	193.33	120.00
D	4% w/v Sucrose	None	70.00	43.33	1466.67
E	6% w/v Mannitol	None	93.33	83.33	193.33
F		0.01% PS80	73.33	150.00	73.33
G		0.01% PS80	83.33	210.00	116.67
H	2% w/v Mannitol	None	40.00	100.00	5436.67
I	6% w/v Sucrose 1% w/v Mannitol	None	33.33	253.33	86.67
J		0.01% PS80	93.33	50.00	153.33
K		0.01% PS80	150.00	280.00	196.67
L	4% w/v Sucrose 2% w/v Mannitol	None	56.67	76.67	93.33
M		0.01% PS80	283.33	193.33	60.00
N		0.01% PS80	126.67	190.00	93.33

Table 67: HIAC $\geq 5 \mu\text{m}$ Particles per mL

Code	Sugar	Surfactant	2 – 8°C	50°C	Freeze Thaw
A	8% w/v	None	33.33	220.00	56.67
B		0.01% PS80	20.00	16.67	23.33

C	Sucrose	0.01% PS80	53.33	113.33	50.00
D	4% w/v Sucrose	None	13.33	13.33	550.00
E	6% w/v Mannitol	None	43.33	30.00	33.33
F		0.01% PS80	36.67	36.67	23.33
G		0.01% PS80	30.00	46.67	33.33
H	2% w/v Mannitol	None	6.67	26.67	1560.00
I	6% w/v Sucrose 1% w/v Mannitol	None	26.67	56.67	20.00
J		0.01% PS80	33.33	3.33	36.67
K		0.01% PS80	36.67	76.67	70.00
L	4% w/v Sucrose 2% w/v Mannitol	None	20.00	26.67	26.67
M		0.01% PS80	103.33	40.00	16.67
N		0.01% PS80	40.00	23.33	33.33

Table 68: HIAC $\geq 10 \mu\text{m}$ Particles per mL

Code	Sugar	Surfactant	2 – 8°C	50°C	Freeze Thaw
A	8% w/v Sucrose	None	13.33	60.00	20.00
B		0.01% PS80	6.67	3.33	6.67
C		0.01% PS80	13.33	83.33	20.00
D	4% w/v Sucrose	None	6.67	10.00	66.67
E	6% w/v Mannitol	None	10.00	16.67	13.33
F		0.01% PS80	3.33	10.00	3.33
G		0.01% PS80	6.67	16.67	0.00
H	2% w/v Mannitol	None	3.33	6.67	143.33
I	6% w/v Sucrose 1% w/v Mannitol	None	20.00	26.67	3.33
J		0.01% PS80	13.33	0.00	0.00
K		0.01% PS80	16.67	13.33	33.33
L	4% w/v Sucrose 2% w/v Mannitol	None	3.33	16.67	10.00
M		0.01% PS80	46.67	26.67	3.33
N		0.01% PS80	10.00	3.33	20.00

Table 69: HIAC $\geq 25 \mu\text{m}$ Particles per mL

Code	Sugar	Surfactant	2 – 8°C	50°C	Freeze Thaw
A	8% w/v Sucrose	None	3.33	0.00	0.00
B		0.01% PS80	0.00	0.00	0.00
C		0.01% PS80	3.33	6.67	0.00

D	4% w/v Sucrose	None	0.00	0.00	0.00
E	6% w/v Mannitol	None	0.00	0.00	0.00
F		0.01% PS80	0.00	0.00	0.00
G		0.01% PS80	0.00	0.00	0.00
H	2% w/v Mannitol	None	0.00	6.67	0.00
I	6% w/v Sucrose 1% w/v Mannitol	None	3.33	6.67	0.00
J		0.01% PS80	3.33	0.00	0.00
K		0.01% PS80	6.67	0.00	3.33
L	4% w/v Sucrose 2% w/v Mannitol	None	0.00	6.67	3.33
M		0.01% PS80	6.67	3.33	0.00
N		0.01% PS80	0.00	0.00	6.67

Summary and Conclusions

[00636] All the samples were analyzed by visual appearance (2 week), pH (buffer exchange and 2 week), A280 (buffer exchange and 2 week), DLS (2 week), DSF (1 week), SEC (2 week), and HIAC (2 week).

[00637] The concentrations of a majority of the samples were within 10% of the target concentration at the conclusion of the buffer exchange. Both samples E (20 mM citrate, 6% mannitol, pH 6.5) and F (20 mM citrate, 6% mannitol, 0.01% PS80 pH 6.5) had concentrations of 0.89 mg/mL at the conclusion of the buffer exchange. Upon conclusion of incubation at respective conditions (2 – 8°C, 50°C), 10 of the 14 formulations evaluated had concentrations consistent with their starting concentrations. All 3 samples for formulations A (20 mM citrate, 8% sucrose, pH 6.5), B (20 mM citrate, 8% sucrose, 0.01% PS80, pH 6.5), E (20 mM citrate, 6% mannitol, pH 6.5), and F (20 mM citrate, 6% mannitol, 0.01% PS80) had concentrations ranging from 0.87 mg/mL to 0.79 mg/mL.

[00638] Evaluation by pH showed no significant differences between the various samples.

[00639] Evaluation by visual appearance showed no significant differences in the color and clarity of the samples. Most of the samples, based on visual appearance, were free of visible particulates. The 50°C sample for formulation A (20 mM citrate, 8% sucrose, pH 6.5), the freeze thaw sample for formulation D (20 mM citrate, 4% sucrose, pH 6.5), the freeze thaw sample for formulation I (20 mM citrate, 6% sucrose, 1% mannitol, pH 6.5), and the freeze thaw sample for formulation L (20 mM citrate, 4% sucrose, 2% mannitol, pH 6.5) all had some small round particulates visible. The freeze thaw sample for formulation H (20 mM citrate, 2% mannitol, pH

6.5) had numerous small round visible particulates. Notably, all 4 of these formulations lacked surfactant (0.01% PS80).

[00640] Evaluation by differential scanning fluorimetry of the remaining material after buffer exchanged (stored for 1 week at 2 – 8 °C) showed that formulations E (20 mM citrate, 6% mannitol, pH 6.5) and I (20 mM citrate, 6% sucrose, 1% mannitol, pH 6.5) had the highest T_{m1} for the 1 mg/mL samples, while formulations G (20 mM citrate, 6% mannitol, 0.01% PS80, 10 mg/mL), K (20 mM citrate, 6% sucrose, 1% mannitol, 0.01% PS80 pH 6.5, 10 mg/mL), and N (20 mM citrate, 4% sucrose, 2% mannitol, 0.01% PS80, pH 6.5, 10 mg/mL) had the highest T_{m1} for the 10 mg/mL samples. Formulations I (20 mM citrate, 6% sucrose, 1% mannitol, pH 6.5), J (20 mM citrate, 6% sucrose, 1% mannitol, 0.01% PS80, pH 6.5), and L (20 mM citrate, 4% sucrose, 2% mannitol, pH 6.5) had the highest T_{m2} for the 1 mg/mL samples and formulation K (20 mM citrate, 6% sucrose, 1% mannitol, 0.01% PS80, pH 6.5, 10 mg/mL) had the highest T_{m2} for the 10 mg/mL samples. Formulation F (20 mM citrate, 6% mannitol, 0.01% PS80) had a significantly higher T_{agg} than the other 1 mg/mL samples. The 10 mg/mL samples were consistent with respect to T_{agg} . Overall, all samples demonstrated T_{m1} values > 66°C.

[00641] Evaluation by SEC-HPLC of the 2 week material showed minimal variation among the 2 – 8°C samples as well as the freeze thaw samples. The %Main of the 1 mg/mL 50°C samples ranged from 68.8% to 88.9%. Formulations D (20 mM citrate, 4% sucrose, pH 6.5), E (20 mM citrate, 6% mannitol, pH 6.5), I (20 mM citrate, 6% sucrose, 1% mannitol, pH 6.5), and J (20 mM citrate, 6% sucrose, 1% mannitol, 0.01% PS80, pH 6.5) all had %Main above 80%. The %Main of the 10 mg/mL 50°C samples ranged from 57.4% to 76.5%. Formulations C (20 mM citrate, 8% sucrose, 0.01% PS80, pH 6.5, 10 mg/mL), N (20 mM citrate, 4% sucrose, 2% mannitol, 0.01% PS80, pH 6.5, 10 mg/mL) had %Main above 70% while Formulation K (20 mM citrate, 6% sucrose, 1% mannitol, 0.01% PS80, pH 6.5, 10 mg/mL) had the lowest %Main at 57.4%.

[00642] The light scattering data indicated formulations I (20 mM citrate, 6% sucrose, 1% mannitol, pH 6.5) and J (20 mM citrate, 6% sucrose, 1% mannitol, 0.01% PS80, pH 6.5) generally had the smallest average size as well as the smallest monomer size for the 3 tested conditions. A small species was detected in some of the 1 mg/mL samples (formulations A, B, I, J, L, and M), most likely from the sucrose and mannitol. The small species was not detected in the 10 mg/mL samples, and was most likely masked by the higher concentration. Due to the automated analysis, the presence of this species complicated conclusions regarding the polydispersity of the samples. With respect to the average size, formulations I and J consistently had smaller average sizes than most of the 1 mg/mL samples for each of the 3 conditions. Formulations I and J also had a smaller

monomer size for than most of the 1 mg/mL samples for the 2 – 8°C condition as well as the 50°C condition. Formulations D (20 mM citrate, 4% sucrose, pH 6.5), E (20 mM citrate, 6% mannitol, pH 6.5), F (20 mM citrate, 6% mannitol, 0.01% PS80), and H (20 mM citrate, 2% mannitol, pH 6.5) had a smaller monomer size than the rest of the 1 mg/mL samples for the freeze thaw condition. For the 10 mg/mL samples, all of the samples were largely consistent across the 3 tested conditions. The 10 mg/mL 50°C samples displayed an appreciable increase in both average size as well as monomer size. In a number of samples a second species was detected (200 d.nm to 1200 d.nm) and in a majority of samples a third species was detected (1500 d.nm to 5500 d.nm). These larger species were primarily detected in the 1 mg/mL samples at all 3 conditions.

[00643] Evaluation by HIAC largely corroborated the visual appearance data. The 50°C sample for formulation A (20 mM citrate, 8% sucrose, pH 6.5), the freeze thaw sample for formulation D (20 mM citrate, 4% sucrose, pH 6.5), and the freeze thaw sample for formulation H (20 mM citrate, 2% mannitol, pH 6.5) all had higher $\geq 2 \mu\text{m}$ particle counts, $\geq 5 \mu\text{m}$ particle counts, and $\geq 10 \mu\text{m}$ particle counts. With the exception of these three samples, the remaining samples were relatively consistent. None of the samples exceeded the USP <787> specification.

[00644] Performance of the formulation J (20 mM Citrate, 6% w/v Sucrose, 1% w/v Mannitol, 0.01% PS80, pH 6.5) was determined to be the most desirable. This buffer/excipient/surfactant combination at a higher concentration of 10 mg/mL (formulation K) did not perform well by SEC-HPLC after the 2 week incubation at 50°C. Additionally, the 1 mg/mL samples generally performed better or as well as the 10 mg/mL samples.

Example 25: Pharmacokinetic (PK) Analysis of DF hIL12-Fc si

[00645] DF hIL12-Fc si is a monovalent human IL12-Fc fusion protein designed to enhance the efficacy of IL12 without proportionally increasing adverse effects. DF hIL12-Fc si has a substantially longer half-life compared to rhIL12. The extended half-life of DF hIL12-Fc si enables a protracted pharmacodynamic profile without the need for frequent repeat administration and the consequent repeat spikes in IL12 exposure, which cause toxicities. The longer half-life enables significantly greater anti-tumor activity with less frequent dosing in mouse models, suggesting infrequent dosing in patients, such as once every 3 weeks (Q3W), may be efficacious and offer an acceptable safety profile.

[00646] The subcutaneous (SC) route was chosen as the most appropriate for administration of DF hIL12-Fc si because of a better pharmacokinetic (PK) profile, avoiding a spike in drug concentration at a maximum serum concentration observed post-dose (C_{max}) that may result in a better tolerability.

In vitro Pharmacology

In vitro Binding Characteristics

[00647] DF hIL12-Fc si retained the binding affinity of native IL12 and human IgG1 Fc to their respective receptors, IL12R and FcRn. In contrast, the human IgG1 Fc portion of DF hIL12-Fc si was mutated to abrogate binding to FcγRs.

In vitro Cellular Activity

[00648] To compare the potency of DF hIL12-Fc si to that of rhIL12, IFNγ production from human primary immune cells stimulated with either phytohemagglutinin (PHA) or anti-CD3 antibody was analyzed *in vitro*. Both DF hIL12-Fc si and rhIL12 consistently exhibited comparable potencies across IFNγ production assays with activated human PBMCs, isolated human T cells, or isolated human NK cells.

[00649] A separate *in vitro* study was conducted in unstimulated human PBMCs with clinical-grade DF hIL12-Fc si to evaluate for the potential of inducing cytokine release syndrome (CRS). In this study, DF hIL12-Fc si only led to a dose-dependent increase of IFNγ, consistent with expected pharmacology, but did not induce secretion of the other 7 cytokines evaluated.

In vitro Species Cross-reactivity

[00650] The *in vitro* activity of DF hIL12-Fc si in stimulating IFNγ release from mouse, rat, and cynomolgus monkey immune cells was analyzed in order to evaluate the appropriate species for toxicology studies.

[00651] The cynomolgus monkey (*Macaca fascicularis*) was selected as the only pharmacologically relevant species for the conduct of nonclinical safety studies based on: Comparisons of the amino acid sequences of IL12 across species; Binding pattern of DF hIL12-Fc si relative to IL12Rβ1 expression on cynomolgus monkey immune cell subsets in PBMCs compared to binding patterns on human PBMC subsets; and Stimulation of IFNγ release in cynomolgus monkey primary immune cells by DF hIL12-Fc si relative to that of humans.

[00652] Consistent with the literature (Schoenhaut DS, Chua AO, Wolitzky AG, Quinn PM, Dwyer CM, McComas W, et al., *J Immunol.* 1992;148(11):3433-40), neither human DF hIL12-Fc si nor rhIL12 enhanced IFNγ production from mouse splenocytes or rat PBMCs.

In vivo Pharmacology

In vivo Pharmacology in Mice

[00653] As human IL12 was not functional in mouse cells, a surrogate murine IL12-Fc was generated that mirrors the human DF hIL12-Fc si drug candidate, allowing for examination of the PK/PD profile and efficacy in syngeneic mouse *in vivo* tumor models. Mouse IL12 is considered

to have a similar expression pattern and function in mice compared to that observed in humans with native IL12 (Car 1999).

[00654] The surrogate molecule, designated DF-mIL-12-Fc si, utilizes murine IL12, in which the p35 and p40 subunits were fused to the N-termini of 2 different Fc variants. The mouse IgG2a Fc fragment was mutated to abrogate Fc γ R binding while retaining binding to FcRn (Schoenhaut DS, Chua AO, Wolitzky AG, Quinn PM, Dwyer CM, McComas W, et al., *J Immunol.* 1992;148(11):3433-40), which has been found to be most analogous to the human Fc variant utilized in DF hIL12-Fc si (human IgG1 Fc silent).

Ex Vivo Biological Potency of DF-mIL-12-Fc si

[00655] The potency of DF-mIL-12-Fc si (mean 50% effective concentration [EC₅₀] = 2.07 $\pm$ 0.8 pM) was comparable to that of rmIL12 (mean EC₅₀ = 0.69 $\pm$ 0.14 pM). Neither human DF hIL12-Fc si nor rhIL12 enhanced IFN γ production from mouse splenocytes, confirming a lack of species cross-reactivity.

In vivo Characterization and Pharmacokinetics of DF-mIL-12-Fc si

[00656] The PK/PD profile and bioavailability of DF-mIL-12-Fc si were evaluated after a single dose administration of the molecule in BALB/c or C57BL/6 mice.

[00657] DF-mIL-12-Fc si demonstrated a protracted serum t_{1/2} of 29.85 hours, which was approximately 5 times longer than that of rmIL12 (t_{1/2} = 6.05 hours). This 5-fold extended t_{1/2} of DF-mIL-12-Fc si resulted in ~40-fold greater mediated IFN γ production (area under the concentration-time curve from the time of dosing to the time of the last observation [AUC_{0-219h}] = 916,654 h*pg/mL) that was more durable and sustained compared to that of relative to rmIL12 (AUC_{0-219h} = 20,304 h*pg/mL). IFN γ levels remained elevated for over 200 hours following a single administration of DF-mIL-12-Fc si, and this enhancement of IFN γ exposure resulted from administration DF-mIL-12-Fc si at equimolar amounts to that of the rmIL12 group, which yielded approximately the same C_{max}.

[00658] In BALB/c mice, the bioavailabilities of DF-mIL-12-Fc si were 66% and 32% when dosed IP and SC, respectively. Comparable bioavailabilities of 73% and 44% (IP and SC, respectively) were obtained in C57BL/6 mice.

[00659] Approximately 4-fold higher IFN γ secretion was observed in C57BL/6 mice compared to that of BALB/c mice (C_{max} of IFN γ was ~25,000 and ~6,000 pg/mL in C57BL/6 and BALB/c mice, respectively).

[00660] Importantly, the PD response, as measured by serum IFN γ levels, was similar regardless of administration route, despite the differences in bioavailability and a lower C_{max} observed

following SC administration. These results suggest that the IL12-Fc format is able to achieve full PD efficacy by the SC route while exposing to only 1/10th the C_{max} as by the IV route. While certain IL12 toxicities are mediated by IFN γ and therefore may be similar following IV or SC administration, other side effects have been reported to be IFN γ independent (Leonard JP, Sherman ML, Fisher GL, Buchanan LJ, Larsen G, Atkins MB, et al., *Blood*. 1997;90(7):2541-8) and may potentially be less pronounced following SC administration due to the lower IL12 C_{max} .

[00661] In summary, DF-mIL-12-Fc si demonstrated prolonged serum $t_{1/2}$ and extended IFN γ production compared to rmIL12 with favorable bioavailability when dosed IP and SC in C57BL/6 and BALB/c mouse strains. However, both routes of administration yielded similar serum IFN γ levels as with IV dosing, and 4-fold higher IFN γ secretion was observed in C57BL/6 mice compared to that of BALB/c.

DF-mIL-12-Fc si Therapeutic Index

[00662] In the B16F10 melanoma model, IL12 variants DF-mIL-12-Fc si and rmIL12 were dosed to match their IL12 serum exposure levels (DF-mIL-12-Fc si weekly and rmIL12 daily), with PD responses, tolerability, and *in vivo* efficacy analyzed to determine the benefit-risk profile of DF-mIL-12-Fc si in comparison to rmIL12.

[00663] Repeat SC dosing of rmIL12 in naïve C57BL/6 resulted in a profound accumulation of serum IFN γ within 6 days compared to weekly injected DF-mIL-12-Fc si; AUC IFN γ in rmIL12-treated animals was increased 2.8 – 4.5-fold over that in DF-mIL-12-Fc si-treated mice. In addition, subsequent doses of rmIL12 resulted in little or no IFN γ production, suggesting strong negative feedback that limits responses. In contrast, weekly administered DF-mIL-12-Fc si resulted in sustained and moderate IFN γ secretion with the second dose demonstrating a similar PD profile to the first dose. Moreover, inn B16F10 tumor-bearing C57BL/6 mice, daily dosing of 0.5 or 1 μ g rmIL12 was lethal and all mice were euthanized after one week of treatment. Daily dosing of 0.25 μ g rmIL12 (MTD) was less efficacious in controlling tumor progression compared to DF-mIL-12-Fc si dosed weekly at a level equimolar to 1 μ g rmIL12.

[00664] These findings support that the toxicity observed in the clinical trials performed with rhIL12 were, at least partially, the consequence of a frequent administration schedule.

Efficacy of DF-mIL-12-Fc si Monotherapy in Mouse Tumor Models

[00665] Two different mouse models, B16F10 melanoma (derived from C57BL/6) and CT26-20.7 colon carcinoma (derived from BALB/c), were chosen to analyze DF-mIL-12-Fc si efficacy *in vivo*. B16F10 is a “cold” tumor model and it has been reported to be resistant to checkpoint blockade and to monoclonal antibodies with antibody-dependent cellular cytotoxicity (ADCC)

function as single agents (Mosely SI, Prime JE, Sainson RC, Koopmann JO, Wang DY, Greenawalt DM, et al., *Cancer Immunol Res.* 2017;5(1):29-41). CT26 is a well characterized colon carcinoma model known to exhibit an inflammatory tumor microenvironment. CT26-20.7 is a subline of CT26 derived by transduction with a murine Tyrp1 transgene with similar growth and characteristics as the parental line.

[00666] Pharmacology studies were conducted to (1) compare *in vivo* activity of DF-mIL-12-Fc si and rmIL12; (2) assess dose response and frequency of DF-mIL-12-Fc si in established and large 800 mm³ tumors; (3) determine whether IP and SC administration of DF-mIL-12-Fc si mediate similar anti-tumor responses; and (4) evaluate the impact of different dosing frequencies on tumor burden.

Efficacy of DF-mIL-12-Fc si in CT26 Colon Carcinoma Model

[00667] In CT26-20.7 tumor-bearing BALB/c mice were treated IP once weekly for 5 weeks with DF-mIL-12-Fc si, mIgG2a isotype control, or rmIL12 at doses equimolar to 1 µg rmIL12 after mean tumor volume (MTV) reached 270 mm³. Treatment with DF-mIL-12-Fc si resulted in an increased antitumor response ($p < 0.0001$), yielding 100% complete responses (CRs) compared to 10% CR in the rmIL12-treated group.

[00668] Examination of DF-mIL-12-Fc si dose level and frequency in CT26-20.7 tumor-bearing BALB/c mice, it was observed that a single dose of DF-mIL-12-Fc si induced 100% response rate. Although all mice responded to DF-mIL-12-Fc si treatment, responses were less durable, with 20% of the tumors progressing at a later stage. Similar results were obtained when mice were dosed once weekly for 2 weeks.

[00669] Examination of a potential dose-effect revealed a significant ($p < 0.05$) dose-dependent titration of the antitumor effect between groups treated at 1 or 0.1 µg; however, 80% of mice treated with the 10-fold lower DF-mIL-12-Fc si dose concentration (0.1 µg) responded to treatment and survival was prolonged.

[00670] Moreover, similar antitumor efficacy against CT26-20.7 tumors was observed in tumor-bearing BALB/c mice with average tumor volumes of 230 mm³ administered DF-mIL-12-Fc si via different routes (IP or SC) QW for 7 weeks at doses equimolar to 1 µg rmIL12 (**FIGs. 19A-B**). These findings are consistent with published data indicating that IFN γ is a major mediator of anti-tumor efficacy, and these studies with DF-mIL-12-Fc si demonstrated similar levels of IFN γ after IV, IP or SC administration, despite differences in C_{max} with the various routes.

[00671] To analyze whether DF-mIL-12-Fc si monotherapy could induce effective immune responses against larger, end-stage tumors, CT26-20.7 tumor-bearing mice were treated 18 days

post-inoculum, after tumor volumes had reached an average of 815 mm³. DF-mIL-12-Fc si was administered SC once weekly at dose levels equimolar to 1 or 2 µg of rmIL12 for 7 weeks, or equimolar to 2 µg dosed once. In this model with larger tumor volumes, a single dose of 2 µg DF-mIL-12-Fc si was sufficient to induce potent antitumor responses, with a CR rate of 80%. Moreover, weekly dosing at either 1 or 2 µg maintained tumor control, resulting in CR rates of 100% (1 µg weekly) or 90% (2 µg weekly). DF-mIL-12-Fc si was well tolerated, without clinical observations or effects on body weight, while mediating regression of the larger tumors.

Efficacy of DF-mIL-12-Fc si Combination Therapy With PD1 Blockade in B16F10 Melanoma Model

[00672] PD1 blockade is known to have little or no efficacy against established B16F10 tumors (Mosely 2017). Combination therapy of DF-mIL-12-Fc si and PD1 blockade was performed in the B16F10 tumor model in 2 studies to analyze whether an antitumor immune response could be amplified. C57BL/6 mice were treated with DF-mIL-12-Fc si or anti-PD1 as single agents and in combination once average tumor volumes reached ~215 mm³ or ~200 mm³ (Study 1 and Study 2, respectively). Tumor-bearing mice were administered DF-mIL-12-Fc si IP (QW for 8 weeks in Study 1) or SC (QW for 7 weeks in Study 2) at a dose equimolar to 0.5 µg of rmIL12, and anti-PD1 was administered twice weekly IP at 200 µg (twice weekly for 19 or 13 doses, in Study 1 or 2, respectively).

[00673] While administration of DF-mIL-12-Fc si alone delayed tumor regression, combination with PD1 blockade further extended the duration of antitumor responses. Survival was extended with DF-mIL-12-Fc si in combination with PD1 blockade compared to that observed with either monotherapy. Despite the synergistic efficacy, the regimen of DF-mIL-12-Fc si and anti-PD1 combination therapy appeared to be well tolerated by B16F10 tumor-bearing mice. There were no signs of additive or synergistic toxicity with the combination of DF-mIL-12-Fc si and anti-PD1, and there were neither clinical observations nor effects on body weight.

In vivo Pharmacology in Monkeys

[00674] DF hIL12-Fc si was administered IV (1.9 to 40 µg/kg) or SC (1 to 20 µg/kg) in cynomolgus monkeys in the nonclinical toxicology studies. After administration, a substantial secondary peak of PD markers IFNγ and interferon gamma-inducible protein 10 (IP-10) followed, which was dose dependent.

[00675] In non-GLP and GLP studies, monkeys treated with DF hIL12-Fc si demonstrated a generally dose-dependent increase in IFNγ, with peak levels occurring 48 to 72 hrs post-dose. IP10 was also similarly increased. In head to head non-GLP comparisons with rhIL-12 and DF hIL12-

Fc si at equimolar dose levels, DF hIL12-Fc si generally caused a greater increase in IFN γ that lasted longer than the IFN γ response produced by rhIL12. DF hIL12-Fc si produced IFN γ responses that were still detectable 120-168 hrs post-dose, while molar equivalent rhIL12 IFN γ returned to baseline at similar timepoints. The more durable IFN γ response of DF hIL12-Fc si is most likely due to DF hIL12-Fc si's prolonged $t_{1/2}$ in comparison to rhIL12. IP10 was not assessed in the non-GLP head to head comparisons with rhIL12.

[00676] In the GLP study, peak IFN γ levels were generally similar at ≥ 8 $\mu\text{g/kg}$ SC and 12 $\mu\text{g/kg}$ IV, though there was some individual animal variability within groups. Some attenuation of the IFN γ and IP10 response was observed after repeat dosing, but some monkeys in the GLP study demonstrated significant IFN γ response to the first and second dose. After the third dose in the GLP study, there was minimal to no increase in IFN γ either due to attenuation or possible impact of anti-drug antibodies (ADA). IP10 response after the third dose of DF hIL12-Fc si was more significant than the IFN γ response, possibly reflecting greater $t_{1/2}$ of IP10.

Secondary Pharmacology

Binding to Fc γ R

[00677] The potential for DF hIL12-Fc si to bind to Fc γ Rs was evaluated by surface plasmon resonance (SPR). The Biacore™ 8K SPR system was used to evaluate binding of DF hIL12-Fc si to recombinant human (CD64, CD32a H131 and R131 alleles, CD16a V158 and F158 alleles, CD32b, CD16b) and cynomolgus (CD64 and CD16) receptors that were captured on the chip via site-specific biotinylation. Trastuzumab, a well-established IgG1 biologic drug, was used as an isotype-specific experimental control. Qualitative assessment of the data concluded that DF hIL12-Fc si did not demonstrate meaningful binding to any of the Fc γ Rs tested. In contrast, titration of the IgG1 control trastuzumab at the same receptor-specific, physiologically relevant concentrations demonstrated a full range of dose-dependent binding across Fc γ Rs, as expected.

[00678] Furthermore, the human IgG1 Fc domain is known to bind C1q, a component of the classical complement cascade which mediates complement-dependent cytotoxicity (CDC) (Idusogie 2000). To confirm that DF hIL12-Fc si does not elicit CDC, human PBMCs stimulated with PHA for 3 days were incubated with 5% human complement serum in the presence of DF hIL12-Fc si ranging from 0.0823 to 20 nM. The addition of serum did not trigger CDC. In contrast, anti-MHC1 antibody (used as a positive control) induced complement serum-dependent death of T cells.

C-Reactive Protein as a Surrogate for IFN γ secretion

[00679] C-reactive protein (CRP) is a marker of early phase inflammation used to monitor patients that are suspected of having severe infection. Increased CRP levels are used in the cynomolgus studies as a surrogate for the measurement of the secretion of IFN gamma, which is much more challenging to use as a clinical biomarker because of its short half-life. Based on these studies, the measurement of CRP represents a more reliable biomarker for detecting the PD activity of DF hIL12-Fc si in clinical settings.

Binding to FcRn

[00680] An evaluation of the potential for DF hIL12-Fc si to bind to cynomolgus monkey and human FcRn showed that, as expected, human and cynomolgous monkey FcRn binding was not affected by FcγR-silencing mutations. DF hIL12-Fc si and the IgG1 isotype control trastuzumab were comparable ($< 1.5\times$ difference) in their binding affinity values for cynomolgus monkey and human FcRn at pH 6.0. Likewise, both molecules were similar in their lack of quantifiable binding at the concentrations tested at pH 7.4.

Safety Pharmacology

[00681] Safety pharmacology endpoints (*e.g.*, cytokine assessment, body temperature, respiration rate, blood pressure, heart rate, ECG assessments, and FOB assessments) were incorporated in the GLP 3-week repeat-dose toxicology study in cynomolgus monkeys.

[00682] After administration of DF hIL12-Fc si SC up to 20 μg/kg or IV at 12 μg/kg to monkeys QW for 3 weeks, there were no DF hIL12-Fc si-related effects on body temperature, blood pressure, or the central nervous system (as measured by FOB assessments), respiratory system (as measured by respiratory rate), or cardiovascular system (as measured by ECGs and heart rate).

[00683] In the GLP, 3-week repeat-dose toxicology study, IFNγ and IP10 were robustly increased after administration of DF hIL12-Fc si, which is consistent with its expected pharmacology. There were also sporadic and minimal increases of IL-6 in some DF hIL12-Fc si-treated monkeys. In non-GLP studies in monkeys, in addition to expected IFNγ and IP10 increases, there were also minimal and primarily transient increases in IL6, macrophage inflammatory protein (MIP) MIP-1α, MIP-1β, and thymus-and activation-regulated chemokine (TARC), while other measured cytokines were unaffected. These *in vivo* results in monkeys are consistent with the *in vitro* results of the cytokine release assay in unstimulated human PBMCs, in which only a concentration-dependent increase in IFNγ was observed, and other cytokines were unaffected. Therefore, DF hIL12-Fc si has a low potential for CRS, but select cytokines (*e.g.*, IFNγ, IP-10) are expected to increase because of the expected pharmacology of DF hIL12-Fc si.

Pharmacokinetics and Drug Metabolism in Animals

Overview

[00684] The toxicokinetic (TK) profile of DF hIL12-Fc si was investigated after single, repeat, and/or crossover SC (21 to 20 µg/kg) and IV (1.9 to 40 µg/kg) administration to cynomolgus monkeys in 4 non-GLP toxicology studies and 1 GLP toxicology study. Plasma TK was evaluated using DF hIL12-Fc si concentrations obtained by both enzyme-linked immunosorbent assay (ELISA) (measuring IL-12p70 of DF hIL12-Fc si by detecting each IL 12 subunit) and MSD (measuring IL12p40 and Fc of DF hIL12-Fc si). Data derived from the qualified ELISA method were the preferred source for exposure assessment of DF hIL12-Fc si. An anti-drug antibody (ADA) method was also developed to detect anti-DF hIL12-Fc si antibodies in cynomolgus monkey serum after SC dosing; this method was validated for use in the GLP 3-week toxicology study.

[00685] The predominant TK profile of DF hIL12-Fc si after SC or IV administration was characterized by dose-independent (linear) kinetics, although small animal numbers in non-GLP studies and ADA may have contributed to observed variability. There did not appear to be an overall sex-related difference in the plasma TK of DF hIL12-Fc si.

[00686] In the non-GLP studies, after SC administration, bioavailability was approximately 60% based on ELISA data. The estimated $t_{1/2}$ ranged from 12.4 to 56.4 hours across individual animals after single or repeat SC administration in 4 non-GLP studies. The t_{max} ranged from 4 to 36 hours across individual animals after SC administration, was most commonly 8 hours post-dose. Across individual animals after IV administration, the estimated $t_{1/2}$ ranged from 16.2 to 82.4 hours, with t_{max} ranging from 0.25 to 1 hour post-dose.

[00687] The TK profile of DF hIL12-Fc si was confirmed in the GLP toxicology study. In general, sex-related differences in DF hIL12-Fc si mean C_{max} , area under the concentration-time curve from the time of dosing to 24 hours post-dose (AUC_{0-24}), and AUC_{0-168} values were less than 2-fold. After SC dosing, exposure, as assessed by DF hIL12-Fc si mean C_{max} and AUC_{0-168} , generally increased with increasing dose level from 4 µg/kg DF hIL12-Fc si. The increases in mean C_{max} and AUC_{0-168} were dose proportional. No accumulation of DF hIL12-Fc si was observed after multiple doses of DF hIL12-Fc si. Subcutaneous bioavailability of DF hIL12-Fc si was approximately 40%. Mean $t_{1/2}$ for SC administration ranged from 17.5 to 35.8 hours on Days 1 and 15, while mean $t_{1/2}$ for IV administration was 22.2 hours on Day 1 and 45.3 hours on Day 15.

[00688] In the single-dose non-GLP SC study in monkeys, ADA to DF hIL12-Fc si was demonstrated by Day 8, with confirmed ADA up to Day 22; however, overall titers of ADA remained relatively low and close in value to low positive control. In another non-GLP study, an

initial SC dose followed by an IV dose demonstrated a lower than expected IV exposure profile, which may be explained by ADA, although titers were not measured. Overall, data from non-GLP studies suggest that the SC route in monkeys may be more prone to ADA development than the IV route, which was also confirmed in the GLP study. For example, 9 of 10 monkeys at 20 µg/kg SC and 3 of 10 monkeys at 12 µg/kg IV developed ADA by Day 15 in the GLP study. The majority of confirmed ADA samples were seen on Day 15 before the third dose, and maximum concentrations (C_{max}) was impacted in some animals, suggesting the presence of neutralizing antibodies. ADA impacted exposures of individual monkeys in both the non-GLP and GLP studies, but suitable exposure was achieved for a long enough duration to confidently define the toxicology profile of DF hIL12-Fc si. However, ADA in monkeys is not predictive of immunogenicity in humans. For these reasons, the DF hIL12-Fc si-001 first-in-human (FIH) study will evaluate serum titers of anti-DF hIL12-Fc si antibodies throughout the study.

[00689] Additionally, the pharmacologic response to DF hIL12-Fc si, as measured by IFN γ response, was variable across individual animals, with no apparent sex-related differences, but showed some dose-dependency when comparing tolerated doses. The peak IFN γ response ranged from 3 to 5 days post-dose across animals in the non-GLP and GLP studies. In the GLP study, the IFN γ response was attenuated with repeat dosing, although a small percentage of animals demonstrated an IFN γ response after both the first and second doses. Additionally, dose-dependent increases in IP-10 were detectable after the first and third doses, with attenuation. The route of administration did not appear to impact the timing of the peak pharmacologic response.

[00690] Studies to evaluate metabolism and excretion have not been conducted because these studies, which are routinely conducted for small molecule drugs, are not considered necessary or useful for biologics such as monoclonal antibodies. Dedicated studies have not been conducted to evaluate drug-drug interactions (DDIs) because there is no reason to believe that DF hIL12-Fc si, an IL12-Fc fusion protein, is metabolized by cytochrome P450 (CYP) enzymes. Therefore, it is unlikely that a small molecule that inhibits or induces CYP enzymes could impact the PK of DF hIL12-Fc si, and thus, the PK DDI potential for DF hIL12-Fc si is considered to be low.

Absorption and Pharmacokinetics

Single-dose Pharmacokinetics

[00691] Exposures after administration of a single dose of DF hIL12-Fc si as part of the single- or repeat-dose toxicology studies were informative for acute toxicity assessment. Across studies, DF hIL12-Fc si was tolerated up to 20 µg/kg as a single SC dose. The single-dose IV maximum tolerated dose (MTD) was ≤ 19 µg/kg in females and ≤ 20 µg/kg in males; a single IV dose ≥ 20

or ≥ 40 $\mu\text{g/kg}$ in females or males, respectively, resulted in early euthanasia 8 days after DF hIL12-Fc si administration.

[00692] Day 1 exposures (as measured by C_{max} and area under the concentration-time curve from the time of dosing to the time of the last quantifiable sample [AUC_{0-t}]), as well as t_{max} and $t_{1/2}$, derived from ELISA data obtained from the single- and repeat-dose toxicology studies are summarized in Table 70.

Table 70: Comparison of Day 1 Plasma Toxicokinetics in Cynomolgus Monkeys

Study (GLP Status)	Dose Level (µg/kg)	Route of Administration	n	C _{max} (pg/mL)			AUC _{0-∞} (h*pg/mL) ^a			t _{max} (h)		t _{1/2} (h)	
				M	F		M	F		M	F	M	F
RW11CN (Non-GLP)	1	SC	2/sex	2010	1380		85,100 ^b	61,600 ^b		8 ^c	8 ^c	13.8 ^d	N/A
	2	SC	2/sex	3900	1670		161,000 ^b	149,000		6 ^c	8 ^c	17.3	42.4
	4	SC	2/sex	5410	8010		425,000 ^c	515,000 ^c		22 ^d	6 ^c	32.5 ^d	37.8
TD36MM (Non-GLP)	4	SC	2/sex	8860	10,500		562,000 ^c	590,000 ^c		22 ^c	22 ^c	40.2	35.4
	8	SC	2/sex	14,000	20,200		783,000 ^c	1,550,000 ^c		8 ^c	24 ^c	33.9	29.6 ^d
NF37DV (GLP)	4	SC	3/sex	11,800	10,300		480,000	426,000		8 ^c	8 ^c	19.5	17.5
	8	SC	5/sex	16,900	12,800		1,080,000	720,000		8 ^c	8 ^c	18.5	28.0
	20	SC	5/sex	25,700	32,900		2,390,000	2,490,000		24 ^c	48 ^c	31.9	35.8
	12	IV	5/sex	147,000	189,000		3,240,000	4,440,000		1 ^c	1 ^c	25.6	18.8
QW56LH (Non-GLP)	1.9	IV	1/sex	53,600	47,900		1,450,000	921,000		0.25	0.25	30.5	17.4
	19	IV	1/sex	570,000	502,000		20,700,000	15,100,000		0.25	0.25	26.4	19.0
DQ81GX (Non-GLP)	20 ^f	IV	2/sex	498,000	572,000		14,800,000 ^g	16,900,000		0.25 ^c	0.25 ^c	70.0	22.9
	40 ^h	IV	2/sex	1,120,000	994,000		37,300,000	40,300,000		0.25 ^c	0.25 ^c	22.7	37.3

Sources: RW11CN, TD36MM, QW56LH, DQ81GX, and NF37DV.

C_{max}: maximum plasma concentration; AUC_{0-∞}: area under the concentration-time curve from the time of dosing to the time of the last quantifiable sample.

Note: All TK exposures presented here were obtained using DF hIL12-Fc si plasma concentrations measured using a qualified ELISA method (targeting p70 of IL12).

^a AUC exposures were based on concentrations quantified through 168 hours, unless otherwise noted.

^b AUC₀₋₇₂.

^c Median value.

^d n = 1.

^e AUC₀₋₂₄₀.

^f DF hIL12-Fc si was not tolerated in females at 20 µg/kg, resulting in euthanasia on Day 8.

^g AUC₀₋₃₃₆.

^h DF hIL12-Fc si was not tolerated in males and females at 40 µg/kg, resulting in euthanasia on Day 8.

Comparison of Acute Toxicity and Exposures Across All Toxicology Studies After Single-Dose Administration

[00693] Tolerability assessment and exposures after administration of the initial, first dose in repeat-dose toxicity studies are informative for acute toxicity assessment (Studies TD36MM, QW56LH, DQ81GX, and NF37DV). The single-dose IV MTD was ≤ 19 $\mu\text{g/kg}$ in females and ≤ 20 $\mu\text{g/kg}$ in males; a single IV dose ≥ 20 or ≥ 40 $\mu\text{g/kg}$ in females or males, respectively, resulted in early euthanasia 8 days after DF hIL12-Fc si administration (Study DQ81GX). The exposures at 19 and 20 $\mu\text{g/kg}$ IV overlapped with similar IFN γ pharmacology, suggesting animal-to-animal variation in the immune system in response to DF hIL12-Fc si treatment, which may have led to differential tolerability. This is consistent with the known variability of the immune system as a target organ (Brodin P, Davis MM., *Nat Rev Immunol.* 2017;17(1):21-9).

Repeat-dose Pharmacokinetics

Toxicokinetics After Repeat Subcutaneous/Intravenous Administration of DF hIL12-Fc si via a Crossover Design (Studies TD36MM and DX81GX)

[00694] In a non-GLP study (Study TD36MM), cynomolgus monkeys were administered DF hIL12-Fc si, first SC and then IV, with a 14-day washout period after each dose, with samples for TK collected through 336 hours post-dose.

[00695] After SC administration, the systemic exposure of both male and female monkeys to DF hIL12-Fc si appeared to be characterized by dose-independent (linear) kinetics over the dose range of 4 to 8 $\mu\text{g/kg}$. The systemic exposure to DF hIL12-Fc si tended to be slightly higher in females than in males.

[00696] After IV administration to monkeys who had previously received DF hIL12-Fc si by the SC route, the systemic exposure of male and female monkeys to DF hIL12-Fc si appeared to be characterized by dose-dependent (nonlinear) kinetics over the dose range of 2 to 4 $\mu\text{g/kg}$, such that increasing the dose of DF hIL12-Fc si above 2 $\mu\text{g/kg}$ resulted in a lower systemic exposure than was predicted from a linear relationship in males, but a higher systemic exposure in females. These results would be consistent with the production of ADA to DF hIL12-Fc si in the 2 males and 1 female before the IV dose was administered. Consequently, the results after IV administration must be interpreted with caution.

[00697] SC bioavailability of DF hIL12-Fc si appeared to be 60% to 70% (based on 4 animals); however, it should be noted that anomalously high estimates of bioavailability (omitted from the

range presented here) were observed in some animals, consistent with the production of ADA affecting the exposure after IV administration, although ADA was not measured on this study.

[00698] In another non-GLP study (DQ81GX), cynomolgus monkeys were administered DF hIL12-Fc si first via IV bolus and then SC, with a 14-day washout period between doses. A separate group of animals received rhIL12 with the same routes and frequency of administration to allow for comparison. Blood samples for TK analysis were obtained through 240 hours post-dose. Plasma concentrations of DF hIL12-Fc si and rhIL12 were measured by qualified ELISA (targeting p70 of IL12 [ie, measuring rhIL12 and DF hIL12-Fc si]) and MSD (targeting p40 and Fc of DF hIL12-Fc si [ie, measuring DF hIL12-Fc si and not measuring rhIL12]) methods. Because of the toxicity observed after IV administration of DF hIL12-Fc si, the TK profile for SC administration was characterized only for DF hIL12-Fc si (males only) and rhIL12.

[00699] Plasma concentrations of DF hIL12-Fc si after IV administration were lower when measured by the MSD method (as to be expected, given the ELISA method detects the IL12 heterodimer). While the C_{max} values derived from the MSD data were approximately 54% lower than those derived from the ELISA method, the AUC_{0-t} values were similar to those derived from the ELISA method in males and 18% higher in females. The AUC_{0-t} values of DF hIL12-Fc si in female monkeys were similar to those in males after IV administration when measured using the ELISA method but were slightly higher when measured by the MSD method. The relationships between AUC_{0-t} and dose level after IV administration (based on ELISA data) indicated exposures increased in a slightly greater than dose-proportional manner over the dose range of 20 to 40 $\mu\text{g/kg}$. The t_{max} after IV administration was 0.25 hours post-dose (the first sampling time), as was expected, whereas the t_{max} ranged from 4 to 24 hours across individual animals after SC administration.

[00700] The $t_{1/2}$ varied quite widely (ranging from 16.2 to 82.4 hours across individual animals) after IV administration at 20 or 40 $\mu\text{g/kg}$, but could only be estimated adequately for 1 animal after SC administration (14.9 hours based on ELISA data and 62.0 hours based on MSD data). Subcutaneous bioavailability of DF hIL12-Fc si at 20 $\mu\text{g/kg}$ appeared to be variable, with a mean value of 53.5% (range of 38.8% to 68.2%) based on ELISA data and 89.0% (range of 62.8% to 115.3%) based on MSD results.

[00701] The C_{max} and AUC_{0-t} values of rhIL12 in female monkeys were similar to those of males after IV administration; however, the C_{max} and AUC_{0-t} values were lower than those in males after SC administration. The t_{max} after IV administration was also 0.25 hours post-dose, whereas the t_{max} was 8 hours post-dose after SC administration. The $t_{1/2}$ ranged from 9.1 to 17.5 hours across

individual animals after IV administration and 18.9 to 22.9 hours after SC administration. Subcutaneous bioavailability of rhIL12 at 10 µg/kg was approximately 31% in males and 18% in females (overall range of 18.0% to 35.2%).

Toxicokinetics After Repeat Intravenous Administration of DF hIL12-Fc si (Study QW56LH)

[00702] In a non-GLP study, cynomolgus monkeys were administered DF hIL12-Fc si, or rhIL12 via IV bolus on Days 1 and 8, with samples for TK collected through 168 hours postdose. Plasma concentrations were measured by both qualified ELISA (targeting p70 of IL12 [ie, measuring rhIL12 and DF hIL12-Fc si]) and MSD (targeting p40 and Fc of DF hIL12-Fc si [ie, measuring DF hIL12-Fc si and not measuring rhIL12]) methods.

[00703] After repeated IV bolus administration of DF hIL12-Fc si, there was no accumulation of DF hIL12-Fc si. The AUC₀₋₁₆₈ of DF hIL12-Fc si increased in an approximately dose-proportional manner over the dose range of 1.9 to 19 µg/kg on Days 1 and 8 in males, but tended to increase in a greater than dose-proportional-manner in females. At 19 µg/kg, the female AUC₀₋₁₆₈ was approximately 1.8-fold higher than that predicted from a linear relationship. The AUC₀₋₁₆₈ of DF hIL12-Fc si in females was generally similar to that in males, although the female AUC₀₋₁₆₈ derived from the MSD data appeared to be lower than that of the male at 1.9 µg/kg on both Days 1 and 8.

[00704] The terminal t_{1/2} could not be estimated adequately for all animals but, where it could be estimated was in the range of 17.4 to 30.5 hours and generally appeared to be independent of dose and sex. The plasma clearance of DF hIL12-Fc si was low, and the volume of distribution was slightly lower than the blood volume (73.4 mL/kg) and considerably lower than the volume of total body water (693 mL/kg) (Davies B, Morris T., *Pharm Res.* 1993;10(7):1093-5).

[00705] After repeated IV bolus administration of rhIL12, there was no accumulation of rhIL12. The AUC₀₋₁₆₈ of rhIL12 generally increased in an approximately dose-proportional manner over the dose range of 1 to 10 µg/kg on Days 1 and 8, but tended to increase in a greater than dose-proportional manner in males on Day 1. At 10 µg/kg, the male AUC₀₋₁₆₈ on Day 1 was approximately 1.8-fold higher than that predicted from a linear relationship. There did not appear to be any differences in the systemic exposure of males and females to rhIL12. The terminal t_{1/2} (7.2 to 17.0 hours) was shorter than that of DF hIL12-Fc si, the plasma clearance was low, and the volume of distribution was similar to the blood volume and considerably lower than the volume of total body water.

[00706] The shorter half-life of rhIL12 compared to that of DF hIL12-Fc si explains the greater (~4.5× to 7.4×) AUC₀₋₁₆₈ observed with DF hIL12-Fc si.

Table 71: Mean Plasma Toxicokinetics of DF hIL12-Fc si After Subcutaneous or Intravenous Administration to Cynomolgus Monkeys in a 3-Week Study (Study NF37DV)

Dose Level (µg/kg)/ Route	C _{max} (pg/mL)		AUC ₀₋₁₆₈ (h*pg/mL)		t _{max} ^a (h)		t _½ (h)		F (%)	
	M	F	M	F	M	F	M	F	M	F
Day 1^b										
4 (SC)	11,800	10,300	480,000	426,000	8.0	8.0	19.5	17.5 ^c	37.5	33.3
8 (SC)	16,900	12,800	1,080,000	720,000	8.0	8.0	18.5	28.0	42.2	28.1
20 (SC)	25,700	32,900	2,390,000	2,490,000	24.0	48.0	31.9	35.8 ^d	37.4	38.9
12 (IV)	147,000	189,000	3,240,000	4,440,000	1.0	1.0	25.6	18.8	NA	NA
Day 15										
4 (SC)	9580 ^c	7240 ^c	NR	NR	8.0 ^c	8.0 ^c	NR	NR	NA	NA
8 (SC)	17,300 ^d	NA	451,000 ^e	NR	8.0 ^d	NR	NR	NR	NA	NA
20 (SC)	38,000 ^e	37,300 ^e	2,650,000 ^e	NR	8.0 ^e	24.0 ^e	23.9 ^e	NR	NA	NA
12 (IV)	155,000 ^f	173,000 ^d	3,400,000 ^f	2,280,000 ^d	1.0 ^f	1.0 ^d	24.2 ^c	59.8 ^e	NA	NA

Source: Study NF37DV.

AUC₀₋₁₆₈: area under the concentration-time curve from the time of dosing to 168 hours post-dose; ADA: anti-drug antibodies; C_{max}: maximum plasma concentration; ELISA: enzyme-linked immunosorbent assay; F: female(s); M: male(s); NA: not applicable; NR: not reported due to insufficient number of ADA-negative animals; TK: toxicokinetic(s).

Note: All TK parameters were derived from concentrations quantified by validated ELISA. Number of animals per sex per group are denoted in the footnotes. On Day 15, exposures are presented only for animals that did not have ADA, even though there was quantifiable exposure in these ADA-positive animals. Because DF hIL12-Fc si is intended to be dosed once every 3 weeks in patients, Day 1 exposures from monkey studies were used as the best comparison to the intended human dosing schedule.

^a Median values; ^b n = 5/sex/group on Day 1, with the exception of 4 µg/kg SC (n = 3/sex); ^c n = 2/sex; ^d n = 3/sex; ^e n = 1/sex; ^f n = 5/sex.

[00707] In general, sex-related differences in DF hIL12-Fc si mean C_{max}, AUC₀₋₂₄, and AUC₀₋₁₆₈ values were less than 2-fold. After SC dosing, exposure, as assessed by DF hIL12-Fc si mean C_{max} and AUC₀₋₁₆₈, generally increased with increasing dose level from 4 µg/kg DF hIL12-Fc si. The increases in mean C_{max} and AUC₀₋₁₆₈ were dose proportional. No accumulation of DF hIL12-Fc si was observed after multiple doses of DF hIL12-Fc si in monkeys. Subcutaneous bioavailability of DF hIL12-Fc si was approximately 40%. Mean t_½ for SC administration ranged from 17.5 to 35.8 hours on Days 1 and 15, while mean t_½ for IV administration was 22.2 hours on Day 1 and 45.3 hours on Day 15.

[00708] The incidence of ADA induction to DF hIL12-Fc si was 0% (0 out of 10) at 0 µg/kg, 33% (2 out of 6) at 4 µg/kg SC, 80% (8 out of 10) at 8 µg/kg SC, 90% (9 out of 10) at 20 µg/kg SC, and 30% (3 out of 10) at 12 µg/kg IV. Plasma concentrations of DF hIL12-Fc si in the ADA-positive animals on Day 15 were overall lower, but generally still quantifiable, than those in the ADA-negative animals. The effect of ADA was variable, with plasma concentrations in ADA-

positive animals ranging from being similar to those in ADA-negative animals in some cases, to being markedly lower in others. However, this indicates that, in general, the ADA-positive animals were still exposed to DF hIL12-Fc si on Day 15. Thus, ADA did not negatively impact the toxicology study interpretation, as there was adequate exposure during most of the dosing period.

Bioavailability

[00709] After SC administration in cynomolgus monkeys in non-GLP Studies TD36MM and DQ81GX, DF hIL12-Fc si generally demonstrated bioavailability of approximately 60%, although some variability was observed. Although not confirmed, it is believed that ADA development may have impacted the second IV dose in Study TD36MM, which subsequently would have impacted bioavailability calculations; therefore, these values were excluded when considering the overall average bioavailability across animals. Table 72 presents the bioavailability across animals within these studies.

[00710] In GLP Study NF37DV, SC bioavailability of DF hIL12-Fc si, based on AUC_{0-168} , was in the range of 18.2% to 52.8% across individual male and female animals, with mean values of 35.4%, 35.1% and 38.2% after the first dose at 4, 8, and 20 $\mu\text{g/kg}$ SC, respectively.

Table 72: Assessment of DF hIL12-Fc si Bioavailability

Study Number	Route of Administration	Animal ID	Sex	Dose (µg/kg)	Bioavailability (SC/IV AUC ₀₋₄ /dose)
TD36MM	SC then IV	1200	Male	4-SC, 2-IV	67%
TD36MM	SC then IV	1201	Male	4-SC, 2-IV	59%
TD36MM	SC then IV	1700	Female	4-SC, 2-IV	386% ^a
TD36MM	SC then IV	1701	Female	4-SC, 2-IV	64%
TD36MM	SC then IV	2200	Male	8-SC, 4-IV	536% ^a
TD36MM	SC then IV	2201	Male	8-SC, 4-IV	604% ^a
TD36MM	SC then IV	2700	Female	8-SC, 4-IV	62%
TD36MM	SC then IV	2701	Female	8-SC, 4-IV	479% ^a
DQ81GX	IV then SC	2170	Male	20-IV, 20-SC	39%
DQ81GX	IV then SC	2171	Male	20-IV, 20-SC	68%
NF37DV	SC	2360	Male	4	29%
NF37DV	SC	2361	Male	4	33%
NF37DV	SC	2362	Male	4	50%
NF37DV	SC	2860	Female	4	29%
NF37DV	SC	2861	Female	4	53%
NF37DV	SC	2862	Female	4	18%
NF37DV	SC	3360	Male	8	41%
NF37DV	SC	3361	Male	8	42%
NF37DV	SC	3362	Male	8	34%
NF37DV	SC	3363	Male	8	51%
NF37DV	SC	3364	Male	8	42%
NF37DV	SC	3860	Female	8	21%
NF37DV	SC	3861	Female	8	38%
NF37DV	SC	3862	Female	8	38%
NF37DV	SC	3863	Female	8	24%
NF37DV	SC	3864	Female	8	20%

Study Number	Route of Administration	Animal ID	Sex	Dose (µg/kg)	Bioavailability (SC/IV AUC ₀₋₄ /dose)
NF37DV	SC	4360	Male	20	46%
NF37DV	SC	4361	Male	20	35%
NF37DV	SC	4362	Male	20	27%
NF37DV	SC	4363	Male	20	53%
NF37DV	SC	4364	Male	20	26%
NF37DV	SC	4860	Female	20	50%
NF37DV	SC	4861	Female	20	49%
NF37DV	SC	4862	Female	20	14%
NF37DV	SC	4863	Female	20	37%
NF37DV	SC	4864	Female	20	45%

Sources: Study TD36MM, DQ81GX and NF37DV.

ADA: anti-drug antibody; AUC₀₋₄: area under the concentration-time curve from the time of the last quantifiable sample; ID: identification; IV: intravenous(ly); SC: subcutaneous(ly).

^a Although not confirmed, it is believed that ADA development may have impacted the second IV dose in Study TD36MM, which impacted bioavailability calculations. Therefore, these values are not relevant when calculating the average bioavailability.

Distribution

[00711] Volume of distribution at steady state (V_{ss}) could only be calculated in a non-GLP study, Study QW56LH. Mean V_{ss} was 37.6 mL/kg after the first IV dose and 47.55 mL/kg after the second IV dose. In subsequent non-GLP studies in monkeys, V_{ss} could not be calculated because of insufficient characterization of the terminal phase after IV infusion.

[00712] In the GLP study, mean V_{ss} was 91.9 and 71.1 mL/kg in males and females, respectively, after the first IV dose, with an overall mean V_{ss} of 81.5 mL/kg across all 10 animals. On Day 15, the mean V_{ss} was 107 mL/kg in males ($n = 2$) and 108 mL/kg in the single female evaluated, for an overall mean V_{ss} of 108 mL/kg across the 3 animals.

Metabolism

[00713] Metabolism studies of DF hIL12-Fc si have not been conducted. Standard metabolism studies routinely conducted for small molecule drugs are not considered necessary or useful for biologics such as antibodies.

Excretion

[00714] Excretion studies of DF hIL12-Fc si have not been conducted. Standard elimination studies routinely conducted for small molecule drugs are not considered necessary or useful for biologics such as DF hIL12-Fc si.

Drug-Drug Interactions

[00715] No Drug-Drug interaction (DDI) studies have been performed to date. Therapeutic proteins such as cytokines or monoclonal antibodies that act as cytokine modulators are likely to exhibit interactions with small molecule drugs by influencing the expression and stability of specific cytokine p450 (CYP) enzymes and drug transporters (Huang SM, Zhao H, Lee JJ, Reynolds K, Zhang L, Temple R, et al., *Clin Pharmacol Ther.* 2010;87(4):497-503). Among cytokines, IL6 is known to downregulate CYP expression. Modeling shows IL-6 could reduce the intrinsic clearance of CYP3A4 by 28% at approximately 48 hours post-dose (Xu Y, Hijazi Y, Wolf A, Wu B, Sun YN, Zhu M., *CPT Pharmacometrics Syst Pharmacol.* 2015;4(9):507-15). DF hIL12-Fc si induced minimal and sporadic increases of IL6 in monkeys at tolerated doses. Given the de novo synthesis of CYP enzymes ($t_{1/2}$ of 24 to 36 hours) and transient duration of the IL6 cytokine spike with DF hIL12-Fc si, the risk of DDIs is not considered significant.

[00716] Because there is no reason to believe that DF hIL12-Fc si, an IL12-Fc fusion protein, is metabolized by CYP enzymes, it is unlikely that a small molecule that inhibits or induces CYP enzymes could impact the PK of DF hIL12-Fc si. Based on these considerations, the PK DDI potential for DF hIL12-Fc si is considered to be low.

Immunogenicity

[00717] In the single-dose toxicology study in monkeys, 7 of 12 treated animals were found to be positive for anti-drug antibody (ADA). Additionally, although ADA was not evaluated, variability in TK and anomalously high estimates of bioavailability in a 4-week repeat dose study in cynomolgus monkeys were considered consistent with the production of ADA to DF hIL12-Fc si. ADA impacted exposures of individual monkeys in both non-GLP and GLP studies, but suitable exposure was achieved for a long enough duration to confidently define the toxicology profile of DF hIL12-Fc si. ADA in monkeys is not predictive of immunogenicity in humans. For these reasons, serum titers of anti-DF hIL12-Fc si antibodies will be evaluated in clinical studies.

Example 26: Treatment of Cancer using DF hIL12-Fc si

Objectives

[00718] This clinical study is designed with the following phases: Phase 1, Phase 1b, and Phase 2.

[00719] The primary objective of Phase 1 is to assess the safety and tolerability of DF hIL12-Fc si as monotherapy, and to determine the maximum tolerated dose (MTD) of DF hIL12-Fc si in patients with advanced (unresectable, recurrent or metastatic) solid tumors.

[00720] The primary objective of Phase 1b is to assess the safety and tolerability of DF hIL12-Fc si in combination with pembrolizumab, and to determine the maximum tolerated dose (MTD)

of DF hIL12-Fc si in combination with pembrolizumab in patients with advanced (unresectable, recurrent, or metastatic) solid tumors.

[00721] The primary objective of Phase 2 is to assess the Objective Response Rate (ORR) according to the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1) per an Independent Endpoint Review Committee (IERC), for all Efficacy Expansion Cohorts testing the clinical activity of DF hIL12-Fc si as a monotherapy or in combination.

[00722] The secondary objectives of Phase 1 and Phase 1b, with DF hIL12-Fc si as monotherapy and in combination with pembrolizumab, are to: Characterize the PK of DF hIL12-Fc si; evaluate immunogenicity of DF hIL12-Fc si, and to correlate its exposure and clinical activity; assess best overall response (BOR), as determined by the Investigator for DF hIL12-Fc si using RECIST 1.1; assess duration of response (DOR) of DF hIL12-Fc si, using RECIST 1.1; assess progression-free survival (PFS) for DF hIL12-Fc si, using RECIST 1.1; and assess overall survival (OS) time.

[00723] The secondary objectives of Phase 2, with DF hIL12-Fc si as monotherapy and in combination with pembrolizumab, are to: characterize the PK of DF hIL12-Fc si; assess duration of response (DOR) of DF hIL12-Fc si, per an IERC using RECIST 1.1; assess clinical benefit rate (CBR) of DF hIL12-Fc si using RECIST 1.1. CBR is defined as the percentage of patients with complete response (CR), partial response (PR), or stable disease (SD) as best response; assess the safety of DF hIL12-Fc si; evaluate the immunogenicity of DF hIL12-Fc si, and correlate with exposure and clinical activity; assess progression-free survival (PFS) for DF hIL12-Fc si, per an IERC using RECIST 1.1; and Assess overall survival (OS) time.

Exploratory Objectives

[00724] The exploratory objectives, both with DF hIL12-Fc si as monotherapy and in combination with pembrolizumab, are to: evaluate changes from baseline in tumor and peripheral biomarkers, and the relationship to PK; assess the PK of pembrolizumab (Phase 1b and Cohort 2C only); evaluate the activity of DF hIL12-Fc si in the Efficacy Expansion Cohorts Part (Phase 2) per Investigator assessment (ORR, DOR, CBR, and BOR, by RECIST); and evaluate the association between tumor and peripheral biomarkers, and tumor response rate.

Study Design Overview

[00725] This study is a Phase 1/2, open-label, dose-escalation study with a consecutive parallel-group efficacy expansion study, designed to determine the safety, tolerability, PK, pharmacodynamics, and preliminary anti-tumor activity of DF hIL12-Fc si as monotherapy and in combination with pembrolizumab. A schematic diagram of the study design is shown in **FIG. 51A** (for monotherapy) and **51B** (for combination therapy with Pembrolizumab).

[00726] The study consists of 3 parts: **Phase 1:** Dose-escalation as a monotherapy using a 3+3 design, with Phase 1 Cohort Expansion; **Phase 1b:** Dose-escalation as a combination with pembrolizumab using a 3+3 design, with Phase 1b Cohort Expansion; and **Phase 2:** Efficacy Expansion using a group sequential design.

[00727] DF hIL12-Fc si is evaluated as a monotherapy in Efficacy Expansion cohorts in the following indications: Cohort 2A: Advanced (unresectable or metastatic) melanoma; and Cohort 2B: Advanced (unresectable or metastatic) renal cell carcinoma (RCC)

[00728] DF hIL12-Fc si is evaluated in combination with pembrolizumab in an Efficacy Expansion cohort in the following indication: Cohort C: Advanced (unresectable or metastatic) urothelial carcinoma

[00729] In each study phase, patients receive DF hIL12-Fc si on Day 1 every 3 weeks (Q3W). Patients receive DF hIL12-Fc si until confirmed progressive disease (PD), unacceptable toxicity (*i.e.*, dose-limiting toxicity [DLT]), or any reason for withdrawal from the study or Investigational Medicinal Product (IMP) occurs.

Phase 1 Dose Escalation DF hIL12-Fc si Monotherapy

[00730] The Phase 1 Dose-escalation Phase of the study is designed to determine the dose-limiting toxicities (DLTs) and maximum tolerated dose (MTD) of DF hIL12-Fc si as monotherapy using a standard 3+3 design.

[00731] The decision to escalate to the next dose level (DL) is based on safety assessments after all patients of a cohort have had safety evaluations performed through Cycle 2, Day 1 (C2D1), unless due to DLT. In order to assess the safety of DF hIL12-Fc si, a Safety Monitoring Committee (SMC), responsible for dose-escalation decisions, is established.

[00732] After the safety of Dose Level “n” has been established, the SMC has the option to permit enrollment of up to 10 patients at that DL in the Phase 1 Expansion Cohort; no more than 30 patients can be enrolled by this process.

[00733] The MTD is defined as the highest DL at which ≤ 1 patient of 6 evaluable patients experiences a DLT.

Phase 1b: Dose-escalation as a combination with pembrolizumab

[00734] The Phase 1b Dose-escalation Phase of the study is designed to determine the DLTs and MTD of DF hIL12-Fc si when given in combination with pembrolizumab, using a standard 3+3 design, as described for Phase 1.

[00735] Pembrolizumab is administered once every 3 weeks (on Day 1 of each cycle) per its U.S. package insert. The administration of pembrolizumab precedes that of DF hIL12-Fc si.

[00736] DF hIL12-Fc si dose levels tested in combination with pembrolizumab are the same as those tested as a monotherapy.

[00737] Phase 1b starts after any of the following criteria are met with DF hIL12-Fc si monotherapy: a Grade 2 drug-related toxicity occurs at any dose level occurring during the DLT observation period; a DLT occurs at a dose level not defined as the MTD; and dose-escalation is complete, with no MTD defined.

[00738] After one of these criteria are met, Phase 1b (DF hIL12-Fc si in combination with pembrolizumab) starts using a dose of DF hIL12-Fc si two dose levels below the one that met any of the above criteria, or if any of the criteria are met at DL1 or at DL2, the starting dose for combination is DL1, after the safety of DL1 has been established (defined by 3 patients treated at DL2 or 6 patients treated at DL1, with no more than one DLT observed at DL1).

[00739] After the safety of Dose Level “n” has been established, the SMC has the option to permit enrollment of up to 10 patients at that dose level in the Phase 1b Expansion Cohort; no more than 30 patients can be enrolled by this process.

Phase 2 Efficacy Expansion

[00740] The following tumor types are enrolled at the recommended phase 2 dose (RP2D):

[00741] As a monotherapy: Cohort 2A: Advanced (unresectable or metastatic) melanoma; and Cohort 2B: Advanced (unresectable or metastatic) renal cell carcinoma.

[00742] In combination with pembrolizumab: Cohort 2C: Advanced (unresectable or metastatic) urothelial carcinoma.

Inclusion and Exclusion Criteria

[00743] Male or female patients aged ≥ 18 years with an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 at study entry and an estimated life expectancy of at least 3 months are enrolled.

[00744] Key inclusion criteria in each study phase/cohort are as follows:

[00745] Dose-Escalation cohorts in Phase 1/1b: Clinical or radiological evidence of disease

[00746] Dose Expansion Cohorts in Phase 1/1b: Has one of the following tumor types: melanoma, non-small cell lung cancer (NSCLC), small-cell lung cancer (SCLC), head and neck squamous cell carcinoma (HNSCC), classical Hodgkin lymphoma, primary mediastinal large B-Cell lymphoma, urothelial carcinoma, micro-satellite instability high cancer, gastric cancer, oesophageal cancer, cervical cancer, hepatocellular cancer, Merkel cell carcinoma, renal cell carcinoma, endometrial cancer, cutaneous T cell lymphoma, or triple negative breast cancer; has

measurable disease, as determined by the Investigator using the Response Evaluation Criteria for Solid Tumors (RECIST), version 1.1;

[00747] Cohort 2A

[00748] Patients with advanced melanoma who: received treatment with an anti-programmed cell death protein 1 (PD-1) antibody for at least 6 weeks; have a confirmation of PD at least 4 weeks after the initial diagnosis of PD while receiving an anti PD-1 is made. Confirmation of PD can be based on radiological or clinical observations; must have received a BRAF inhibitor if the tumor carries a BRAF activating mutation and have progressed after the last line of treatment.

[00749] Cohort 2B

[00750] Patients with advanced RCC who: have any clear cell histology component; received treatment with an anti PD-1/PD-L1 antibody and an anti-vascular endothelial growth factor therapy as a monotherapy or in combination; received ≤ 3 prior lines of therapy

[00751] Cohort 2C

[00752] Patients with advanced urothelial carcinoma who: have histologically or cytologically documented locally advanced or metastatic transitional cell carcinoma of the urothelium (including renal pelvis, ureters, urinary urothelial, urethra); have received one (and no more than one) platinum-containing regimen (e.g., platinum plus another agent such as gemcitabine, methotrexate, vinblastine, doxorubicin, etc.) for inoperable locally advanced or metastatic urothelial carcinoma with radiographic progression or with recurrence within 6 months after the last administration of a platinum-containing regimen as an adjuvant, which would be considered failure of a first-line, platinum-containing regimen; have received no more than 2 lines of therapy (including the platinum-containing regimen) for the treatment of the metastatic disease; have not received treatment with a checkpoint inhibitor (CPI) (*i.e.*, anti-PD-1 or anti-PD-L1 as a monotherapy or in combination with a platinum-based chemotherapy).

Dose/ Mode of Administration/ Dosing Schedule

[00753] DF hIL12-Fc si is administered as a subcutaneous (SC) injection Q3W (*i.e.*, on Day 1 of each cycle). Patients receive the drug SC in a volume of not more than 1 mL in a maximum of 2 injection sites. The second administration is completed within 10 minutes after the completion of the first administration, if applicable.

[00754] In Phase 1/1b, patients are hospitalized for the night following the first administration of DF hIL12-Fc si.

[00755] The DF hIL12-Fc si DLs ($\mu\text{g/kg}$) are as follows in Table 73.

TABLE 73: DF hIL12-Fc si DLs ($\mu\text{g/kg}$)

	DL1	DL2	DL3	DL4	DL5	DL6	DL7	DL8	DL9	DL 10
Dose of DF hIL12-Fc si ($\mu\text{g/kg}$)	0.05	0.10	0.20	0.40	0.60	0.80	1.00	1.20	1.40	1.75
Equivalent IL12 ($\mu\text{g/kg}$)	0.021	0.053	0.105	0.211	0.316	0.421	0.526	0.632	0.737	0.921

[00756] The dose of DF hIL12-Fc si is calculated based on the weight of the patient at baseline. The patient's calculated dose is only recalculated if the patient's weight changes by 10% or more since the time of their last dose calculation.

Exploratory Biomarkers

Peripheral Biomarkers

[00757] Peripheral biomarkers are assessed in the periphery in all patients, including: cellular parameters: peripheral blood mononuclear cell (PBMCs) for immunophenotyping (IPT) by flow cytometry; soluble factors: Cytokines and chemokines in serum samples; *ex vivo* IL12 response assay: PBMCs for *ex vivo* stimulation followed by analysis of IFN γ production; circulating tumor (ct) deoxyribonucleic acid (DNA).

[00758] IPT assessments are performed on PBMCs derived from whole blood samples are collected 2 hours prior to administration of DF hIL12-Fc si C1 through C3 and at each of the following study visits: C1D3, C1D8, C2D8, and C3D3.

[00759] Soluble factors are determined in serum samples collected within 2 hours prior to DF hIL12-Fc si administration on D1 of each treatment cycle, and on C1D2, C1D3, C1D5, C1D8, C1D15, C2D3, C3D3, and C4D3, and at the EOT and SFU visits.

[00760] In order to complete all the assessments on tumor materials, blood (*e.g.*, whole blood, plasma, and serum samples), is collected from patients.

Biomarkers Derived From Tumor Tissue

[00761] Tissue derived biomarkers are evaluated on the pre-treatment and on treatment biopsies in patients participating in the Dose-escalation phase (optional biopsies), the Phase 1/1b Expansion Cohorts part (mandatory biopsies), and the Phase 2 Efficacy Expansion Cohorts phase (mandatory biopsies).

[00762] A panel of putative markers including molecular, soluble and cellular markers is analyzed at baseline from archived tumor tissue (or fresh tumor biopsy, if available), whole blood,

and serum samples to investigate a possible correlation between clinical efficacy and analyzed markers.

[00763] For patients enrolled in the Dose-escalation Phase, the level of PD-L1 expression is determined using a commercially available kit (Dako PD-L1 IHC 22C3 pharmDx) and analysis of CD3 positivity (T cell infiltration) is determined by immunohistochemistry (IHC).

[00764] For patients enrolled in the Phase 1/1b Expansion Cohorts and the Efficacy Expansion Cohorts, fresh mandatory tumor biopsies is performed at Screening (*i.e.*, within 30 days before first study drug dose) and at pre-specified time points during the treatment period.

[00765] Other biomarkers that are assessed include: frequency and localization of tumor-infiltrated leukocytes (eg, CD8, CD4 T-cells, Treg, NK cells, macrophage [M1/2 profile] by IHC or IF), gene expression profile, and pharmacogenomics (PGx).

Reference Therapy: Dose/Mode of Administration/ Dosing Schedule

[00766] In Phase 1b and Cohort 2C, pembrolizumab is administered at a dose of 200 mg, once every 3 weeks via intravenous (IV) infusion, in accordance with the U.S. package insert. The administration of pembrolizumab precedes that of DF hIL12-Fc si. DF hIL12-Fc si is administered within 1 hour after the completion of the administration of pembrolizumab.

Planned Treatment Duration per Patient

[00767] Patients receive study treatment until development of progressive disease (PD) or unacceptable toxicity, or any criterion for withdrawal from the study or DF hIL12-Fc si occurs.

[00768] Any patients who have experienced a confirmed complete response (CR) are treated for at least 12 months after confirmation, unless a criterion for discontinuation is met, at the discretion of the Investigator. If the Investigator believes that such a patient may benefit from treatment beyond 12 months, it may be permissible to continue the treatment after discussion with the Sponsor Medical Monitor. The maximum treatment duration is 24 months.

Statistical Methods (Includes Sample Size Calculation)

[00769] The number of the evaluable patients for this study is derived from the dose-escalation “3+3” design and the expansion cohort sizes. The final sample size may vary depending on the total number of DLs that are evaluated, patient replacement for DLT evaluation, if applicable, and expansion from 3 to 6 patients if a DLT is observed.

[00770] In the event that rapid recruitment in the expansion phase impacts supply of IMP, the screening of new patients for any cohort may be temporarily paused with 24-hours notice to Investigators.

[00771] The final sample size may vary depending on the total number of DLs that are evaluated, patient replacement for DLT evaluation, if applicable, and expansion from 3 to 6 patients if a DLT is observed.

Efficacy Expansion as a Monotherapy (Cohorts 2A and 2B)

[00772] The primary endpoint for this phase is the ORR. For each of these cohorts, the null hypothesis is that the objective response rate (ORR) does not exceed 5% (H_0 : ORR < 5%) and the alternative hypothesis is that the ORR is greater than 10% (H_1 : ORR $\geq$ 10%).

[00773] The target ORR of DF hIL12-Fc si as a monotherapy is 20%. It is expected to enroll 40 patients for each of these cohorts (*i.e.*, approximately 80 patients in total).

[00774] Using a group sequential design, with 40 patients in each of the indication cohorts, the efficacy cohort provides ~90% study power to detect a 15% difference at a 1-sided overall type I error rate of 0.025, assuming the target ORR of 20% for DF hIL12-Fc si.

[00775] For each of Cohorts 2A and 2B, a futility interim analysis, with Lan-DeMets O'Brien Fleming boundary, is planned at 50% information fraction (*i.e.*, at ~20 patients).

[00776] The enrollment may be stopped for futility once 20 patients have completed 3 months follow-up or have withdrawn from the study, if none of the enrolled patients have achieved an unconfirmed BOR of PR or CR according to RECIST 1.1. At the end of the study, a cohort is declared successful if at least 5 patients achieve a confirmed BOR of PR or CR according to RECIST 1.1.

Efficacy Expansion in Combination with Pembrolizumab (Cohort 2C)

[00777] The Phase 2 portion for efficacy expansion in combination with pembrolizumab determines the clinical activity of DF hIL12-Fc si in combination in patients with UBC who have progressed after one line of platinum-based chemotherapy.

[00778] The primary endpoint is the ORR. The study enrolls 40 patients so that the observation of 15 responses (CR or PR) out of the 40 patients enroll will lead to a 95% CI (0.2317; 0.5419) that excludes the value of the percentage of responses reported for pembrolizumab in a similar population, that was enrolled in KEYNOTE-045. In that study, the ORR was 21.7% (Bellmunt J, de Wit R, Vaughn DJ, Fradet Y, Lee JL, Fong L, et al., *N Engl J Med.* 2017;376(11):1015-1026.).

[00779] A minimum 4-week metric is used to qualify SD and to confirm CR, PR or PD. DOR is defined from the time between the first observation of a CR or PR and disease progression. PFS, is defined according to RECIST 1.1 and defined from first administration of study treatment until first observation of progressive disease or death, whichever comes first. OS is defined as the time from first administration of study treatment to death.

[00780] Safety analyses are summarized by descriptive statistical presentation, by cohort and/or across cohorts.

Example 27: Treatment of Cancer using DF hIL12-Fc si in a single dose

[00781] The primary objective of this study is to assess the safety and tolerability of DF hIL12-Fc si as a monotherapy when administered in a single dose, and to determine the maximum tolerated dose (MTD) of DF hIL12-Fc si in patients with advanced (unresectable, recurrent or metastatic) solid tumors. DF hIL12-Fc si is administered as a subcutaneous (SC) injection in a single dose. Patients receive the drug SC in a volume of not more than 1 mL in a maximum of 2 injection sites. The second administration is completed within 10 minutes after the completion of the first administration, if applicable. The patients receive only the single dose of DF hIL12-Fc si.

Example 28: Treatment of Cancer using DF hIL12-Fc si

[00782] This is a Phase 1/2, open-label, dose-escalation study with a consecutive parallel-group efficacy expansion study, designed to determine the safety, tolerability, PK, pharmacodynamics, and preliminary anti-tumor activity of DF-hIL-12-Fc si as monotherapy and in combination with pembrolizumab.

Table 74: Phase 1/2, Open-Label, Dose-Escalation Study

Condition or Disease	Intervention/Treatment	Phase
Solid Tumor; Melanoma; renal cell carcinoma; urothelial carcinoma	Biological/Vaccine: DF-hIL-12-Fc si; Biological/Vaccine: Pembrolizumab	Phase 1/Phase 2

[00783] The study consists of 3 parts: Phase 1: Dose-escalation as a monotherapy using a 3+3 design, with Phase 1 Cohort Expansion; Phase 1b: Dose-escalating as a combination with pembrolizumab using a 3+3 design, with Phase 1b cohort Expansion; Phase 2: Efficacy Expansion using a group sequential design.

[00784] In phase 2, DF-hIL-12-Fc si is evaluated as a monotherapy in the following indications: Cohort 2A: Advanced (unresectable or metastatic) melanoma; Cohort 2B: Advanced (unresectable or metastatic) renal cell carcinoma (RCC).

[00785] In phase 2, DF-hIL-12-Fc si is evaluated in combination with pembrolizumab in the following indication: Cohort C: Advanced (unresectable or metastatic) urothelial carcinoma.

[00786] In each study phase, patients receive DF-hIL-12-Fc si on Day 1 every 3 weeks (Q3W). Patients receive DF-hIL-12-Fc si until confirmed progressive disease (PD), unacceptable toxicity (i.e., dose-limiting toxicity [DSL]), or any reason for withdrawal from the study or Investigational Medicinal Product (IMP) occurs.

[00787] The arms and interventions are presented in Table 75 below.

Table 75: Arms and Interventions

Arm	Intervention/Treatment
Experimental DF-hIL-12-Fc si Monotherapy Dose Escalation; 3+3 dose escalation of subcutaneous DF-hIL-12-Fc si as monotherapy in patients with solid tumors.	Biological/Vaccine DF-hIL-12-Fc si; DF-hIL-12-Fc si is a monovalent human interleukin-12 (IL-12) constant fragment (Fc) fusion protein that binds to the IL-12 receptor.
Experimental: DF-hIL-12-Fc si Monotherapy Expansion (Melanoma); Dose expansion of up to 40 patients with melanoma receiving subcutaneous DF-hIL-12-Fc si as monotherapy.	Biological/Vaccine: DF-hIL-12-Fc si; DF-hIL-12-Fc si is a monovalent human interleukin 12 (IL-12)-constant fragment (Fc) fusion protein that binds to the IL-12 receptor
Experimental: DF-hIL-12-Fc si Monotherapy Expansion (Renal Cell); Dose expansion of up to 40 patients with renal cell carcinoma receiving subcutaneous DF-hIL-12-Fc si as monotherapy	Biological/Vaccine: DF-hIL-12-Fc si; DF-hIL-12-Fc si is a monovalent human interleukin 12 (IL-12)-constant fragment (Fc) fusion protein that binds to the IL-12 receptor
Experimental: DF-hIL-12-Fc si In Combination with Keytruda Escalation; 3+3 dose escalation of subcutaneous DF-hIL-12-Fc si in combination with intravenous Keytruda.	Biological/Vaccine: DF-hIL-12-Fc si; DF-hIL-12-Fc si is a monovalent human interleukin-12 (IL-12) constant fragment (Fc) fusion protein that binds to the IL-12 receptor; Biological/Vaccine: Pembrolizumab; Pembrolizumab is a potent and highly selective humanized mAb of the Immunoglobulin (IgG4)/kappa isotype designed to directly block the interaction between PD-1 and its ligands, PD-L1 and PD-L2; Other names: Keytruda
Experimental: DF-hIL-12-Fc si in Combination with Keytruda Expansion (Urothelial); Dose expansion of up to 40 patients with urothelial carcinoma receiving subcutaneous DF-hIL-12-Fc si in combination with intravenous Keytruda.	Biological/Vaccine: DF-hIL-12-Fc si; DF-hIL-12-Fc si is a monovalent human interleukin-12 (IL-12)-constant fragment (Fc) fusion protein that binds to the IL-12 receptor; Biological/Vaccine: Pembrolizumab; Pembrolizumab is a potent and highly selective humanized mAb of the Immunoglobulin (IgG4)/kappa isotype designed to directly block the interaction between PD-1 and its ligands, PD-L1 and PD-L2; Other names: Keytruda

[00788] The primary outcome measures include: 1. Assessment of the number of dose limiting toxicities experienced on study with monotherapy DF-hIL-12-Fc si as defined per criteria in the study protocol [Time Frame: First 3 weeks on treatment for each subject]; To assess the number of adverse events experienced during treatment with monotherapy DF-hIL-12-Fc si that meet dose limiting toxicity criteria per the study protocol; 2. Assessment of the number of dose limiting toxicities experienced on study with combination therapy of DF-hIL-12-Fc si and pembrolizumab as defined per criteria in the study protocol [Time Frame: First 3 weeks on treatment for each subject in the combination therapy cohort]; To assess the number of adverse events experienced during treatment with combination therapy of DF-hIL-12-Fc si and pembrolizumab that meet dose limiting toxicity criteria per the study protocol; 3. Assess overall response rate [Time Frame: Through 90 days after completion of the study, an average of 1 year]; To assess the Overall Response Rate (ORR) per RECIST version 1.1 criteria of patients in the Phase 2 expansion cohorts.

[00789] The secondary outcome measures include: 1. Assess number of treatment emergent adverse events throughout study [Time Frame: Until 30 days after the last treatment of the last subject enrolled in the Phase 2 portion of the study]; Characterize the safety profile of DF-hIL-12-Fc si by assessing the number of adverse events occurring while on treatment with DF-hIL-12-Fc si; 2. Determine serum concentrations of DF-hIL-12-Fc si at various timepoints [Time Frame: From start of treatment up through 28 days after last treatment]; Concentration vs time of DF-hIL-12-Fc si will be measured using blood samples taken at various time points on study; 3. Assess Duration of Response [Time Frame: From time of initiation of therapy until the date of first documented tumor progression, assessed up to 24 months]; To assess duration of response using RECIST 1.1 criteria; 4. Assess Best Overall Response [Time Frame: Through 90 days after completion of the study, an average of 1 year]; To assess best overall response using RECIST 1.1 criteria; 5. Assess Overall Survival [Time Frame: Time from enrollment in the study until death, measured up to 2 years after last treatment on study]; To assess overall survival following treatment; 6. Assess Overall Response Rate [Time Frame: Time from enrollment in the study until up to 2 years after last treatment on study]; To assess overall response rate according to Investigator judgment.

Eligibility Criteria

[00790] The inclusion criteria (general phase 1) are: 1. Signed written informed consent; 2. Male or female patients aged ≥ 18 years; 3. Histologically or cytologically proven locally advanced or metastatic solid tumors, for which no standard therapy exists, or standard therapy has failed; 4. ECOG performance status of 0 or 1 at study entry and an estimated life expectancy of at least 3

months; 5. Clinical or radiological evidence of disease; 6. Archived tumor biopsy available, not older than 6 months, at least 8 slides available; or optional fresh biopsy obtained within the Screening window; 7. Adequate hematological function defined by white blood cell (WBC) count $\geq 3 \times 10^9/L$ with absolute neutrophil count (ANC) $\geq 1.5 \times 10^9/L$, lymphocyte count $\geq 0.5 \times 10^9/L$, platelet count $\geq 75 \times 10^9/L$, and hemoglobin ≥ 9 g/dL (may have been transfused); 8. Adequate hepatic function defined by a total bilirubin level $\leq 1.5 \times$ the upper limit of normal (ULN), an AST level $\leq 2.5 \times$ ULN, and an ALT level $\leq 2.5 \times$ ULN or, for patients with documented metastatic disease to the liver, AST and ALT levels $\leq 5 \times$ ULN; 9. Adequate renal function defined by an estimated creatinine clearance 50 ml/min according to the Cockcroft-Gault formula; 10. Experienced resolution of toxic effect(s) of the most recent prior anti-cancer therapy to $\leq$ Grade 1 (except alopecia) per NCI CTCAE v5.0 If a patient underwent major surgery or radiation therapy of > 30 Gray, the patient must have recovered from the toxicity and/or complications from the intervention (Patients with $\leq$ Grade 2 neuropathy or $\leq$ Grade 2 alopecia are an exception to this criterion and may qualify for the study.); 11. Effective contraception for women of child bearing potential (WOCBP) patients as defined by World Health Organization (WHO) guidelines for 1 "highly effective" method or 2 "effective" methods; 12. Eligible to receive pembrolizumab per its approved label. (Combination cohorts only.).

[00791] Additional Phase 1 Monotherapy Expansion Inclusion Criteria are: 1. Has one of the following tumor types: melanoma, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), head and neck squamous cell carcinoma (HNSCC), classical Hodgkin lymphoma, primary mediastinal large B-Cell lymphoma, urothelial carcinoma, micro satellite instability high cancer, gastric cancer, oesophageal cancer, cervical cancer, hepatocellular cancer, Merkel cell carcinoma, renal cell carcinoma, endometrial cancer, cutaneous T cell lymphoma, or triple negative breast cancer; 2. Measurable disease, as determined by the Investigator using RECIST, version 1.1; 3. Agrees to undergo a pre-treatment biopsy and another biopsy while on treatment; 4. Has a clinical/radiological presentation of their disease consistent with the execution of a pre-treatment biopsy and another biopsy while on treatment.

[00792] Additional Phase 1b Combination Therapy Expansion Cohort Criteria are: 1. Measurable disease, as determined by the Investigator using RECIST, version 1.1; 2. Agrees to undergo a pre-treatment biopsy and another biopsy while on treatment; 3. Has a clinical/radiological presentation of their disease consistent with the execution of a pre-treatment biopsy and another biopsy while on treatment.

[00793] Inclusion criteria (General Phase 2) are: 1. Signed written informed consent; 2. Male or female patients aged ≥ 18 years; 3. ECOG performance status of 0 or 1 at study entry and an estimated life expectancy of at least 3 months; 4. Measurable disease, as determined by the Investigator using RECIST, version 1.1; 5. Agrees to undergo a pre-treatment biopsy and another biopsy while on treatment; 6. Adequate hematological function defined by WBC count $\geq 3 \times 10^9/L$ with ANC $\geq 1.5 \times 10^9/L$, lymphocyte count $\geq 0.5 \times 10^9/L$, platelet count $\geq 75 \times 10^9/L$, and hemoglobin ≥ 9 g/dL (may have been transfused); 7. Adequate hepatic function defined by a total bilirubin level $\leq 1.5 \times \text{ULN}$, an AST level $\leq 2.5 \times \text{ULN}$, and an ALT level $\leq 2.5 \times \text{ULN}$ or, for patients with documented metastatic disease to the liver, AST and ALT levels $\leq 5 \times \text{ULN}$; 8. Adequate renal function defined by an estimated creatinine clearance > 50 ml/min according to the Cockcroft-Gault formula; 9. Experienced resolution of toxic effect(s) of the most recent prior anti-cancer therapy to Grade ≤ 1 (except alopecia) per NCI CTCAE v5.0 If a patient underwent major surgery or radiation therapy of > 30 Gray, the patient must have recovered from the toxicity and/or complications from the intervention (Patients with $\leq$ Grade 2 neuropathy or $\leq$ Grade 2 alopecia are an exception to this criterion and may qualify for the study.); 10. Has a clinical/radiological presentation of their disease consistent with the execution of a pre-treatment biopsy and another biopsy while on treatment; 11. Effective contraception for WOCBP patients as defined by WHO guidelines for 1 "highly effective" method or 2 "effective" methods.

[00794] Additional Phase 2 Inclusion Criteria (Melanoma only) are: 1. Received treatment with an anti PD-1 antibody for at least 6 weeks; 2. Have a confirmation of PD at least 4 weeks after the initial diagnosis of PD while receiving an anti PD-1 is made. Confirmation of PD can be based on radiological or clinical observations; 3. Must have received a BRAF inhibitor if the tumor carries a BRAF activating mutation and have progressed after the last line of treatment.

[00795] Additional Phase 2 Inclusion Criteria (Renal Cell only) are: 1. Any clear cell histology component; 2. Received treatment with an anti PD-1 /PD-L 1 antibody or an anti-vascular endothelial growth factor therapy as monotherapy or in combination; 3. Received ≤ 3 prior lines of therapy.

[00796] Additional Phase 2 Inclusion Criteria (Urothelial Carcinoma only) are: 1. Histologically or cytologically documented locally advanced or metastatic transitional cell carcinoma of the urothelium (including renal pelvis, ureters, urinary urothelial, urethra); 2. Must have received one (and no more than one) platinum-containing regimen (e.g., platinum plus another agent such as gemcitabine, methotrexate, vinblastine, doxorubicin) for inoperable locally advanced or metastatic urothelial carcinoma with radiographic progression or with recurrence within 6 months after the

last administration of a platinum-containing regimen as an adjuvant, which would be considered failure of a first-line, platinum-containing regimen; 3. Have received no more than 2 lines of therapy (including the platinum-containing regimen) for the treatment of the metastatic disease; 4. Must NOT have received treatment with a CPI (i.e., anti-PD-1 or anti-PD-L1 as a monotherapy or in combination with a platinum-based chemotherapy.

[00797] The Exclusion Criteria are: 1. Concurrent treatment with a non-permitted drug; 2. Prior treatment with rhIL2 or any recombinant long acting drug containing an IL2 moiety; 3. Concurrent anticancer treatment (eg, cytoreductive therapy, radiotherapy [with the exception of palliative bone directed radiotherapy], immune therapy, or cytokine therapy except for erythropoietin), major surgery (excluding prior diagnostic biopsy), concurrent systemic therapy with steroids or other immunosuppressive agents, or use of any investigational drug within 28 days before the start of study treatment. Short-term administration of systemic steroids (ie, for allergic reactions or the management of irAEs) is allowed; Note: Patients receiving bisphosphonates are eligible provided treatment was initiated at least 14 days before the first dose of DF-hIL-12-Fc si; 4. Previous malignant disease other than the target malignancy to be investigated in this study within the last 3 years, with the exception of basal or squamous cell carcinoma of the skin, localized prostate cancer or cervical carcinoma in situ; 5. Rapidly progressive disease; 6. Any Grade 2 and higher neurological or pulmonary toxicity during a treatment with an anti-PD-1 or PD-L1 agent administered as a monotherapy; 7. Active or history of central nervous system (CNS) metastases. Melanoma patients with brain metastasis(es) are eligible if they have been stable for 4 weeks after treatment; 8. Receipt of any organ transplantation including autologous or allogeneic stem-cell transplantation; 9. Significant acute or chronic infections (including historic positive test for human immunodeficiency virus [HIV], or active or latent hepatitis B or active hepatitis C tested during the Screening window); 10. Preexisting autoimmune disease (except for patients with vitiligo) needing treatment with systemic immunosuppressive agents for more than 28 days within the last 3 years or clinically relevant immunodeficiencies (eg, dys-gammaglobulinemia or congenital immunodeficiencies), or fever within 7 days of Day 1; 11. Known severe hypersensitivity reactions to monoclonal antibodies (mAbs) ($\geq$ Grade 3 NCI CTCAE v5.0), any history of anaphylaxis, or uncontrolled asthma (ie, 3 or more features of partly controlled asthma); 12. Persisting toxicity related to prior therapy $\geq$ Grade 2 NCI CTCAE v5.0, however alopecia and sensory neuropathy $\leq$ Grade 2 is acceptable; 13. Pregnancy or lactation in females during the study; 14. Known alcohol or drug abuse; 15. Serious cardiac illness or medical conditions including but not limited to: a. History of New York Heart Association class III or IV heart failure or systolic dysfunction (left

ventricular ejection fraction [LVEF] <55%); b. High-risk uncontrolled arrhythmias ie, tachycardia with a heart rate >100/min at rest; c. Significant ventricular arrhythmia (ventricular tachycardia) or higher-grade atrioventricular (AV)-block (second degree AV-block Type 2 [Mobitz 2] or third-degree AV-block); d. Angina pectoris requiring anti-anginal medication; e. Clinically significant valvular heart disease; f. Evidence of transmural infarction on ECG; g. Poorly controlled hypertension (defined by: systolic >180 mm Hg or diastolic >100 mmHg); h. Clinically relevant uncontrolled cardiac risk factors, clinically relevant pulmonary disease or any clinically relevant medical condition in the opinion of the Investigator that may limit participation in this study; 16. All other significant diseases (e.g., inflammatory bowel disease), which, in the opinion of the Investigator, might impair the patient's ability to participate; 17. Any psychiatric condition that would prohibit the understanding or rendering of informed consent; 18. Legal incapacity or limited legal capacity; 19. Incapable of giving signed informed consent, which includes compliance with the requirements and restrictions listed in the informed consent form (ICF) and in this protocol.

Example 29: Treatment of Cancer using DF hIL12-Fc si

Objectives

[00798] This clinical study is designed with the following phases: Phase 1, Phase 1b, and Phase 2.

[00799] The primary objective of Phase 1 is to assess the safety and tolerability of DF hIL12-Fc si (also referred to as DF6002) as monotherapy, and to determine the maximum tolerated dose (MTD) of DF hIL12-Fc si in patients with advanced (unresectable, recurrent or metastatic) solid tumors.

[00800] The primary objective of Phase 1b is to assess the safety and tolerability of DF hIL12-Fc si in combination with Nivolumab, and to determine the maximum tolerated dose (MTD) of DF hIL12-Fc si in combination with Nivolumab in patients with advanced (unresectable, recurrent, or metastatic) solid tumors.

[00801] The primary objective of Phase 2 is to assess the Objective Response Rate (ORR) according to the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1) per an Independent Endpoint Review Committee (IERC), for all Efficacy Expansion Cohorts testing the clinical activity of DF hIL12-Fc si as a monotherapy or in combination.

[00802] The secondary objectives of Phase 1 and Phase 1b, with DF hIL12-Fc si as monotherapy and in combination with Nivolumab, are to: characterize the PK of DF hIL12-Fc si; evaluate immunogenicity of DF hIL12-Fc si, and to correlate its exposure and clinical activity; assess best overall response (BOR), as determined by the Investigator for DF hIL12-Fc si using RECIST 1.1;

assess best overall response (BOR), as determined by the Investigator for DF hIL12-Fc si using RECIST 1.1; assess duration of response (DOR) of DF hIL12-Fc si, using RECIST 1.1; assess progression-free survival (PFS) for DF hIL12-Fc si, using RECIST 1.1; assess overall survival (OS) time.

[00803] The secondary objectives of Phase 2, with DF hIL12-Fc si as monotherapy and in combination with Nivolumab, are to: characterize the PK of DF hIL12-Fc si; assess duration of response (DOR) of DF hIL12-Fc si, per an IERC using RECIST 1.1; assess clinical benefit rate (CBR) of DF hIL12-Fc si using RECIST 1.1. CBR is defined as the percentage of patients with complete response (CR), partial response (PR), or stable disease (SD) as best response; assess the safety of DF hIL12-Fc si; evaluate the immunogenicity of DF hIL12-Fc si, and correlate with exposure and clinical activity; assess progression-free survival (PFS) for DF hIL12-Fc si, per an IERC using RECIST 1.1; and assess overall survival (OS) time.

[00804] The arms and interventions are presented in Table 76 below:

Table 76: Arms and Interventions

Arm	Intervention/Treatment
Experimental: DF6002 Monotherapy Dose Escalation; 3+3 dose escalation of subcutaneous DF6002 as monotherapy in patients with solid tumors.	Biological: DF6002; DF6002 is a monovalent human interleukin-12 (IL12)-constant fragment (Fc) fusion protein that binds to the IL12 receptor.
Experimental: DF6002 Monotherapy Expansion (Melanoma); Dose expansion of up to 40 patients with melanoma receiving subcutaneous DF6002 as monotherapy.	Biological: DF6002; DF6002 is a monovalent human interleukin-12 (IL12)-constant fragment (Fc) fusion protein that binds to the IL12 receptor.
Experimental: DF6002 Monotherapy Expansion (NSCLC); Dose expansion of up to 40 patients with non-small cell lung cancer receiving subcutaneous DF6002 as monotherapy.	Biological: DF6002; DF6002 is a monovalent human interleukin-12 (IL12)-constant fragment (Fc) fusion protein that binds to the IL12 receptor
Experimental: DF6002 In Combination with Opdivo Escalation; 3+3 dose escalation of subcutaneous DF6002 in combination with intravenous Opdivo.	Biological: DF6002 DF6002 is a monovalent human interleukin-12 (IL12)-constant fragment (Fc) fusion protein that binds to the IL12 receptor. Biological: Nivolumab Nivolumab is a human immunoglobulin G4 (IgG4) monoclonal antibody, which binds to the programmed death-1 receptor (PD-1). Other Name: Opdivo
Experimental: DF6002 in Combination with Opdivo Expansion (Melanoma) Dose expansion of up to 40 patients with melanoma receiving subcutaneous DF6002 in combination with intravenous Opdivo.	Biological: DF6002 DF6002 is a monovalent human interleukin-12 (IL12)-constant fragment (Fc) fusion protein that binds to the IL12 receptor. Biological: Nivolumab

	Nivolumab is a human immunoglobulin G4 (IgG4) monoclonal antibody, which binds to the programmed death-1 receptor (PD-1). Other Name: Opdivo
Experimental: DF6002 in Combination with Opdivo Expansion (NSCLC) Dose expansion of up to 40 patients with non-small cell lung cancer receiving subcutaneous DF6002 in combination with intravenous Opdivo.	Biological: DF6002 DF6002 is a monovalent human interleukin-12 (IL12)-constant fragment (Fc) fusion protein that binds to the IL12 receptor. Biological: Nivolumab Nivolumab is a human immunoglobulin G4 (IgG4) monoclonal antibody, which binds to the programmed death-1 receptor (PD-1). Other Name: Opdivo

[00805] The primary outcome measures include:

[00806] 1. Assessment of the number of dose limiting toxicities experienced on study with monotherapy DF6002 as defined per criteria in the study protocol [Time Frame: First 3 weeks on treatment for each subject.] To assess the number of adverse events experienced during treatment with monotherapy DF6002 that meet dose limiting toxicity criteria per the study protocol;

[00807] 2. Assessment of the number of dose limiting toxicities experienced on study with combination therapy of DF6002 and nivolumab as defined per criteria in the study protocol [Time Frame: First 3 weeks on treatment for each subject in the combination therapy cohort.] To assess the number of adverse events experienced during treatment with combination therapy of DF6002 and nivolumab that meet dose limiting toxicity criteria per the study protocol; and

[00808] 3. Assess overall response rate [Time Frame: Through 90 days after completion of the study, an average of 1 year.] To assess the Overall Response Rate (ORR) per RECIST version 1.1 criteria of patients in the Phase 2 expansion cohorts. The secondary outcome measures include: 1. Assess number of treatment emergent adverse events throughout study [Time Frame: Until 30 days after the last treatment of the last subject enrolled in the Phase 2 portion of the study.] Characterize the safety profile of DF6002 by assessing the number of adverse events occurring while on treatment with DF6002; 2. Determine serum concentrations of DF6002 at various timepoints [Time Frame: From start of treatment up through 28 days after last treatment] Concentration vs time of DF6002 will be measured using blood samples taken a various time points on study;

[00809] 4. Assess Duration of Response [Time Frame: From time of initiation of therapy until the date of first documented tumor progression, assessed up to 24 months] To assess duration of response using RECIST 1.1 criteria;

[00810] 5. Assess Best Overall Response [Time Frame: Through 90 days after completion of the study, an average of 1 year] To assess best overall response using RECIST 1.1 criteria;

[00811] 6. Assess Overall Survival [Time Frame: Time from enrollment in the study until death, measured up to 2 years after last treatment on study] To assess overall survival following treatment; and

[00812] 7. Assess Overall Response Rate [Time Frame: Time from enrollment in the study until up to 2 years after last treatment on study] To assess overall response rate according to Investigator judgment.

[00813] The inclusion criteria (General Phase 1 and Phase 1b) include: 1. Male or female patients aged ≥ 18 years; 2. Histologically or cytologically proven locally advanced or metastatic solid tumors, for which no standard therapy exists or standard therapy has failed among the following tumor types: melanoma, non-small cell lung cancer, small cell lung cancer, head and neck squamous cell, urothelial, gastric, esophageal, cervical, hepatocellular, merkel cell, cutaneous squamous cell carcinoma, renal cell, endometrial, triple-negative breast, ovarian, and prostate; 3. ECOG performance status of 0 or 1; 4. Clinical or radiological evidence of disease; 5. Adequate hematological, hepatic and renal function; 6. Resolution of toxic effect(s) of prior anti-cancer therapy to $\leq$ Grade 1 (Patients with $\leq$ Grade 2 neuropathy, $\leq$ Grade 2 endocrinopathy or $\leq$ Grade 2 alopecia are exceptions); and 7. Effective contraception for women of child-bearing potential as defined by World Health Organization guidelines for 1 "highly effective" method or 2 "effective" methods.

[00814] Additional Phase 1 Monotherapy and Phase 1b Combination With Nivolumab Expansion Inclusion Criteria include: 1. Has one of the following tumor types: melanoma, non-small cell lung cancer, or triple negative breast cancer; and 2. Agrees to undergo a pre-treatment biopsy and another biopsy while on treatment.

[00815] Expansion Inclusion Criteria specific to Melanoma include: 1. Histologically confirmed, unresectable Stage III or Stage IV melanoma, as specified in the American Joint Committee on Cancer staging system; 2. Participants with ocular or uveal melanoma are ineligible; 3. PD-L1 status must be documented if available; 4. BRAF (V600) mutation status must be known. Both BRAF-mutated and wild type participants are permitted in this cohort; 5. BRAF-mutated participants must have been treated with approved targeted therapies; 6. Must

have documented progressive or recurrent disease on or after discontinuation of anti-PD-(L)1 therapy (administered as monotherapy or as part of a combination) as per RECIST 1.1 criteria; and 7. Participants who received anti-PD-(L)1 in the adjuvant setting must have documented progressive or recurrent disease on or within 6 months of discontinuation of anti-PD-(L)1 therapy (administered as monotherapy or as part of a combination) as per RECIST 1.1 criteria.

[00816] Expansion Inclusion Criteria specific to NSCLC include: 1. Histologically confirmed NSCLC meeting stage criteria for stage IIIB, stage IV, or recurrent disease; 2. Participants must have recurrent or progressive disease during or after platinum doublet-based chemotherapy or at least two prior lines of systemic therapy for advanced or metastatic disease OR Must have recurrent or progressive disease within 6 months after completing platinum-based chemotherapy for local disease; 3. Participants must have received and progressed on or after anti-PD-(L)1 therapy, if available; and 4. Status for actionable mutations (e.g., EGFR, ALK, ROS1, RET, etc.) must be known (when testing is available as per country/region standard of care practices); participants with actionable mutations must have received and progressed on, have been intolerant to, or not be a candidate for, standard tyrosine kinase inhibitors (as available per country/region standard of care practices).

[00817] Expansion Inclusion Criteria specific to TNBC include: 1. Histologically confirmed unresectable, locally advanced or metastatic triple negative breast cancer. PD-L1 status, HER2-negative, estrogen receptor-negative, and progesterone receptor-negative status must be evaluated by local institutions before enrolment per guidelines of the American Society of Clinical Oncology and the College of American Pathologists; 2. Patients must not have received an anti PD-1/PD-L1 for the treatment of the metastatic disease, but the administration of an anti PD-1/PD-L1 in the adjuvant setting is acceptable; 3. Patients must have received one line of chemotherapy for the treatment of their metastatic disease, and experience progression during or after that line of chemotherapy; and 4. Patients must have not received more than one line of chemotherapy for the treatment of their unresectable, recurrent or metastatic disease.

[00818] Inclusion Criteria for Phase 2 (General) include: 1. Male or female patients aged ≥ 18 years; 2. ECOG performance status of 0 or 1; 3. Clinical or radiological evidence of measurable disease; 4. Adequate hematological, hepatic and renal function; 5. Resolution of toxic effect(s) of prior anti-cancer therapy to $\leq$ Grade 1. (Patients with $\leq$ Grade 2 neuropathy, $\leq$ Grade 2 endocrinopathy or $\leq$ Grade 2 alopecia are exceptions.); 6. Participants must have received and progressed on or after anti-PD-(L)1 therapy; and 7. Effective contraception for women of child-

bearing potential as defined by World Health Organization guidelines for 1 "highly effective" method or 2 "effective" methods.

[00819] Additional Inclusion Criteria for Phase 2 (Advanced Melanoma Patients) include: 1. Participants who received anti-PD-(L)1 in the advanced/metastatic setting, must have documented progressive or recurrent disease on or within 3 months of discontinuation of anti-PD-(L)1 therapy; 2. Participants who received anti-PD-(L)1 in the adjuvant setting must have documented progressive or recurrent disease on or within 6 months of discontinuation of anti-PD-(L)1 therapy; 3. Disease progression was confirmed at least 4 weeks after the initial diagnosis of disease progression while receiving an anti PD-1 antibody; 4. BRAF mutation status must be known and treated with approved targeted therapies; 5. Received a BRAF inhibitor if the tumor carries a BRAF activating mutation and progressed after the last line of treatment; 6. Participants with ocular or uveal melanoma are ineligible; and 7. Confirmation of radiographic progression on prior anti-PD-(L)1 therapy is required with a scan confirming progression at least 4 weeks after the initial progression.

[00820] Additional Inclusion Criteria for Phase 2 (Non-small Cell Lung Cancer) include: 1. Participants must have recurrent or progressive disease during or after platinum doublet-based chemotherapy or at least two prior lines of systemic therapy for advanced or metastatic disease OR must have recurrent or progressive disease within 6 months after completing platinum-based chemotherapy for local disease; and 2. Status for actionable mutations must be known; participants with actionable mutations must have received and progressed on, have been intolerant to, or not be a candidate for, standard tyrosine kinase inhibitors.

[00821] Exclusion Criteria for All Patients (All Phases): 1. Prior treatment with rhIL2 or any recombinant long acting drug containing an IL2 moiety; 2. Concurrent anticancer treatment (with the exception of palliative bone directed radiotherapy), immune therapy, or cytokine therapy (except for erythropoietin), major surgery (excluding prior diagnostic biopsy), concurrent systemic therapy with steroids or other immunosuppressive agents, or use of any investigational drug within 28 days before the start of study treatment; 3. Previous malignant disease other than the current target malignancy within the last 3 years, with the exception of basal or squamous cell carcinoma of the skin, localized prostate cancer or cervical carcinoma in situ; 4. Rapidly progressive disease; 5. Any Grade 2 and higher neurological or pulmonary toxicity during a treatment with an anti-PD-1 or PD-L1 agent administered as a monotherapy; 6. Active or history of central nervous system (CNS) metastases unless all of the following criteria are met: a. CNS lesions are asymptomatic and previously treated; b. Patient does not require ongoing steroid

treatment daily for replacement for adrenal insufficiency; c. Imaging demonstrates stability of disease 28 days from last treatment for CNS metastases; 7. Receipt of any organ transplantation, autologous or allogeneic stem-cell transplantation; 8. Significant acute or chronic infections, or active or latent hepatitis B or active hepatitis C; 9. Preexisting autoimmune disease needing treatment with systemic immunosuppressive agents for more than 28 days within the last 3 years, clinically relevant immunodeficiencies, or fever within 7 days of Day 1; 10. Known severe hypersensitivity reactions to monoclonal antibodies and any history of anaphylaxis, or uncontrolled asthma; 11. Serious cardiac illness or medical conditions; and 12. History of life-threatening toxicity related to prior immune therapy except those that are unlikely to re-occur with standard countermeasures.

Exploratory Objectives

[00822] The exploratory objectives, both with DF hIL12-Fc si as monotherapy and in combination with Nivolumab, are to: evaluate changes from baseline in tumor and peripheral biomarkers, and the relationship to PK; assess the PK of Nivolumab (Phase 1b and Cohort 2C only); evaluate the activity of DF hIL12-Fc si in the Efficacy Expansion Cohorts Part (Phase 2) per Investigator assessment (ORR, DOR, CBR, and BOR, by RECIST); evaluate the association between tumor and peripheral biomarkers, and tumor response rate.

Study Design Overview

[00823] This study is a Phase 1/2, open-label, dose-escalation study with a consecutive parallel-group efficacy expansion study, designed to determine the safety, tolerability, PK, pharmacodynamics, and preliminary anti-tumor activity of DF hIL12-Fc si as monotherapy and in combination with Nivolumab. A schematic diagram of the study design is shown in **FIG. 52A** (for monotherapy) and **52B** (for combination therapy with Nivolumab).

[00824] The study consists of 3 parts: **Phase 1:** Dose-escalation as a monotherapy using a 3+3 design, with Phase 1 Cohort Expansion; **Phase 1b:** Dose-escalation as a combination with Nivolumab using a 3+3 design, with Phase 1b Cohort Expansion; **Phase 2:** Efficacy Expansion using a group sequential design.

[00825] DF hIL12-Fc si is evaluated as a monotherapy in Efficacy Expansion cohorts in the following indications: Cohort 2A: Advanced (unresectable or metastatic) melanoma; Cohort 2B: Advanced (unresectable or metastatic) renal cell carcinoma (RCC).

[00826] DF hIL12-Fc si is evaluated in combination with Nivolumab in an Efficacy Expansion cohort in the following indication: Cohort 2C: Advanced (unresectable or metastatic) urothelial carcinoma.

[00827] In each study phase, patients receive DF hIL12-Fc si on Day 1 every 4 weeks (Q4W). Patients receive DF hIL12-Fc si until confirmed progressive disease (PD), unacceptable toxicity (*i.e.*, dose-limiting toxicity [DLT]), or any reason for withdrawal from the study or Investigational Medicinal Product (IMP) occurs.

Phase 1 Dose Escalation DF hIL12-Fc si Monotherapy

[00828] The Phase 1 Dose-escalation Phase of the study is designed to determine the dose-limiting toxicities (DLTs) and maximum tolerated dose (MTD) of DF hIL12-Fc si as monotherapy using a standard 3+3 design.

[00829] The decision to escalate to the next dose level (DL) is based on safety assessments after all patients of a cohort have had safety evaluations performed through Cycle 1, Day 21 (C1D21), unless due to DLT. In order to assess the safety of DF hIL12-Fc si, a Safety Monitoring Committee (SMC), responsible for dose-escalation decisions, is established.

[00830] After the safety of Dose Level “n” has been established, the SMC has the option to permit enrollment in the Phase I expansion cohort up to that dose level; ; no more than 50 patients can be enrolled by this process.

[00831] The MTD is defined as the highest DL at which ≤ 1 patient of 6 evaluable patients experiences a DLT.

Phase 1b: Dose-escalation as a combination with Nivolumab

[00832] The Phase 1b Dose-escalation Phase of the study is designed to determine the DLTs and MTD of DF hIL12-Fc si when given in combination with nivolumab, using a standard 3+3 design, as described for Phase 1.

[00833] Nivolumab is administered once every 4 weeks (on Day 1 of each cycle) per its U.S. package insert. The administration of nivolumab precedes that of DF hIL12-Fc si.

[00834] DF hIL12-Fc si dose levels tested in combination with Nivolumab are the same as those tested as a monotherapy.

[00835] Phase 1b starts after the SMC has established the safety of DL2 monotherapy (defined as the agreement to initiate enrollment into DL3). Phase 1b starts at a DF hIL12-Fc si dose at least 1 level below the safe dose established with monotherapy at the time the Phase 1b is initiated.

[00836] After the safety of Dose Level “n” has been established, the SMC has the option to permit enrollment in the Phase 1b Expansion Cohort; no more than 50 patients can be enrolled by this process across dose levels.

Phase 2 Efficacy Expansion

[00837] The following tumor types are enrolled at the recommended phase 2 dose (RP2D): As a monotherapy: Cohort 2A: Advanced (unresectable or metastatic) melanoma; Cohort 2B: Advanced (unresectable or metastatic) renal cell carcinoma. In combination with Nivolumab, Cohort 2C: Advanced (unresectable or metastatic) urothelial carcinoma.

Safety Oversight

[00838] Male or female patients aged ≥ 18 years with an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 at study entry and an estimated life expectancy of at least 3 months are enrolled. Key inclusion criteria in each study phase/cohort are as follows:

Dose-Escalation cohorts in Phase 1/1b: histologically or cytologically proven locally advanced or metastatic solid tumors for which no standard therapy exists or for which standard therapy has failed in the following indications: melanoma, non-small cell lung (NSCLC), small cell lung, head and neck squamous cell, urothelial, gastric, esophageal, cervical, hepatocellular, merkel cell, cutaneous squamous cell carcinoma, renal cell, endometrial, triple negative breast (TNBC), ovarian, and prostate cancers; clinical or radiological evidence of disease.

Dose Expansion Cohorts in Phase 1/1b: histologically or cytologically proven locally advanced or metastatic solid tumors for which no standard therapy exists or for which standard therapy has failed; Has measurable disease, as determined by the Investigator using the Response Evaluation Criteria for Solid Tumors (RECIST), version 1.1.

Cohort 2A

Patients with advanced melanoma who: received treatment with an anti-programmed cell death protein 1 (PD-1) antibody for at least 6 weeks; have a confirmation of PD at least 4 weeks after the initial diagnosis of PD while receiving an anti PD-1 is made. Confirmation of PD can be based on radiological or clinical observations; must have received a BRAF inhibitor if the tumor carries a BRAF activating mutation and have progressed after the last line of treatment.

Cohort 2B

Patients with advanced RCC who: have any clear cell histology component; received treatment with an anti PD-1/PD-L1 antibody and an anti-vascular endothelial growth factor therapy as a monotherapy or in combination; received ≤ 3 prior lines of therapy.

Cohort 2C

Patients with advanced urothelial carcinoma who: have histologically or cytologically documented locally advanced or metastatic transitional cell carcinoma of the urothelium (including renal pelvis, ureters, urinary urothelial, urethra); have received one (and no more than one)

platinum-containing regimen (*e.g.*, platinum plus another agent such as gemcitabine, methotrexate, vinblastine, doxorubicin, etc.) for inoperable locally advanced or metastatic urothelial carcinoma with radiographic progression or with recurrence within 6 months after the last administration of a platinum-containing regimen as an adjuvant, which would be considered failure of a first-line, platinum-containing regimen; have received no more than 2 lines of therapy (including the platinum-containing regimen) for the treatment of the metastatic disease; have not received treatment with a checkpoint inhibitor (CPI) (*i.e.*, anti-PD-1 or anti-PD-L1 as a monotherapy or in combination with a platinum-based chemotherapy).

Dose/ Mode of Administration/ Dosing Schedule

[00839] DF hIL12-Fc si is administered as a subcutaneous (SC) injection Q4W (*i.e.*, on Day 1 of each cycle) in both monotherapy and combination cohorts. Patients receive the drug SC in a volume of not more than 1 mL in a maximum of 2 injection sites. The second administration is completed within 10 minutes after the completion of the first administration, if applicable.

[00840] In Phase 1/1b, patients are hospitalized for the night following the first administration of DF hIL12-Fc si.

[00841] The DF hIL12-Fc si DLs ($\mu\text{g/kg}$) are as follows in Table 77.

TABLE 77: DF hIL12-Fc si DLs ($\mu\text{g/kg}$)

	DL1	DL2	DL3	DL4	DL5	DL6	DL7	DL8	DL9	DL 10
Dose of DF hIL12-Fc si ($\mu\text{g/kg}$)	0.05	0.10	0.20	0.40	0.60	0.80	1.00	1.20	1.40	1.75
Equivalent IL12 ($\mu\text{g/kg}$)	0.021	0.053	0.105	0.211	0.316	0.421	0.526	0.632	0.737	0.921

[00842] The dose of DF hIL12-Fc si is calculated based on the weight of the patient at baseline. The patient's calculated dose is only recalculated if the patient's weight changes by 10% or more since the time of their last dose calculation.

[00843] In Phase 1b and Cohort 2C, Nivolumab is administered at a dose of 480 mg, once every 4 weeks (Q4W) via intravenous (IV) infusion, in accordance with the package insert. The administration of nivolumab precedes that of DF hIL12-Fc si. DF hIL12-Fc si is administered within 1 hour after the completion of the administration of nivolumab.

Efficacy Expansion as a Monotherapy (Cohorts 2A and 2B)

[00844] The primary endpoint for this phase is the ORR. For each of these cohorts, the null hypothesis is that the objective response rate (ORR) does not exceed 5% (H_0 : ORR < 5%) and the alternative hypothesis is that the ORR is greater than 5% (H_1 : ORR $\geq$ 5%).

[00845] The target ORR of DF hIL12-Fc si as a monotherapy is 20%. It is expected to enroll 40 patients for each of these cohorts (*i.e.*, approximately 80 patients in total).

[00846] Using a group sequential design, with 40 patients in each of the indication cohorts, the efficacy cohort provides ~90% study power to detect a 15% difference at a 1-sided overall type I error rate of 0.025, assuming the target ORR of 20% for DF hIL12-Fc si.

[00847] For each of Cohorts 2A and 2B, a futility interim analysis, with Lan-DeMets O'Brien Fleming boundary, is planned at 50% information fraction (*i.e.*, at ~20 patients).

Efficacy Expansion in Combination with Nivolumab (Cohort 2C)

[00848] The Phase 2 portion for efficacy expansion in combination with nivolumab determines the clinical activity of DF hIL12-Fc si in combination in patients with UBC who have progressed after one line of platinum-based chemotherapy.

[00849] The study enrolls 40 patients so that the observation of 14 responses (CR or PR) out of the 40 patients enroll will lead to a 95% CI (0.206; 0.517) that excludes the value of the percentage of responses reported for nivolumab in a similar population, that was enrolled in Checkmate 275. In that study, the ORR was 19.6% (Sharma P, Retz M, Siefker-Radtke A, Baron A, Necchi A, Bedke J, et al. Nivolumab in metastatic urothelial carcinoma after platin therapy (CheckMate 275): a multicentre, single-arm phase 2 trial. *Lancet Oncol.* 2017 Jan 25; S1470-2045(17):30065-7).

Exploratory Biomarkers

Peripheral Biomarkers

[00850] Peripheral biomarkers are assessed in the periphery in all patients, including: cellular parameters: peripheral blood mononuclear cell (PBMCs) for immunophenotyping (IPT) by flow cytometry; soluble factors: Cytokines and chemokines in serum samples; *ex vivo* IL12 response assay: PBMCs for *ex vivo* stimulation followed by analysis of IFN γ production; circulating tumor (ct) deoxyribonucleic acid (DNA).- Gene expression profile performed using Nanostring: total RNA isolated from peripheral blood collected during screening and on C1D15; IPT assessments are performed on PBMCs derived from whole blood samples collected 2 hours prior to administration of DF hIL12-Fc si C1 through C3 and at each of the following study visits: C1D3, C1D8, C2D8, and C3D3; Soluble factors are determined in serum samples collected within 2 hours

prior to DF hIL12-Fc si administration on D1 of each treatment cycle, and on C1D2, C1D3, C1D5, C1D8, C1D15, C2D3, C3D3, and C4D3, and at the EOT and SFU visits.

[00851] In order to complete all the assessments on tumor materials, blood (*e.g.*, whole blood, plasma, and serum samples), is collected from patients.

Biomarkers Derived From Tumor Tissue

[00852] Tissue derived biomarkers are evaluated on the pre-treatment and on treatment biopsies in patients participating in the Dose-escalation phase (optional biopsies), the Phase 1/1b Expansion Cohorts part (mandatory biopsies), and the Phase 2 Efficacy Expansion Cohorts phase (mandatory biopsies).

[00853] A panel of putative markers including molecular, soluble and cellular markers is analyzed at baseline from archived tumor tissue (or fresh tumor biopsy, if available), whole blood, and serum samples to investigate a possible correlation between clinical efficacy and analyzed markers.

[00854] For patients enrolled in the Dose-escalation Phase, the level of PD-L1 expression is determined using a commercially available kit (Dako PD-L1 IHC 22C3 pharmDx) and analysis of CD3 positivity (T cell infiltration) is determined by immunohistochemistry (IHC).

[00855] For patients enrolled in the Phase 1/1b Expansion Cohorts and the Efficacy Expansion Cohorts, fresh mandatory tumor biopsies are performed at Screening (*i.e.*, within 30 days before first study drug dose) and at pre-specified time points during the treatment period.

[00856] Other biomarkers that are assessed include: frequency and localization of tumor-infiltrated leukocytes (*e.g.*, CD8, CD4 T-cells, Treg, NK cells, macrophage [M1/2 profile] by IHC or IF), gene expression profile, and pharmacogenomics (PGx).

[00857] Germline DNA may be investigated on DNA extracted from whole blood and/or archival tumors. This extracted DNA is used for Whole Exome Sequencing and/or genotyping. For this purpose, an additional 6 mL of whole blood is collected at baseline (*i.e.*, prior to the first administration of study treatment) for all indications; no additional tumor samples are needed because a part of the archived tumor sample is used for the extraction of DNA to study tumor genetics if required.

INCORPORATION BY REFERENCE

[00858] The entire disclosure of each of the patent documents and scientific articles referred to herein is incorporated by reference for all purposes.

EQUIVALENTS

[00859] The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes that come within the meaning and range of equivalency of the claims are intended to be embraced therein.

WHAT IS CLAIMED IS:

1. A pharmaceutical formulation comprising:
 - (a) a heterodimeric Fc-fused protein comprising:
 - (i) a first polypeptide comprising a first antibody Fc domain polypeptide and a first subunit of a multisubunit cytokine; and
 - (ii) a second polypeptide comprising a second antibody Fc domain polypeptide and a second, different subunit of the multisubunit cytokine,
 - (b) citrate;
 - (c) a sugar;
 - (d) a sugar alcohol; and
 - (e) a non-ionic surfactant,at pH 6.0 to 7.0,
wherein the first and second antibody Fc domain polypeptides each comprise different mutations promoting heterodimerization, and
wherein the first subunit and second, different subunit of the multisubunit cytokine are bound to each other.
2. The pharmaceutical formulation of claim 1, wherein the first and/or second antibody Fc domain polypeptides comprise one or more mutation(s) that reduce(s) an effector function of an Fc.
3. The pharmaceutical formulation of claim 1 or 2, wherein the concentration of citrate in the pharmaceutical formulation is about 10 mM to about 30 mM.
4. The pharmaceutical formulation of claim 3, wherein the concentration of citrate in the pharmaceutical formulation is about 20 mM.
5. The pharmaceutical formulation of any one of claims 1-4, wherein the concentration of the sugar in the pharmaceutical formulation is about 3% to about 12% (w/v).
6. The pharmaceutical formulation of claim 5, wherein the concentration of the sugar in the pharmaceutical formulation is about 6% (w/v).

7. The pharmaceutical formulation of claim 5 or 6, wherein the sugar is a disaccharide.
8. The pharmaceutical formulation of claim 7, wherein the disaccharide is sucrose.
9. The pharmaceutical formulation of any one of claims 1-8, wherein the concentration of the sugar alcohol in the pharmaceutical formulation is between about 0.5% to about 6% (w/v).
10. The pharmaceutical formulation of claim 9, wherein the concentration of the sugar alcohol in the pharmaceutical formulation is about 1% (w/v).
11. The pharmaceutical formulation of any one of claims 1-10, wherein the sugar alcohol is derived from a monosaccharide.
12. The pharmaceutical formulation of claim 11, wherein the sugar alcohol is mannitol.
13. The pharmaceutical formulation of any one of claims 1-12, wherein the concentration of the non-ionic surfactant in the pharmaceutical formulation is between about 0.005% to about 0.02% (w/v).
14. The pharmaceutical formulation of claim 13, wherein the concentration of polysorbate 80 in the pharmaceutical formulation is about 0.01% (w/v).
15. The pharmaceutical formulation of claim 13 or 14 wherein the non-ionic surfactant is a polysorbate.
16. The pharmaceutical formulation of claim 15, wherein the polysorbate is polysorbate 80.
17. The pharmaceutical formulation of any one of claims 1-16, wherein the pH is between about 6.1 and about 6.9.
18. The pharmaceutical formulation of claim 17, wherein the pH is between about 6.2 and about 6.8.

19. The pharmaceutical formulation of claim 18, wherein the pH is between about 6.3 and about 6.7.
20. The pharmaceutical formulation of claim 19, wherein the pH is between about 6.4 and about 6.6.
21. The pharmaceutical formulation of claim 20, wherein the pH is about 6.5.
22. The pharmaceutical formulation of any one of claims 1-21, further comprising water.
23. The pharmaceutical formulation of claim 22, wherein the water is Water for Injection, USP.
24. The pharmaceutical formulation of any one of claims 1-23, wherein the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 1 g/L to about 10 g/L.
25. The pharmaceutical formulation of claim 24, wherein the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 2 g/L to about 8 g/L.
26. The pharmaceutical formulation of claim 25, wherein the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 4 g/L to about 6 g/L.
27. The pharmaceutical formulation of claim 26, wherein the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about about 5 g/L.
28. The pharmaceutical formulation of any one of claims 1-27, wherein the pharmaceutical formulation comprises a concentration of the protein for administration of about 0.5 g/L to about 1.5 g/L.
29. The pharmaceutical formulation of claim 28, wherein the pharmaceutical formulation comprises a concentration of the protein for administration of about 0.75 g/L to about 1.25 g/L.

30. The pharmaceutical formulation of claim 29, wherein the pharmaceutical formulation comprises a concentration of the protein for administration of about 1 g/L.
31. The pharmaceutical formulation of any one of 1-30, wherein the formulation is designed to be stored at a temperature between about 2°C and about 8°C.
32. The pharmaceutical formulation of any one of claims 1-31, wherein the pharmaceutical formulation is a clear, colorless solution and free of visible particulates.
33. The pharmaceutical formulation of any one of claims 1-32, wherein the formulation has a thermal stability profile as defined by:
- (a) a T_{m1} of greater than about 60°C, greater than about 61°C, greater than about 62°C, greater than about 63°C, greater than about 64°C, greater than about 65°C, or greater than about 66°C; and/or
 - (b) a T_{m2} of greater than about 70°C, greater than about 71°C, greater than about 72°C, greater than about 73°C, greater than about 74°C, greater than about 75°C, greater than about 76°C, or greater than about 77°C,
- as measured by differential scanning fluorimetry.
34. The pharmaceutical formulation of claim 33, wherein the formulation has a thermal stability profile as defined by a T_{m1} of about 67.0°C and a T_{m2} of about 77.3°C.
35. The pharmaceutical formulation of claim 34, wherein the thermal stability profile of the pharmaceutical formulation, as defined by T_{m1} and/or T_{m2} is changed by less than about 2°C or less than about 1°C when the pharmaceutical formulation is incubated for 1 week at 50°C, as compared to the same pharmaceutical formulation that is incubated for 1 week at 5°C, as measured by differential scanning fluorimetry.
36. The pharmaceutical formulation of any one of 1-35, wherein the formulation has a thermal stability profile as defined by a T_{agg} of greater than about 60°C, greater than about 61°C, greater than about 62°C, greater than about 63°C, greater than about 64°C, greater than about 65°C, greater than about 66°C, or greater than about 67°C, as measured by differential scanning fluorimetry.

37. The pharmaceutical formulation of claim 36, wherein the thermal stability profile of the pharmaceutical formulation, as defined by T_{agg} is changed by less than about 2°C or less than about 1°C when the pharmaceutical formulation is incubated for 1 week at 50°C, as compared to the same pharmaceutical formulation that is incubated for 1 week at 5°C, as measured by differential scanning fluorimetry.

38. The pharmaceutical formulation of any one of claims 1-37, wherein the pH of the pharmaceutical formulation does not change by more than about 0.2 or about 0.1 in pH value after the pharmaceutical formulation is incubated for 1 week at 5°C.

39. The pharmaceutical formulation of any one of claims 1-38, wherein the pH of the pharmaceutical formulation does not change by more than about 0.2 or about 0.1 in pH value after the pharmaceutical formulation is incubated for 1 week at 50°C.

40. The pharmaceutical formulation of any one of claims 1-39, wherein the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less than about 15 nm, less than about 14 nm, less than about 13 nm, or less than about 12 nm, as measured by dynamic light scattering at 25°C.

41. The pharmaceutical formulation of claim 40, wherein the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of about 11.6 nm.

42. The pharmaceutical formulation of any one of claims 1-41, wherein the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less than about 20 nm, less than about 19 nm, less than about 18 nm, less than about 17 nm, less than about 16 nm, or less than about 15 nm, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is incubated for 2 weeks at 50°C.

43. The pharmaceutical formulation of claim 42, wherein the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of about 14.4 nm.

44. The pharmaceutical formulation of any one of claims 1-43, wherein the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less

than about 20 nm, less than about 19 nm, less than about 18 nm, less than about 17 nm, or less than about 16 nm, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is subjected to five freeze thaw cycles.

45. The pharmaceutical formulation of claim 44, wherein the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of about 15.3 nm.

46. The pharmaceutical formulation of any one of claims 1-45, wherein the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is less than about 0.30, less than about 0.29, less than about 0.28, or less than about 0.27, as measured by dynamic light scattering at 25°C.

47. The pharmaceutical formulation of any one of claims 1-46, wherein the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is about 0.26.

48. The pharmaceutical formulation of any one of claims 1-47, wherein the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is less than about 0.30, less than about 0.29, less than about 0.28, less than about 0.27, or less than about 0.26, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is incubated for 2 weeks at 50°C.

49. The pharmaceutical formulation of any one of claims 1-48, wherein the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is about 0.25.

50. The pharmaceutical formulation of any one of claims 1-49, wherein the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is less than about 0.40, less than about 0.35, or less than about 0.34, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is subjected to five freeze thaw cycles.

51. The pharmaceutical formulation of any one of claims 1-50, wherein the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is about 0.33.

52. The pharmaceutical formulation of any one of claims 1-51, wherein the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is greater than about 90%, greater than about 91%, greater than about 92%, greater than about 93%, greater than about 94%, greater than about 95%, greater than about 96%, greater than about 97%, greater than about 98%, or greater than about 99%.

53. The pharmaceutical formulation of claim 52, wherein the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is about 99.0%.

54. The pharmaceutical formulation of any one of claims 1-53, wherein the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is greater than about 75%, greater than about 80%, greater than about 81%, greater than about 82%, greater than about 83%, greater than about 84%, or greater than about 85%, after the pharmaceutical formulation is incubated for 2 weeks at 50°C.

55. The pharmaceutical formulation of claim 54, wherein the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is about 85.2%.

56. The pharmaceutical formulation of any one of claims 1-55, wherein the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is greater than about 90%, greater than about 91%, greater than about 92%, greater than about 93%, greater than about 94%, greater than about 95%, greater than about 96%, greater than about 97%, or greater than about 98%, after the pharmaceutical formulation is subjected to five freeze thaw cycles.

57. The pharmaceutical formulation of claim 56, wherein the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is about 98.9%.

58. A method comprising administering to a subject in need thereof, the pharmaceutical formulation of any one of claims 1-57, as a single-dose therapy.

59. A method comprising administering to a subject in need thereof, the pharmaceutical formulation of any one of claims 1-57, in a multiple-dose therapy at an interval of at least three weeks between the doses or at least four weeks between the doses.

60. The method of claim 59, wherein the pharmaceutical formulation is administered to the subject once every three weeks.

61. The method of claim 59, wherein the pharmaceutical formulation is administered to the subject once every four weeks.

62. The method of claim 59, wherein the pharmaceutical formulation is administered to the subject once every six weeks.

63. The method of any one of claims 59-62, further comprising stopping the multi-dose therapy if the subject develops progressive disease, unacceptable toxicity, or meets a criterion for withdrawal.

64. The method of any one of claims 59-63, wherein if the subject experiences a complete response (CR) during the multi-dose therapy, then the multi-dose therapy is further administered for at least 12 months after the confirmation of the complete response.

65. The method of claim 64, wherein the total duration of the multi-dose therapy is equal to or less than 24 months.

66. The method of claim 64, wherein the total treatment duration is greater than 24 months.

67. The method of any one of claims 58-66, wherein the pharmaceutical formulation is administered by subcutaneous injection.

68. The method of any one of claims 58-67, wherein the pharmaceutical formulation is administered to the subject in an amount sufficient to provide the heterodimeric Fc-fused protein at a dosage of between about 0.05 µg/kg to about 1.75 µg/kg, based on the subject's weight.

69. The method of any one of claims 58-68, wherein the pharmaceutical formulation is administered to the subject in an amount sufficient to provide the heterodimeric Fc-fused protein at a dosage of about 0.05 µg/kg, about 0.10 µg/kg, about 0.20 µg/kg, about 0.40 µg/kg, about 0.60 µg/kg, about 0.80 µg/kg, about 1.00 µg/kg, about 1.20 µg/kg, about 1.40 µg/kg, or about 1.75 µg/kg, based on the subject's weight.

70. The method of any one of claims 58-67, wherein the pharmaceutical formulation is administered to the subject in an amount sufficient to provide the heterodimeric Fc-fused protein at a dosage of greater than 0.00 µg/kg and less than about 0.05 µg/kg, based on the subject's weight.

71. The method of any one of claims 58-67, wherein the pharmaceutical formulation is administered to the subject in an amount sufficient to provide the heterodimeric Fc-fused protein at a dosage of greater than about 1.75 µg/kg, based on the subject's weight.

72. The method of any one of claims 58-71, wherein the subject has cancer.

73. The method of claim 72, wherein the subject has a locally advanced or metastatic solid tumor.

74. The method of claim 72 or 73, wherein the presence of the cancer in the subject is confirmed using the Response Evaluation Criteria for Solid Tumors (RECIST), version 1.1.

75. The method of any one of claims 72-74, wherein the cancer is selected from the group consisting of: melanoma, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), head and neck squamous cell carcinoma (HNSCC), classical Hodgkin lymphoma, primary mediastinal large B-Cell lymphoma, bladder cancer, urothelial carcinoma, micro-satellite instability high cancer, colorectal cancer, gastric cancer, oesophageal cancer, cervical cancer, hepatocellular carcinoma, Merkel cell carcinoma, renal cell carcinoma (RCC), endometrial carcinoma, cutaneous T cell lymphoma, and triple negative breast cancer.

76. The method of any one of claims 72-75, wherein the subject is anti-PD-1 refractory.

77. The method of claim 75, wherein the subject has melanoma.
78. The method of claim 77, wherein the subject has previously been treated with an anti-PD-1 antibody for at least 6 weeks.
79. The method of claim 78, wherein the subject has been confirmed of progression of disease at least 4 weeks after the initial diagnosis of progression of disease while receiving an anti-PD-1 antibody.
80. The method of claim 79, wherein progression of disease is confirmed by radiological or clinical observation.
81. The method of claim 77, wherein, if the subject has a tumor comprising a BRAF activating mutation, then the subject has previously been treated with a BRAF inhibitor.
82. The method of claim 75, wherein the subject has RCC.
83. The method of claim 82, wherein the RCC has clear cell histology.
84. The method of claim 82, wherein the patient has previously been treated with an anti-PD-1/PD-L1 antibody and/or an anti-vascular endothelial growth factor therapy.
85. The method of claim 82, wherein the subject has previously received three or fewer lines of therapy.
86. The method of claim 75, wherein the subject has urothelial carcinoma.
87. The method of claim 86, wherein the subject has locally advanced or metastatic transitional cell carcinoma of the urothelium.
88. The method of claim 86, wherein the subject has previously been treated with a single treatment comprising a platinum-containing regimen and has shown radiographic progression recurrence within 6 months after the last administration of the platinum-containing regimen.

89. The method of claim 86, wherein the subject has previously received two or less lines of therapy.
90. The method of claim 86, wherein the subject has not previously received a checkpoint inhibitor (e.g., anti-PD-1 or anti-PD-L1 antibody) therapy as a monotherapy or in combination with a platinum based chemotherapy.
91. The method of any one of claims 58-90, wherein the pharmaceutical formulation is administered to the subject as a monotherapy.
92. The method of any one of claims 58-90, wherein the pharmaceutical formulation is administered to the subject as a combination therapy.
93. The method of claim 92, further comprising administering to the subject an anti-PD-1 antibody.
94. The method of claim 93, wherein the anti-PD-1 antibody is pembrolizumab.
95. The method of claim 94, wherein pembrolizumab is administered intravenously.
96. The method of claim 94 or 95, wherein pembrolizumab is administered at a dose of 200 mg.
97. The method of any one of claims 94-96, wherein administration of pembrolizumab precedes each administration of the pharmaceutical formulation.
98. The method of claim 97, wherein the pharmaceutical formulation is administered within 1 hour after completion of administration of pembrolizumab.
99. The method of claim 92, wherein the anti-PD-1 antibody is nivolumab.
100. The method of claim 99, wherein nivolumab is administered intravenously.

101. The method of claim 99 or 100, wherein nivolumab is administered at a dose of 480 mg.
102. The method of any one of claims 99-101, wherein administration of nivolumab precedes each administration of the pharmaceutical formulation.
103. The method of claim 102, wherein the pharmaceutical formulation is administered within 1 hour after completion of administration of nivolumab.
104. The method of any one of claims 99-103, wherein the cancer is selected from the group consisting of: melanoma, NSCLC, SCLC, RCC, classical Hodgkin lymphoma, HNSCC, urothelial carcinoma, colorectal cancer, hepatocellular carcinoma, bladder cancer, and oesophageal cancer.
105. The method of claim 104, wherein the cancer is melanoma.
106. The method of claim 105, wherein the melanoma is unresectable.
107. The method of claim 104, wherein the cancer is colorectal cancer.
108. The method of claim 107, wherein the colorectal cancer is microsatellite instability-high (MSI-H) or mismatch repair deficient metastatic (dMMR) colorectal cancer.
109. The method of any one of claims 92-98, further comprising performing a surgical intervention to lyse cancer cells, remove a tumor, or debulk a tumor in the subject.
110. The method of claim 109, wherein the surgical intervention comprises cryotherapy.
111. The method of claim 109, wherein the surgical intervention comprises hyperthermic therapy.
112. The method of claim 109, wherein the surgical intervention comprises administering to the subject a radiotherapy.

113. The method of claim 112, wherein the radiotherapy is a stereotactic body radiation therapy (SBRT).

114. The method of any one of claims 92-113, further comprising administering to the subject an NK cell-targeting therapy.

115. The method of claim 114, wherein the subject is administered a multi-specific binding protein.

116. The method of any one of claims 92-115, further comprising administering to the subject a chimeric antigen receptor therapy.

117. The method of any one of claims 92-116, further comprising administering to the subject a cytokine therapy.

118. The method of any one of claims 92-117, further comprising administering to the subject an innate immune system agonist therapy.

119. The method of any one of claims 92-118, further comprising administering to the subject a chemotherapy.

120. The method of any one of claims 92-119, further comprising administering to the subject a targeted antigen therapy.

121. The method of any one of claims 92-120, further comprising administering to the subject an oncolytic virus therapy.

122. A method of detecting toxicity in a subject receiving a pharmaceutical formulation comprising measuring the concentration of C-reactive protein (CRP) in the subject's blood, wherein the pharmaceutical formulation comprises a heterodimeric Fc-fused protein and a pharmaceutically acceptable carrier, and wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately

to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization.

123. The method of claim 122, wherein

- (1) if the CRP concentration in the subject's blood is higher than a threshold CRP concentration, then the subject is identified as being at risk for developing an adverse drug reaction; and
- (2) if the CRP concentration in the subject's blood is about the same or lower than the threshold C-reactive protein concentration, the subject is not identified as being at risk for developing an adverse drug reaction.

124. The method of claim 122, wherein if the CRP concentration in the subject's blood is higher than the threshold CRP concentration, then (1) the administration of the pharmaceutical formulation is paused; (2) the heterodimeric Fc-fused protein is administered at a lower dose; or (3) a remedial action is taken to reduce or alleviate the formulation's toxicity effects in the subject.

125. The pharmaceutical formulation of any one of claims 1-57 or the method of any one of claims 58-124, wherein the first and second antibody Fc domain polypeptides are human IgG1 Fc domain polypeptides.

126. The pharmaceutical formulation or the method of claim 125, wherein the multisubunit cytokine is a human IL12.

127. The pharmaceutical formulation or the method of claim 126, wherein the human IgG1 Fc domain polypeptides comprise one or more mutation(s) that reduce(s) an effector function of an Fc.

128. The pharmaceutical formulation or the method of claim 127, wherein the first and second antibody Fc domain polypeptides comprise mutations selected from L234A, L235A or L235E, G237A, P329A, A330S, and P331S, numbered according to the EU numbering system.

129. The pharmaceutical formulation or the method of claim 128, wherein the first and second antibody Fc domain polypeptides each comprise mutations L234A, L235A, and P329A.

130. The pharmaceutical formulation or the method of claim 129, wherein the first subunit of a multisubunit cytokine is a p40 subunit of IL12 and the second subunit of a multisubunit cytokine is a p35 subunit of IL12.

131. The pharmaceutical formulation or the method of claim 130, wherein the first subunit of a multisubunit cytokine comprises the amino acid sequence of SEQ ID NO: 127 and the second subunit of a multisubunit cytokine comprises the amino acid sequence of SEQ ID NO: 128.

132. The pharmaceutical formulation or the method of claim 131, wherein the second subunit of a multisubunit cytokine is fused to the second antibody Fc domain by a linker comprising the amino acid sequence of SEQ ID NO: 108.

133. The pharmaceutical formulation or the method of claim 132, wherein

- (a) the first antibody Fc domain comprises mutations L234A, L235A, P329A, Y349C, K360E, and K409W, and
- (b) the second antibody Fc domain comprises mutations L234A, L235A, P329A, Q347R, S354C, D399V, and F405T.

134. The pharmaceutical formulation or the method of claim 133, wherein

- (a) the first antibody Fc domain comprises the amino acid sequence of SEQ ID NO:215, and
- (b) the second antibody Fc domain comprises the amino acid sequence of SEQ ID NO:216.

135. The pharmaceutical formulation or the method of claim 134, wherein the first antibody Fc domain peptide comprises the amino acid sequence of SEQ ID NO:290 and the second antibody Fc domain peptide comprises the amino acid sequence of SEQ ID NO:291.

136. A kit comprising one or more vessels comprising a pharmaceutical formulation, wherein the pharmaceutical formulation comprises:

- (a) a heterodimeric Fc-fused protein comprising a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12,

wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization; and

(b) a pharmaceutically acceptable carrier,

and wherein the one or more vessels collectively comprise about 0.1 mg – about 2 mg of heterodimeric Fc-fused protein.

137. The kit of claim 136, wherein the one or more vessels collectively comprise about 0.5 mg to about 2 mg of heterodimeric Fc-fused protein.

138. The kit of claim 137, wherein the one or more vessels collectively comprise about 1 mg of heterodimeric Fc-fused protein.

139. The kit of claim 138, wherein the kit comprises one vessel comprising about 1 mg of heterodimeric Fc-fused protein.

140. The kit of any one of claims 136-139, wherein the pharmaceutical formulation is a lyophilized formulation or a liquid formulation.

141. The kit of claim 140, wherein the pharmaceutical formulation is a liquid formulation supplied in a volume of 1 mL.

142. Use of a heterodimeric Fc-fused protein in the manufacture of a medicament for treating a cancer, wherein the medicament is manufactured in a liquid pharmaceutical formulation comprising about 0.5 g/L to about 1.5 g/L of the heterodimeric Fc-fused protein contained in one or more vessels,

wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein

CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization.

143. The use of a heterodimeric Fc-fused protein of claim 142, wherein the liquid pharmaceutical formulation comprises about 1.0 g/L of the heterodimeric Fc-fused protein.

144. Use of a heterodimeric Fc-fused protein in the manufacture of a medicament for treating a cancer, wherein the medicament is manufactured in a liquid pharmaceutical formulation comprising about 0.1 mg – about 2 mg of heterodimeric Fc-fused protein contained in one or more vessels,

wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization.

145. The use of a heterodimeric Fc-fused protein of claim 144, wherein the liquid pharmaceutical formulation comprises about 1 mg of heterodimeric Fc-fused protein.

146. The use of a heterodimeric Fc-fused protein of any one of claims 142-145, wherein the medicament is contained in one vessel.

147. The use of a heterodimeric Fc-fused protein of any one of claims 142-146, wherein each vessel contains 1 mg of heterodimeric Fc-fused protein.

148. The use of any one of claims 146-147, wherein the medicament is administered to the subject on day 1, every 3 weeks.

149. The use of any one of claims 146-147, wherein the medicament is administered to the subject on day 1, every 4 weeks.

150. The use of any one of claims 146-148, wherein the medicament is administered subcutaneously.

151. The use of any one of claims 146-150, wherein the medicament is administered in a volume of about 0.1 mL to about 1 mL.

152. The use of claim 151, wherein the medicament is administered in a volume of about 1 mL.

153. The use of any one of claims 146-152, wherein the medicament is administered to a maximum of two injection sites.

154. The use of claim 153, wherein a second injection is completed within 10 minutes after a first injection.

155. The use of any one of claims 146-154, wherein the medicament is administered at a dose of about 0.05 mg/kg to about 1.75 mg/kg.

156. The use of claim 155, wherein the medicament is administered at a dose of about 1 mg/kg.

157. The use of any one of claims 146-156, wherein the medicament is diluted prior to administration in a solution of 0.9% saline (sodium chloride for injection) and 0.01% polysorbate 80.

158. A method of manufacturing a heterodimeric Fc-fused protein for the preparation of a pharmaceutical formulation thereof, the method comprising adding acetic acid to a solution comprising the heterodimeric Fc-fused protein obtained from a Chinese Hamster Ovary (CHO) cell culture expressing the heterodimeric Fc-fused protein for 30 minutes to 90 minutes, wherein the acetate adjusts and maintains the pH of the solution at pH 3.55 to 3.75, and

wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein

CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization.

159. The method of claim 158, wherein the acetic acid is added to the solution comprising the heterodimeric Fc-fused protein for about 60 minutes.

160. The method of claim 158 or 159, wherein the acetic acid adjusts and maintains the pH of the solution to about 3.65.

161. The method of claim 158, wherein the CHO cell culture expressing the heterodimeric Fc-fused protein is maintained in suspension.

162. The method of claim 161, wherein the CHO cell culture expressing the heterodimeric Fc-fused protein is cultured for 7-21 days in a bioreactor.

163. The method of claim 161 or 162, wherein the CHO cell culture expressing the heterodimeric Fc-fused protein is cultured for 14 days in a bioreactor.

164. The method of any one of claims 158-163, wherein the CHO cell culture expressing the heterodimeric Fc-fused protein is harvested by depth filtration to yield a CHO harvest medium.

165. The method of claim 164, wherein the depth filtration is a two-stage single-use depth filtration consisting of DOHC and XOHC filters.

166. The method of claim 164 or 165, wherein the heterodimeric Fc-fused protein is purified from the CHO harvest medium using Protein A capture chromatography, mixed mode chromatography, and cation exchange chromatography to yield the solution comprising the heterodimeric Fc-fused protein.

167. The method of claim 166, wherein Protein A capture chromatography comprises:
equilibrating a Protein A resin with 20 mM Tris, 150 mM NaCl at pH 7.5;
loading CHO harvest medium onto the Protein A resin;
washing the loaded Protein A resin with 20 mM Tris, 150 mM NaCl at pH 7.5;

washing the loaded Protein A resin with 50 mM acetate at pH 5.4; and
eluting the heterodimeric Fc-fused protein from the Protein A resin with 50 mM acetate, 100 mM arginine at pH 3.7 and collecting by 280 nm UV starting at 1.25 AU/cm ascending and ending at 1.25 AU/cm descending.

168. The method of claim 167, wherein the acetic acid is added at a concentration of 0.5M to the solution comprising the heterodimeric Fc-fused protein eluted from the Protein A resin, wherein the acetic acid acidifies the pH of the solution to pH 3.65 for 60 minutes, followed by neutralization of the solution to pH 5.2 by adding 2M Tris.

169. The method of claim 168, wherein following acidification and neutralization of the solution, the solution comprising the heterodimeric Fc-fused protein is passed through a 0.2 μ m filter.

170. The method of claim 169, wherein the filtered solution comprising the heterodimeric Fc-fused protein eluted from the Protein A resin is passed through X0SP depth filtration.

171. The method of claim 170, wherein mixed mode chromatography comprises:
equilibrating a mixed mode chromatography column with 50 mM acetate at pH 5.2;
loading the solution passed through X0SP filtration onto the mixed mode chromatography column;
washing the loaded mixed mode chromatography column with 50 mM acetate at pH 5.2;
and
eluting the heterodimeric Fc-fused protein from the mixed mode chromatography column with 50 mM Acetate, 250 mM NaCl at pH 5.2 and collecting by 280 nm UV starting at 0.625 AU/cm ascending and ending at 1.50 AU/cm descending.

172. The method of claim 171, wherein the solution comprising the heterodimeric Fc-fused protein eluted from the mixed mode chromatography column is passed through a 0.2 μ m filter.

173. The method of claim 172, wherein cation exchange chromatography comprises:
equilibrating a cation exchange chromatography resin with 50 mM Tris at pH 7.4;

loading the filtered solution eluted from the mixed mode chromatography column onto the cation exchange chromatography resin;

washing the loaded cation exchange chromatography resin with 50 mM Tris at pH 7.4; and

eluting the heterodimeric Fc-fused protein from the cationic exchange chromatography resin with a gradient of 50 mM Tris at pH 7.4 and 50 mM Tris, 0.5 M NaCl at pH 7.4, and collecting by 280 nm UV starting at 2.5 AU/cm ascending and ending at 4.5 AU/cm descending.

174. The method of claim 173, wherein the solution comprising the heterodimeric Fc-fused protein eluted from the cation exchange chromatography resin is passed through a 0.2 μ m filter.

175. The method of claim 174, wherein the filtered solution comprising the heterodimeric Fc-fused protein eluted from the cation exchange chromatography resin is nanofiltrated through a prefilter, a 20 nm nominal filter, and a 0.2 μ m membrane.

176. The method of claim 175, wherein the nanofiltrated solution comprising the heterodimeric Fc-fused protein is ultrafiltrated and diafiltrated, wherein ultrafiltration and diafiltration comprises:

equilibrating an ultrafiltration system with 50 mM Tris, 265 mM NaCl at pH 7.4;

concentrating the nanofiltrated solution comprising the heterodimeric Fc-fused protein to a concentration of about 5.0 g/L;

exchanging the buffer using 7 diavolumes of 20 mM citrate at pH 6.5;

concentration the diafiltrated solution comprising the heterodimeric Fc-fused protein to a concentration of about 11.0 g/L;

diluting the concentration solution comprising the heterodimeric Fc-fused protein to a concentration of about 5 g/L to about 10 g/L with 20 mM citrate at pH 6.5; and

adding 20 mM citrate, 18% (w/v) sucrose, 3% (w/v) mannitol, 0.03% (w/v) polysorbate-80 at pH 6.5 to achieve a final concentration of the ultrafiltration/diafiltration retentate solution comprising the heterodimeric Fc-fused protein of 20 mM citrate, 6% (w/v) sucrose, 1% (w/v) mannitol, 0.01% (w/v) polysorbate-80.

177. The method of claim 176, wherein the ultrafiltrated/diafiltrated solution comprising the heterodimeric Fc-fused protein is passed through a 0.2 μ m membrane to yield a bulk drug substance.

178. The method of claim 177, wherein the bulk drug substance is diluted to an 80% drug product solution in a 0.2 μ m filtered buffer comprising 20 mM citrate, 6% (w/v) sucrose, 1% (w/v) mannitol, and 0.01% (w/v) polysorbate-80 at pH 6.5.

179. The method of claim 177 or 178, wherein the bulk drug substance or 80% drug product is diluted to a concentration for administration of 1 mg/mL of the heterodimeric Fc-fused protein in a 0.2 μ m filtered buffer comprising 20 mM citrate, 6% (w/v) sucrose, 1% (w/v) mannitol, and 0.01% (w/v) polysorbate-80 at pH 6.5.

180. A method of treating cancer in a subject who has received treatment with a checkpoint inhibitor antibody for at least 6 weeks, the method comprising administering a pharmaceutical formulation comprising a heterodimeric Fc-fused protein and a pharmaceutically acceptable carrier to the subject, wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization.

181. The method of claim 180, wherein the checkpoint inhibitor antibody is an anti-programmed cell death protein 1 (PD-1) antibody.

182. The method of claim 180 or 181, wherein the cancer is melanoma.

183. The method of claim 182, wherein the melanoma is unresectable or metastatic.

184. The method of claim 182 or 183, wherein the subject is confirmed to have progressive disease at least 4 weeks after the initial diagnosis of progressive disease while receiving the anti-PD-1 antibody.

185. The method of any one of claims 182-184, wherein the subject is confirmed to have progressive disease at least 4 weeks after the initial diagnosis of progressive disease while receiving the anti-PD-1 antibody.

186. The method of any one of claims 184 or 185, wherein progressive disease is confirmed by radiological or clinical observation.

187. A method of treating cancer in a subject who has received treatment with a checkpoint inhibitor antibody or an anti-vascular endothelial growth factor therapy as a monotherapy, or in combination, the method comprising administering a pharmaceutical formulation comprising a heterodimeric Fc-fused protein and a pharmaceutically acceptable carrier to the subject, wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization.

188. The method of claim 187, wherein the checkpoint inhibitor antibody is an anti-PD-1 antibody or an anti-PD-L1 antibody.

189. The method of claim 187 or 188, wherein the cancer is advanced renal cell carcinoma (RCC).

190. The method of claim 189, wherein the RCC is unresectable or metastatic.

191. The method of claim 189 or 190, wherein the RCC has a clear cell component.

192. The method of any one of claims 189-191, wherein the subject received no more than 3 previous lines of therapy.

193. The method of any one of claims 189-192, wherein the subject has not received treatment with a checkpoint inhibitor.

194. The method of claim 193, wherein the checkpoint inhibitor comprises an anti-PD-1 antibody or anti-PD-L1 antibody as a monotherapy or in combination with a platinum based chemotherapy.

195. A method of treating cancer in a subject who has received treatment with only one platinum-containing regimen, the method comprising administering a pharmaceutical formulation comprising a heterodimeric Fc-fused protein and a pharmaceutically acceptable carrier to the subject, wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization.

196. The method of claim 195, wherein the platinum containing regimen is platinum in combination with an agent selected from gemcitabine, methotrexate, vinblastine, and doxorubicin.

197. The method of claim 195 or 196, wherein the cancer is locally advanced or metastatic transitional cell urothelial carcinoma.

198. The method of claim 197, wherein the urothelial carcinoma includes one or more of the group consisting of the renal pelvis, ureters, urinary urothelium, and urethra.

199. The method of claim 197 or 198, wherein the urothelial carcinoma is inoperable.

200. The method of any one of claims 197-199, wherein the urothelial carcinoma is characterized with radiographic progression or with recurrence within 6 months after the last administration of a platinum-containing regimen as an adjuvant.

201. The method of any one of claims 197-200, wherein the urothelial carcinoma is considered failure of a first-line, platinum-containing regimen.
202. The method of any one of claims 197-201, wherein the subject has received no more than 2 lines of therapy (including the platinum-containing regimen) for the treatment of the urothelial carcinoma prior to administration of the pharmaceutical formulation.
203. The method of any one of claims 197-202, wherein the subject has not received treatment with a checkpoint inhibitor (CPI) as a first-line therapy.
204. The method of claim 203, wherein the checkpoint inhibitor is an anti-PD-1 antibody or anti-PD-L1 antibody.
205. The method of claim 203 or 204, wherein the checkpoint inhibitor is a monotherapy or in combination with a platinum based chemotherapy.
206. The method of any one of claims 195-205, wherein the pharmaceutical formulation is administered in combination with pembrolizumab.
207. The method of claim 206, wherein pembrolizumab is administered once every 3 weeks.
208. The method of claim 206 or 207, wherein pembrolizumab is administered before administration of the pharmaceutical formulation.
209. The method of claim 208, wherein the pharmaceutical formulation is administered within one hour after the completion of administration of pembrolizumab.
210. The method of any one of claims 206-209, wherein pembrolizumab is administered at a dose of 200 mg.
211. The method of any one of claims 206-210, wherein pembrolizumab is administered intravenously.

212. The method of any one of claims 206-211, wherein the cancer is selected from the group consisting of: melanoma, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), head and neck squamous cell carcinoma (HNSCC), classical Hodgkin lymphoma, primary mediastinal large B-cell lymphoma, urothelial carcinoma, microsatellite instability-high cancer, gastric cancer, oesophageal cancer, cervical cancer, hepatocellular carcinoma, Merkel cell carcinoma, renal cell carcinoma, and endometrial carcinoma.

213. The method of any one of claims 195-205, wherein the pharmaceutical formulation is administered in combination with nivolumab.

214. The method of claim 213, wherein nivolumab is administered before administration of the pharmaceutical formulation.

215. The method of claim 214, wherein the pharmaceutical formulation is administered within one hour after the completion of administration of nivolumab.

216. The method of any one of claims 213-215, wherein nivolumab is administered at a dose of about 480 mg.

217. The method of any one of claims 213-216, wherein nivolumab is administered intravenously.

218. The method of any one of claims 213-217, wherein the cancer is selected from the group consisting of: melanoma, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), renal cell carcinoma, classical Hodgkin lymphoma, head and neck squamous cell carcinoma (HNSCC), colorectal cancer, hepatocellular carcinoma, bladder cancer, and oesophageal cancer.

219. The method of claim 218, wherein the cancer is melanoma.

220. The method of claim 219, wherein the melanoma is unresectable or metastatic.

221. The method of claim 218, wherein the cancer is colorectal cancer.

222. The method of claim 221, wherein the colorectal cancer is microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer.

223. The method of any one of claims 180-211, wherein the pharmaceutical formulation is administered to the subject on day 1, every 3 weeks.

224. The method of any one of claims 180-222, wherein the pharmaceutical formulation is administered to the subject on day 1, every 4 weeks.

225. The method of any one of claims 180-223, wherein the pharmaceutical formulation is administered subcutaneously.

226. The method of any one of claims 180-225, wherein the pharmaceutical formulation is administered in a volume of about 0.1 mL to about 1 mL.

227. The method of claim 226, wherein the pharmaceutical formulation is administered in a volume of about 1 mL.

228. The method of any one of claims 180-227, wherein the pharmaceutical formulation is administered to a maximum of two injection sites.

229. The method of claim 228, wherein a second injection is completed within 10 minutes after a first injection.

230. The method of any one of claims 180-229, wherein the pharmaceutical formulation is administered at a dose of about 0.05 mg/kg to about 1.75 mg/kg.

231. The method of claim 230, wherein the pharmaceutical formulation is administered at a dose of about 1 mg/kg.

232. The method of any one of claims 180-231, wherein the pharmaceutical formulation is diluted prior to administration in a solution of 0.9% saline (sodium chloride for injection) and 0.01% polysorbate 80.

233. The method of any one of claims 180-232, wherein the presence of the cancer is determined using the Response Evaluation Criteria for Solid Tumors (RECIST), version 1.1.

234. The method of any one of claims 180-233, wherein a subject who has a confirmed complete response is treated with the pharmaceutical formulation for at least 12 months after confirmation unless a criterion for discontinuation is met.

235. A method of treating a subject whose blood concentration of C-reactive protein (CRP) is monitored, the method comprising administering to the subject a pharmaceutical formulation comprising a heterodimeric Fc-fused protein and a pharmaceutically acceptable carrier, wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization.

236. The method of claim 235, wherein

- (1) if the CRP concentration in the subject's blood is higher than a threshold CRP concentration, then the subject is identified as being at risk for developing an adverse drug reaction; and
- (2) if the CRP concentration in the subject's blood is about the same or lower than the threshold C-reactive protein concentration, the subject is not identified as being at risk for developing an adverse drug reaction.

237. The method of claim 235, wherein if the CRP concentration in the subject's blood is higher than the threshold CRP concentration, then (1) the administration of the pharmaceutical formulation is paused; (2) the heterodimeric Fc-fused protein is administered at a lower dose; or (3) a remedial action is taken to reduce or alleviate the formulation's toxicity effects in the subject.

238. A method of treating cancer in a subject in need thereof, the method comprising subcutaneous administration of a pharmaceutical formulation comprising a heterodimeric Fc-fused protein and pharmaceutically acceptable carrier to the subject,

wherein the heterodimeric Fc-fused protein comprises a first Fc region and a second Fc region of an immunoglobulin Fc (fragment crystallizable) pair and the p40 and p35 subunits of IL-12, wherein the p40 and p35 subunits of IL-12 are linked separately to the first Fc region and the second Fc region, or to the second Fc region and the first Fc region, respectively, wherein the p40 and p35 subunits are each linked to the N-terminus or C-terminus of the Fc regions, and wherein CH3 domains of the first Fc region and the second Fc region each comprise one or more mutations promoting heterodimerization; and the pharmaceutical formulation comprises citrate; a sugar; a sugar alcohol; and a non-ionic surfactant, and the pH of the formulation is between 5.5 and 7.0.

239. The kit of any one of claims 136-141, the use of any one of claims 142-157, or the method of any one of claims 158-238, wherein the first Fc region and second Fc region are human IgG1 Fc regions.

240. The kit, the use, or the method of claim 239, wherein human IgG1 Fc regions comprise one or more mutation(s) that reduce(s) an effector function of an Fc.

241. The kit, the use, or the method of claim 240, wherein the first Fc region and second Fc region comprise one or more mutation(s) selected from L234A, L235A or L235E, G237A, P329A, A330S, and P331S, numbered according to the EU numbering system.

242. The kit, the use, or the method of claim 241, wherein the first Fc region and second Fc region each comprise mutations L234A, L235A, and P329A.

243. The kit, the use, or the method of claim 242, wherein the p40 subunit of IL12 comprises the amino acid sequence of SEQ ID NO: 127 and the p35 subunit of IL-12 comprises the amino acid sequence of SEQ ID NO: 128.

244. The kit, the use, or the method of claim 243, wherein the p35 subunit of IL-12 is fused to the second Fc region by a linker comprising the amino acid sequence of SEQ ID NO: 108.

245. The kit, the use, or the method of claim 244, wherein
- (a) the first Fc region comprises mutations L234A, L235A, P329A, Y349C, K360E, and K409W, and
 - (b) the second Fc region comprises mutations L234A, L235A, P329A, Q347R, S354C, D399V, and F405T.
246. The kit, the use, or the method of claim 245, wherein
- (a) the first Fc region comprises the amino acid sequence of SEQ ID NO:215, and
 - (b) the second Fc region comprises the amino acid sequence of SEQ ID NO:216.
247. The kit, the use, or the method of claim 246, wherein the first Fc region linked to the p40 subunit of IL12 comprises the amino acid sequence of SEQ ID NO:290 and the second Fc region linked to the p35 subunit of IL-12 comprises the amino acid sequence of SEQ ID NO:291.
248. The kit, the use, or the method of any one of claims 239-247, wherein the pharmaceutical formulation comprises: (a) citrate; (b) a sugar; (c) a sugar alcohol; and (d) a non-ionic surfactant, further wherein the pH of the formulation is between about 6.0 and about 7.0.
249. The kit, the use, or the method of claim 248, wherein the concentration of citrate in the pharmaceutical formulation is about 10 mM to about 30 mM.
250. The kit, the use, or the method of claim 249, wherein the concentration of citrate in the pharmaceutical formulation is about 20 mM.
251. The kit, the use, or the method of any one of claims 248-250, wherein the concentration of the sugar in the pharmaceutical formulation is about 3% to about 12% (w/v).
252. The kit, the use, or the method of claim 251, wherein the concentration of the sugar in the pharmaceutical formulation is about 6% (w/v).
253. The kit, the use, or the method of claim 251 or 252, wherein the sugar is a disaccharide.
254. The kit, the use, or the method of claim 253, wherein the disaccharide is sucrose.

255. The kit, the use, or the method of any one of claims 248-254, wherein the concentration of the sugar alcohol in the pharmaceutical formulation is between about 0.5% to about 6% (w/v).

256. The kit, the use, or the method of claim 255, wherein the concentration of the sugar alcohol in the pharmaceutical formulation is about 1% (w/v).

257. The kit, the use, or the method of any one of claims 248-256, wherein the sugar alcohol is derived from a monosaccharide.

258. The kit, the use, or the method of claim 257, wherein the sugar alcohol is mannitol.

259. The kit, the use, or the method of any one of claims 248-258, wherein the concentration of the non-ionic surfactant in the pharmaceutical formulation is between about 0.005% to about 0.02% (w/v).

260. The kit, the use, or the method of claim 259, wherein the concentration of polysorbate 80 in the pharmaceutical formulation is about 0.01% (w/v).

261. The kit, the use, or the method of claim 259 or 260, wherein the non-ionic surfactant is a polysorbate.

262. The kit, the use, or the method of claim 261, wherein the polysorbate is polysorbate 80.

263. The kit, the use, or the method of any one of claims 248-262, wherein the pH is between about 6.1 and about 6.9.

264. The kit, the use, or the method of claim 263, wherein the pH is between about 6.2 and about 6.8.

265. The kit, the use, or the method of claim 264, wherein the pH is between about 6.3 and about 6.7.

266. The kit, the use, or the method of claim 265, wherein the pH is between about 6.4 and about 6.6.
267. The kit, the use, or the method of claim 266, wherein the pH is about 6.5.
268. The kit, the use, or the method of any one of claims 248-267, wherein the pharmaceutical formulation further comprising water.
269. The kit, the use, or the method of claim 268, wherein the water is Water for Injection, USP.
270. The kit, the use, or the method of any one of claims 248-269, wherein the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 1 g/L to about 10 g/L.
271. The kit, the use, or the method of claim 270, wherein the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 2 g/L to about 8 g/L.
272. The kit, the use, or the method of claim 271, wherein the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 4 g/L to about 6 g/L.
273. The kit, the use, or the method of claim 272, wherein the pharmaceutical formulation comprises a bulk concentration of heterodimeric Fc-fused protein of about 5 g/L.
274. The kit, the use, or the method of any one of claims 248-273, wherein the pharmaceutical formulation comprises a concentration of the protein for administration of about 0.5 g/L to about 1.5 g/L.
275. The kit, the use, or the method of claim 274, wherein the pharmaceutical formulation comprises a concentration of the protein for administration of about 0.75 g/L to about 1.25 g/L.
276. The kit, the use, or the method of claim 275, wherein the pharmaceutical formulation comprises a concentration of the protein for administration of about 1 g/L.

277. The kit, the use, or the method of any one of 248-276, wherein the formulation is designed to be stored at a temperature between 2°C and 8°C.

278. The kit, the use, or the method of any one of claims 248-277, wherein the pharmaceutical formulation is a clear, colorless solution and free of visible particulates.

279. The kit, the use, or the method of any one of claims 248-278, wherein the pharmaceutical formulation has a thermal stability profile as defined by:

(a) a T_{m1} of greater than about 60°C, greater than about 61°C, greater than about 62°C, greater than about 63°C, greater than about 64°C, greater than about 65°C, or greater than about 66°C; and/or

(b) a T_{m2} of greater than about 70°C, greater than about 71°C, greater than about 72°C, greater than about 73°C, greater than about 74°C, greater than about 75°C, greater than about 76°C, or greater than about 77°C,

as measured by differential scanning fluorimetry.

280. The kit, the use, or the method of claim 279, wherein the formulation has a thermal stability profile as defined by a T_{m1} of about 67.0°C and a T_{m2} of about 77.3°C.

281. The kit, the use, or the method of claim 280, wherein the thermal stability profile of the pharmaceutical formulation, as defined by T_{m1} and/or T_{m2} is changed by less than about 2°C or less than about 1°C when the pharmaceutical formulation is incubated for 1 week at 50°C, as compared to the same pharmaceutical formulation that is incubated for 1 week at 5°C, as measured by differential scanning fluorimetry.

282. The kit, the use, or the method of any one of 248-281, wherein the formulation has a thermal stability profile as defined by a T_{agg} of greater than 60°C, greater than about 61°C, greater than about 62°C, greater than about 63°C, greater than about 64°C, greater than about 65°C, greater than about 66°C, or greater than about 67°C, as measured by differential scanning fluorimetry.

283. The kit, the use, or the method of claim 282, wherein the thermal stability profile of the pharmaceutical formulation, as defined by T_{agg} is changed by less than about 2°C or less than about 1°C when the pharmaceutical formulation is incubated for 1 week at 50°C, as compared to the

same pharmaceutical formulation that is incubated for 1 week at 5°C, as measured by differential scanning fluorimetry.

284. The kit, the use, or the method of any one of claims 248-283, wherein the pH of the pharmaceutical formulation does not change by more than about 0.2 or about 0.1 in pH value after the pharmaceutical formulation is incubated for 1 week at 5°C.

285. The kit, the use, or the method of any one of claims 248-284, wherein the pH of the pharmaceutical formulation does not change by more than about 0.2 or about 0.1 in pH value after the pharmaceutical formulation is incubated for 1 week at 50°C.

286. The kit, the use, or the method of any one of claims 248-285, wherein the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less than about 15 nm, less than about 14 nm, less than about 13 nm, or less than about 12 nm, as measured by dynamic light scattering at 25°C.

287. The kit, the use, or the method of claim 286, wherein the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of about 11.6 nm.

288. The kit, the use, or the method of any one of claims 248-287, wherein the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less than about 20 nm, less than about 19 nm, less than about 18 nm, less than about 17 nm, less than about 16 nm, or less than about 15 nm, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is incubated for 2 weeks at 50°C.

289. The kit, the use, or the method of claim 288, wherein the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of about 14.4 nm.

290. The kit, the use, or the method of any one of claims 248-289, wherein the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of less than about 20 nm, less than about 19 nm, less than about 18 nm, less than about 17 nm, or less than about 16 nm, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is subjected to five freeze thaw cycles.

291. The kit, the use, or the method of claim 290, wherein the heterodimeric Fc-fused protein in the pharmaceutical formulation has a Z-average hydrodynamic diameter of about 15.3 nm.

292. The kit, the use, or the method of any one of claims 248-291, wherein the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is less than about 0.30, less than about 0.29, less than about 0.28, or less than about 0.27, as measured by dynamic light scattering at 25°C.

293. The kit, the use, or the method of any one of claims 248-292, wherein the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is about 0.26.

294. The kit, the use, or the method of any one of claims 248-293, wherein the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is less than about 0.30, less than about 0.29, less than about 0.28, less than about 0.27, or less than about 0.26, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is incubated for 2 weeks at 50°C.

295. The kit, the use, or the method of any one of claims 248-294, wherein the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is about 0.25.

296. The kit, the use, or the method of any one of claims 248-295, wherein the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is less than about 0.40, less than about 0.35, or less than about 0.34, as measured by dynamic light scattering at 25°C, after the pharmaceutical formulation is subjected to five freeze thaw cycles.

297. The kit, the use, or the method of any one of claims 248-296, wherein the polydispersity index of the heterodimeric Fc-fused protein in the pharmaceutical formulation is about 0.33.

298. The kit, the use, or the method of any one of claims 248-297, wherein the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is greater than about 90%, greater than about 91%, greater

than about 92%, greater than about 93%, greater than about 94%, greater than about 95%, greater than about 96%, greater than about 97%, greater than about 98%, or greater than about 99%.

299. The kit, the use, or the method of claim 298, wherein the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is about 99.0%.

300. The kit, the use, or the method of any one of claims 248-299, wherein the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is greater than about 75%, greater than about 80%, greater than about 81%, greater than about 82%, greater than about 83%, greater than about 84%, or greater than about 85%, after the pharmaceutical formulation is incubated for 2 weeks at 50°C.

301. The kit, the use, or the method of claim 300, wherein the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is about 85.2%.

302. The kit, the use, or the method of any one of claims 248-301, wherein the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is greater than about 90%, greater than about 91%, greater than about 92%, greater than about 93%, greater than about 94%, greater than about 95%, greater than about 96%, greater than about 97%, or greater than about 98%, after the pharmaceutical formulation is subjected to five freeze thaw cycles.

303. The kit, the use, or the method of claim 302, wherein the purity profile of the pharmaceutical formulation, as measured by the area of the main peak as a percentage of total detected area in a SEC-HPLC analysis, is about 98.9%.

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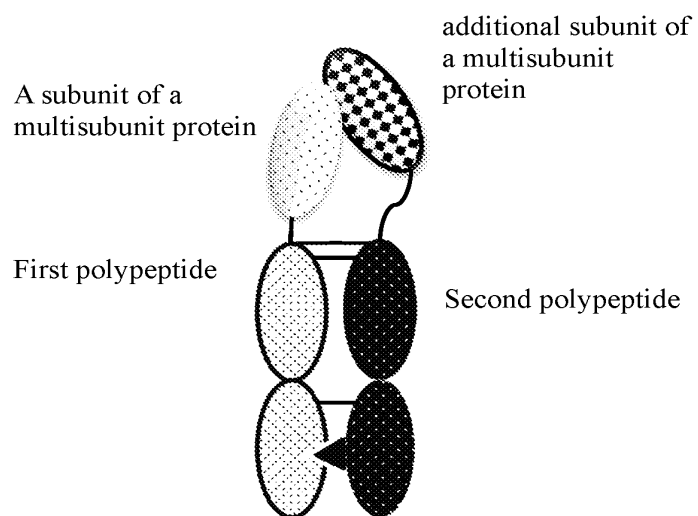
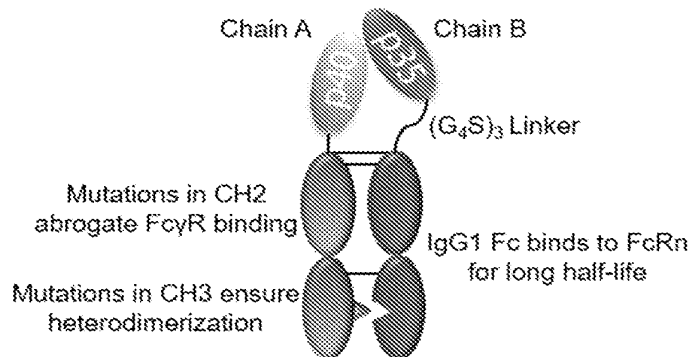
FIG. 1A**FIG. 1B**

FIG. 1C

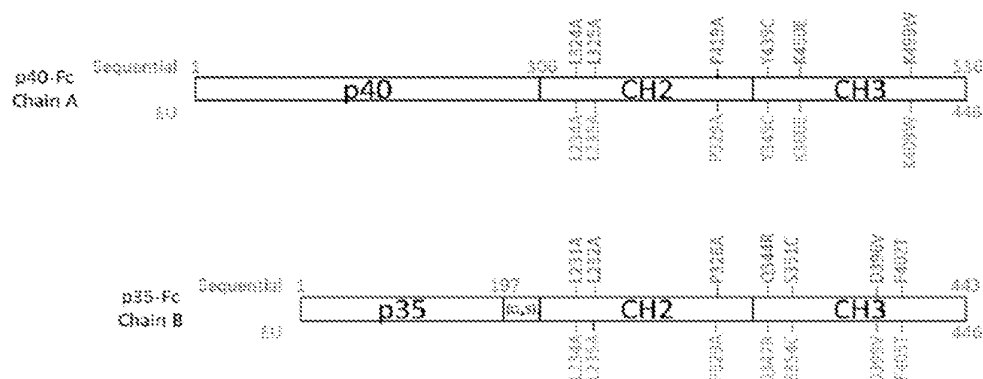
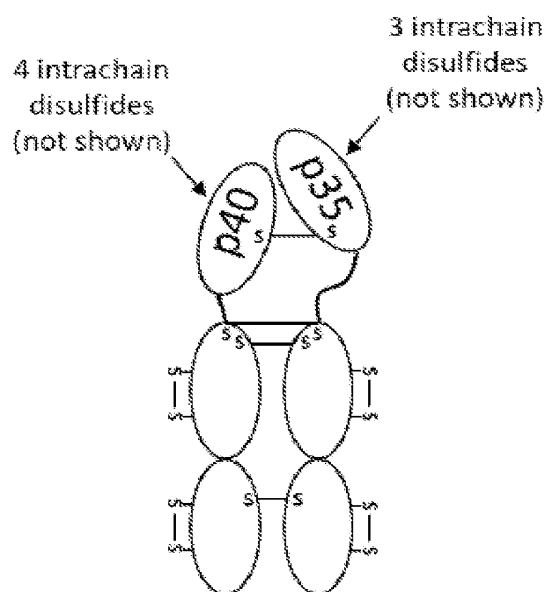


FIG. 1D



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FIG. 2A

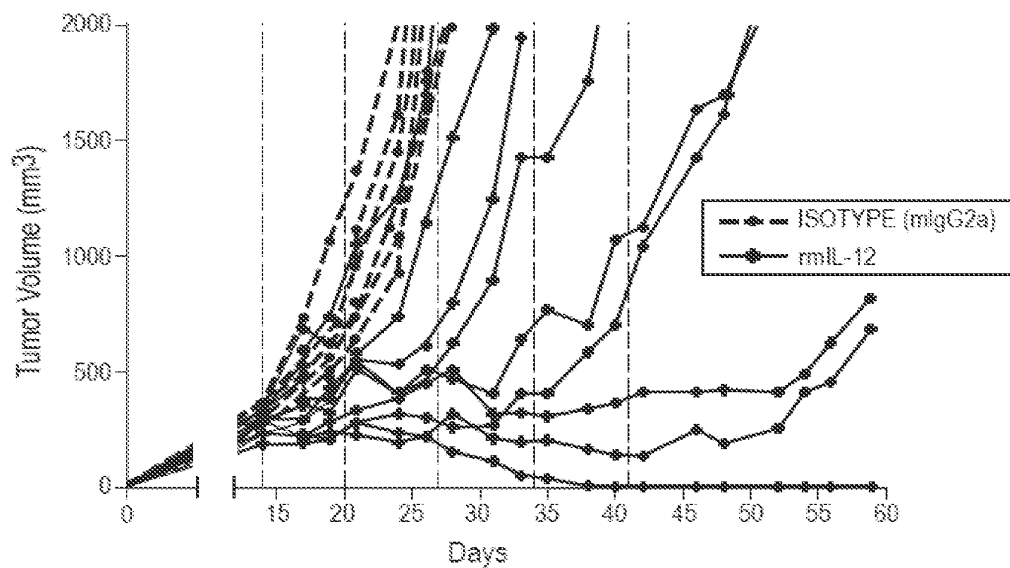
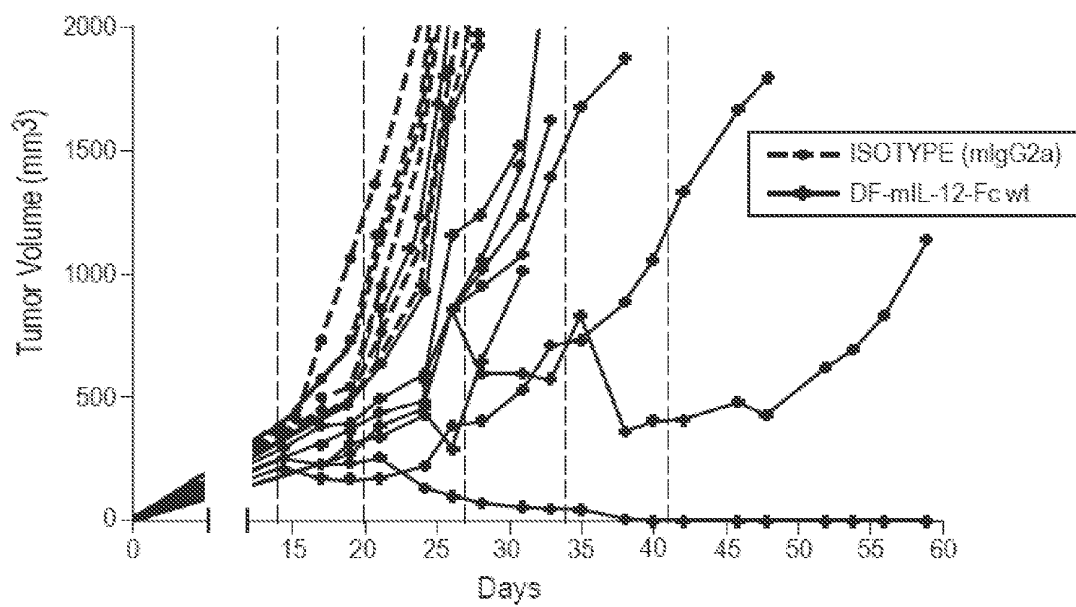


FIG. 2B



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FIG. 2C

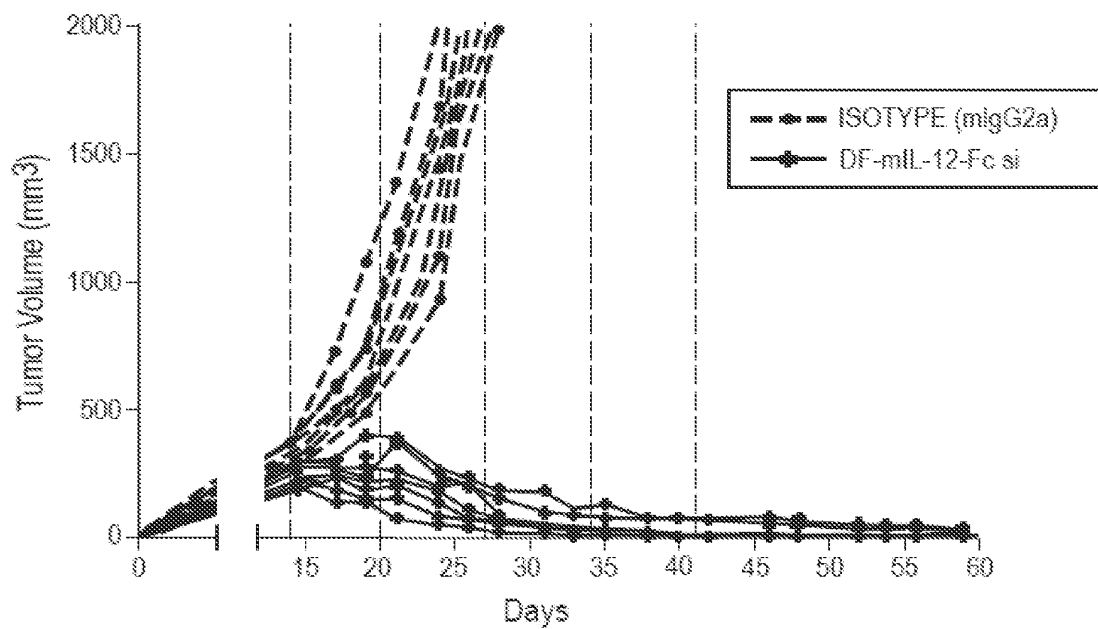
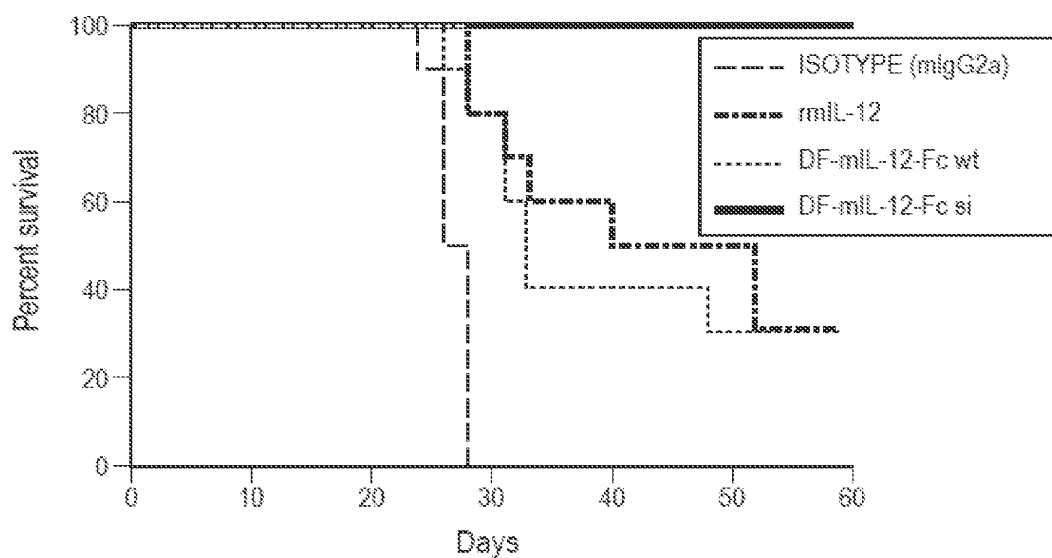


FIG. 3



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FIG. 4A

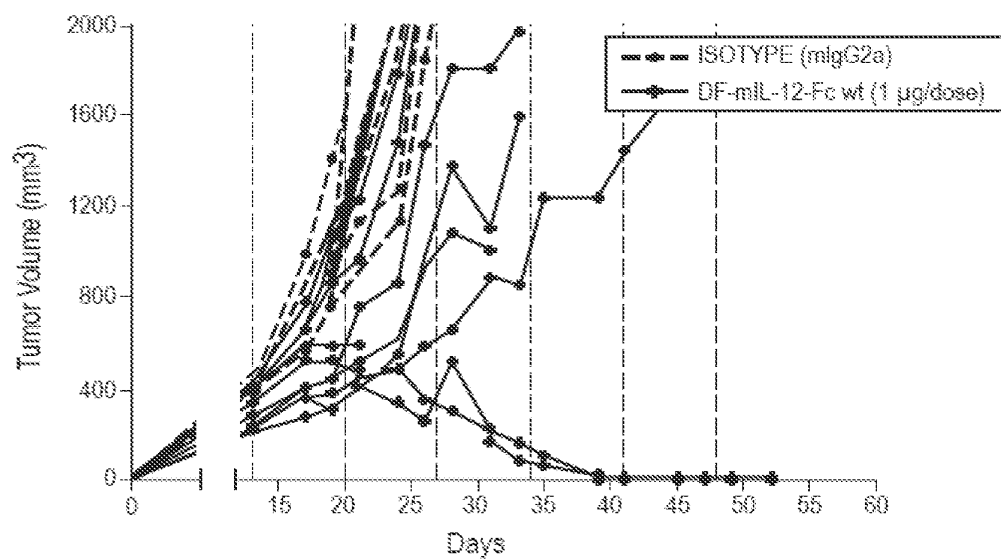
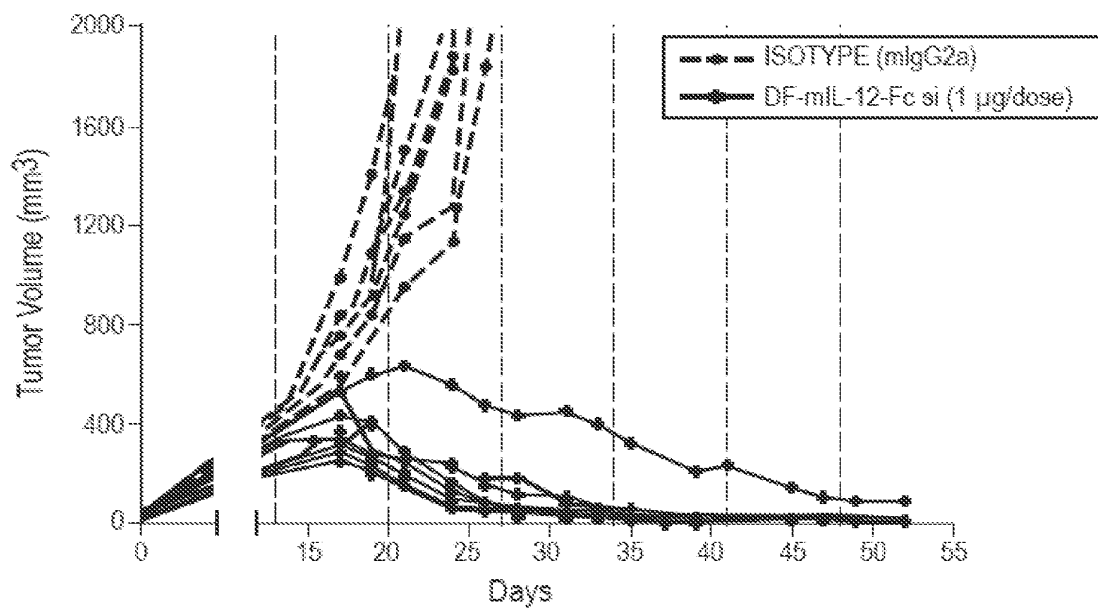
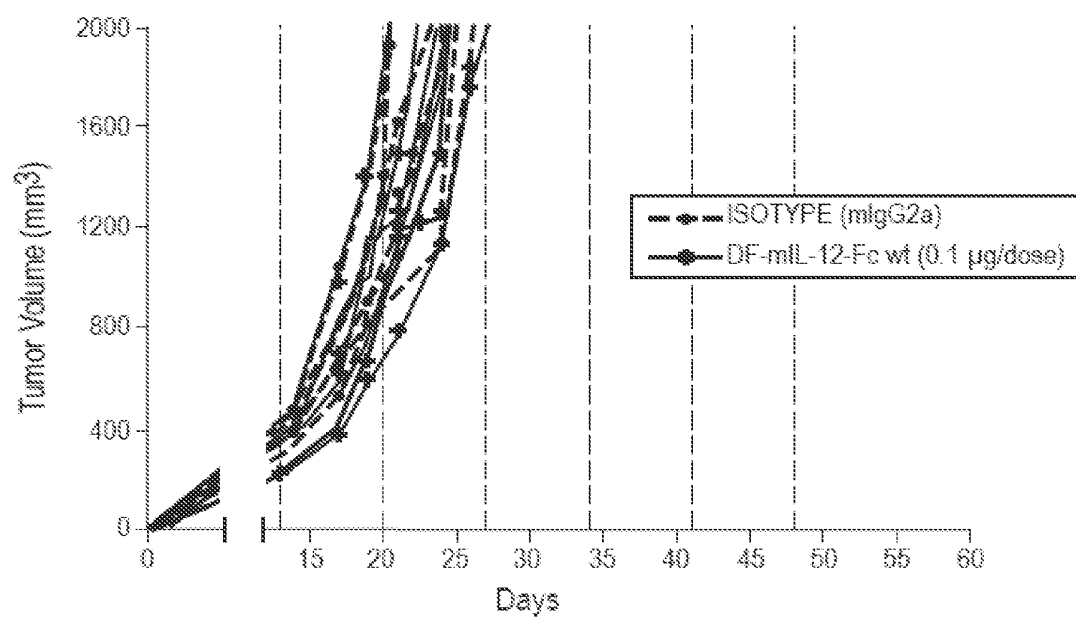


FIG. 4B



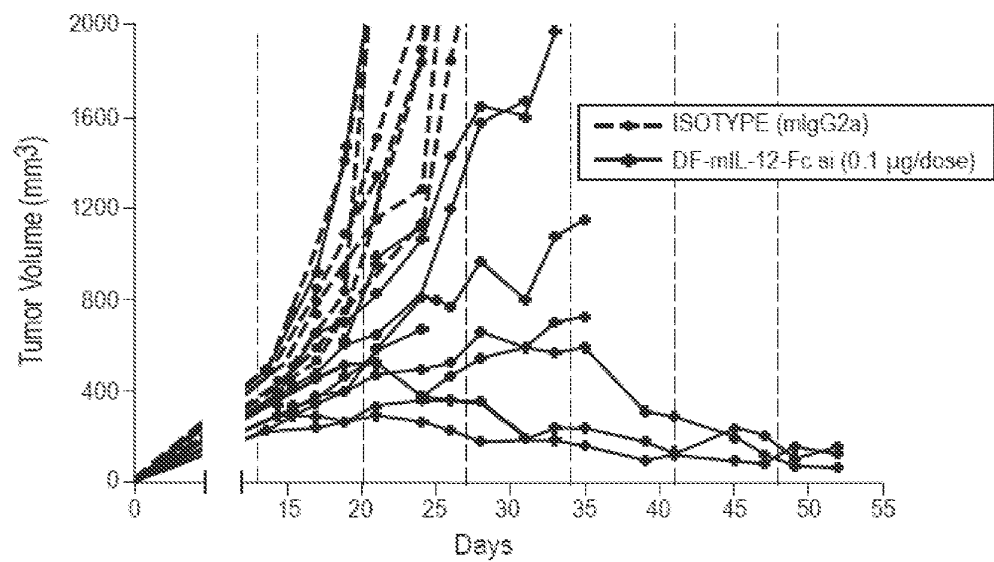
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FIG. 4C



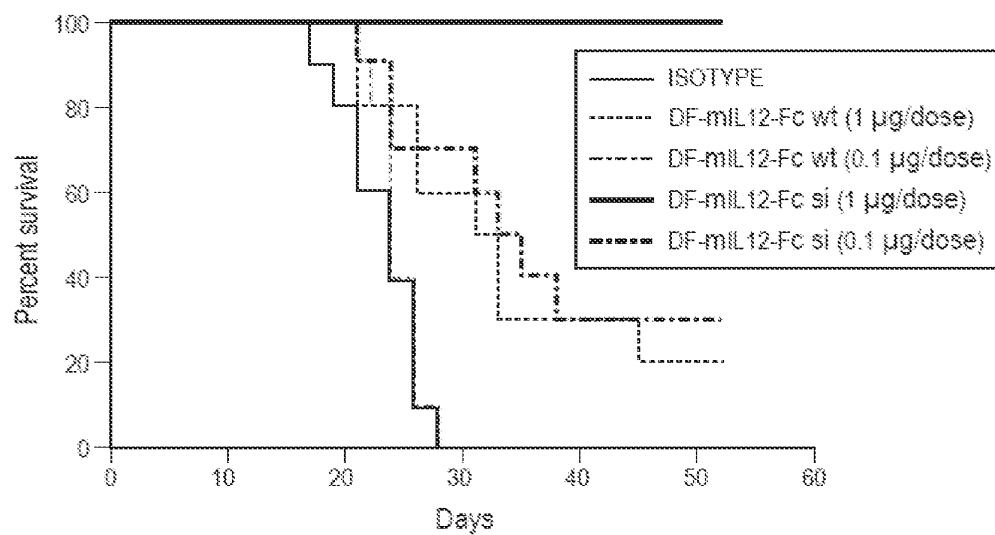
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FIG. 4D



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FIG. 5



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FIG. 6A

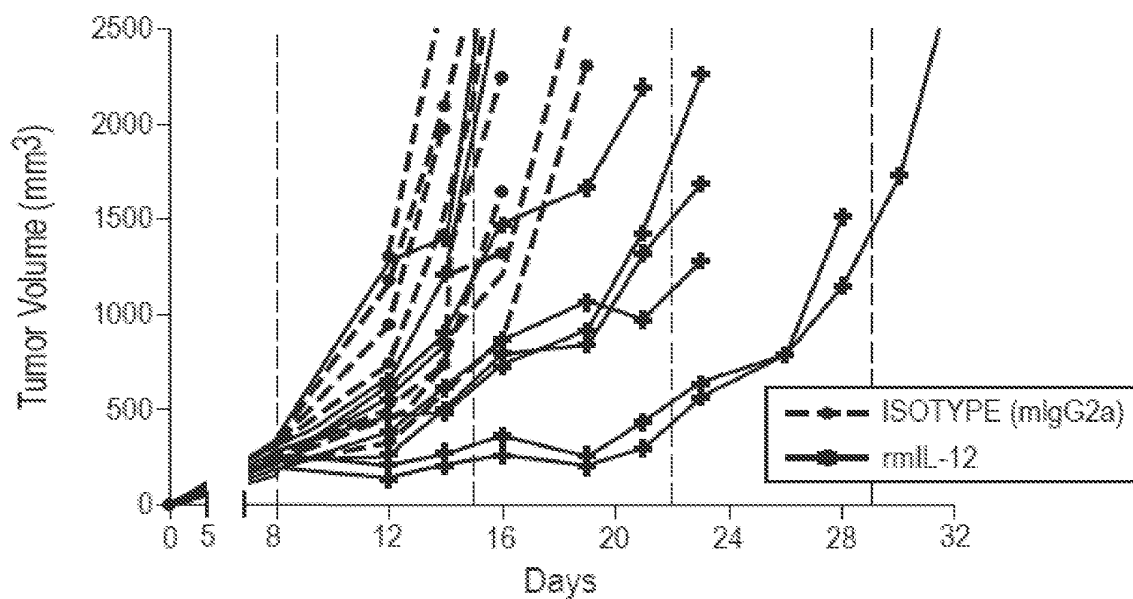
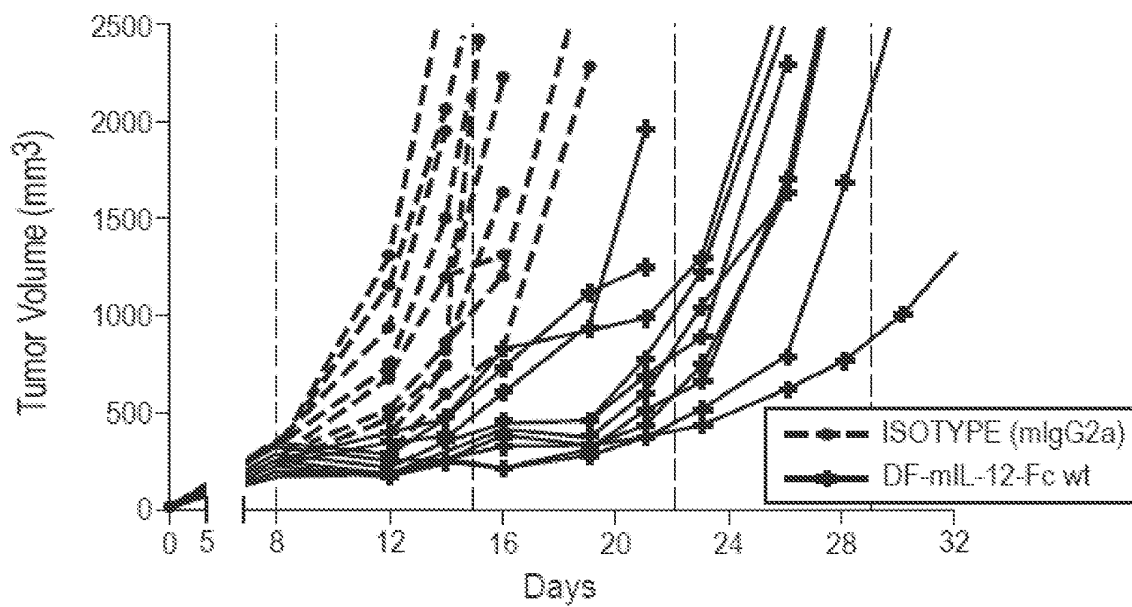


FIG. 6B



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FIG. 6C

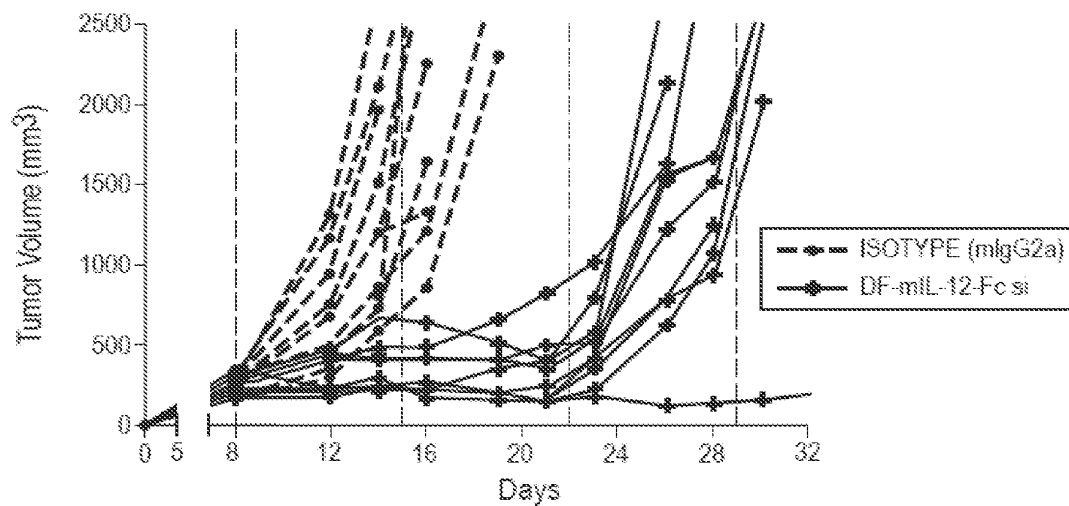
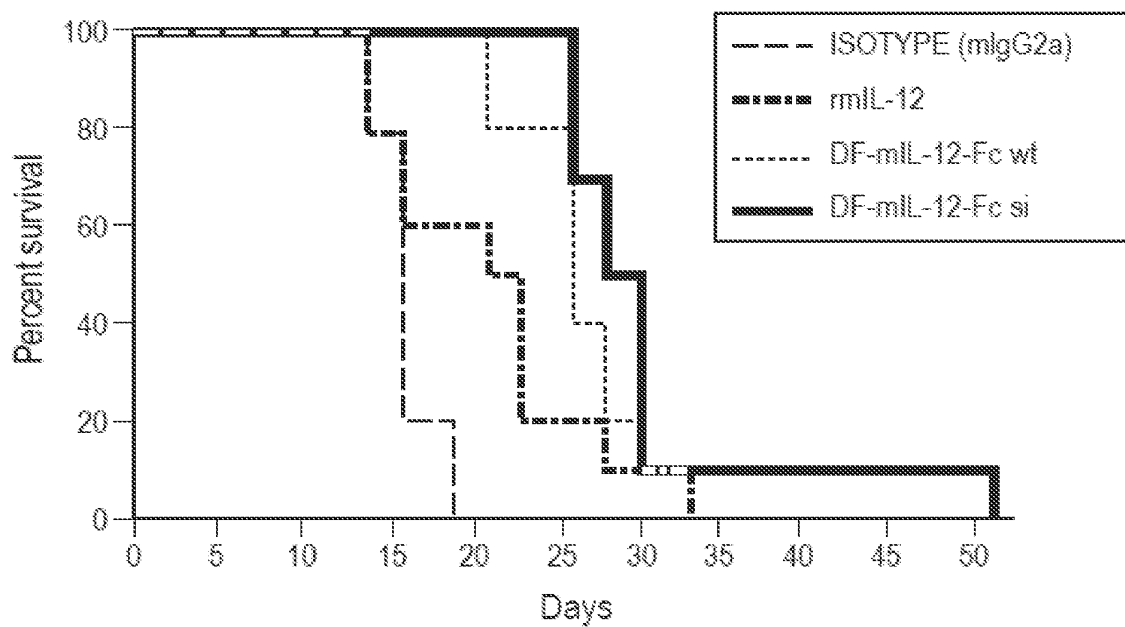


FIG. 7



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FIG. 8A

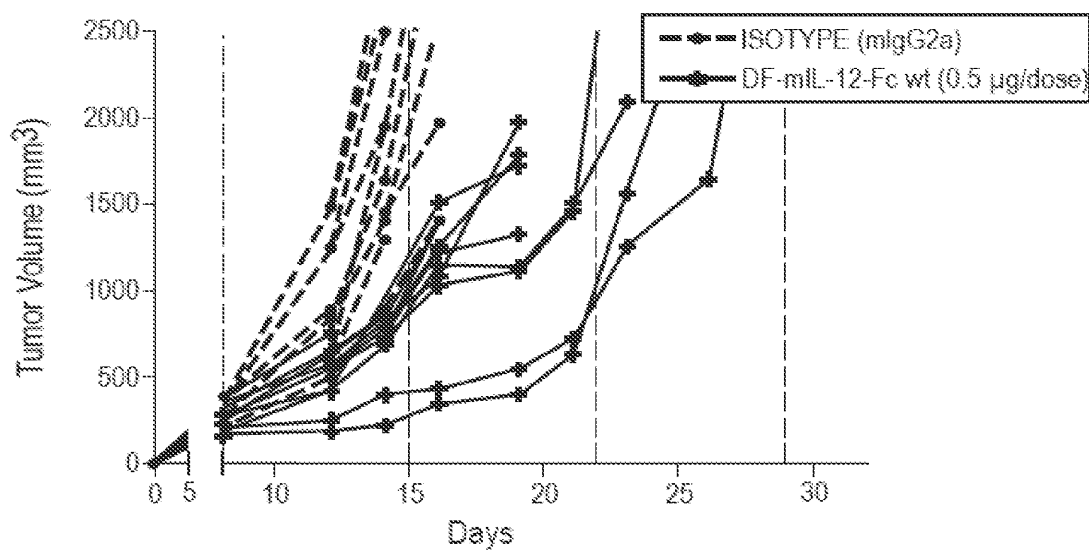
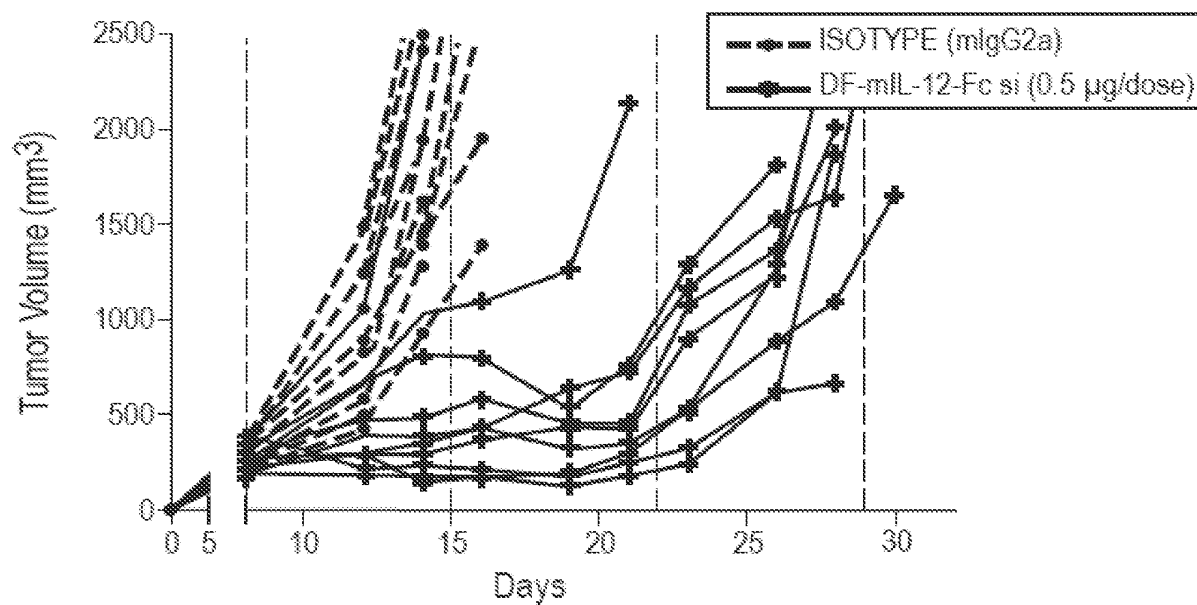


FIG. 8B



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FIG. 8C

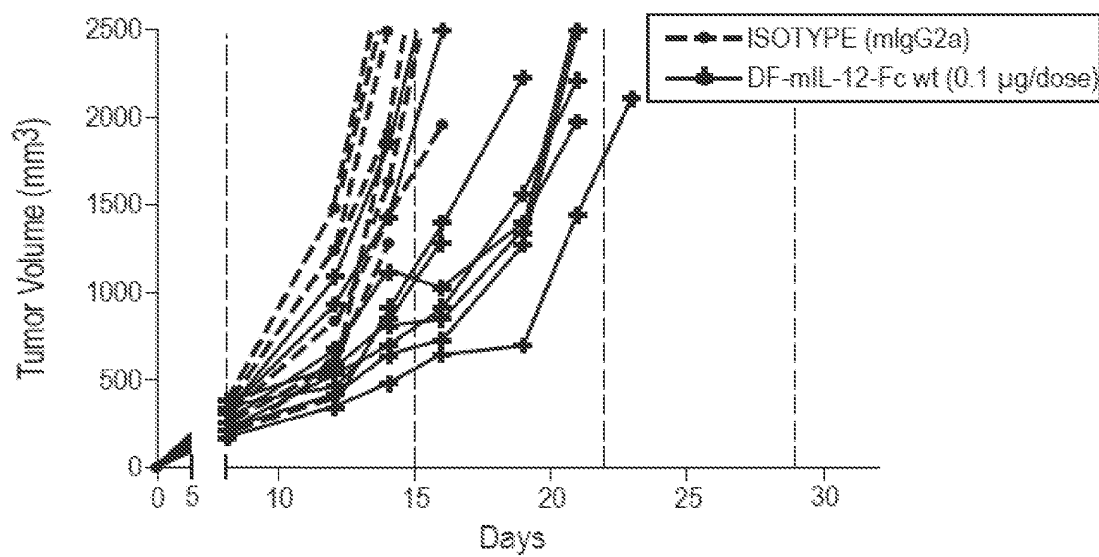
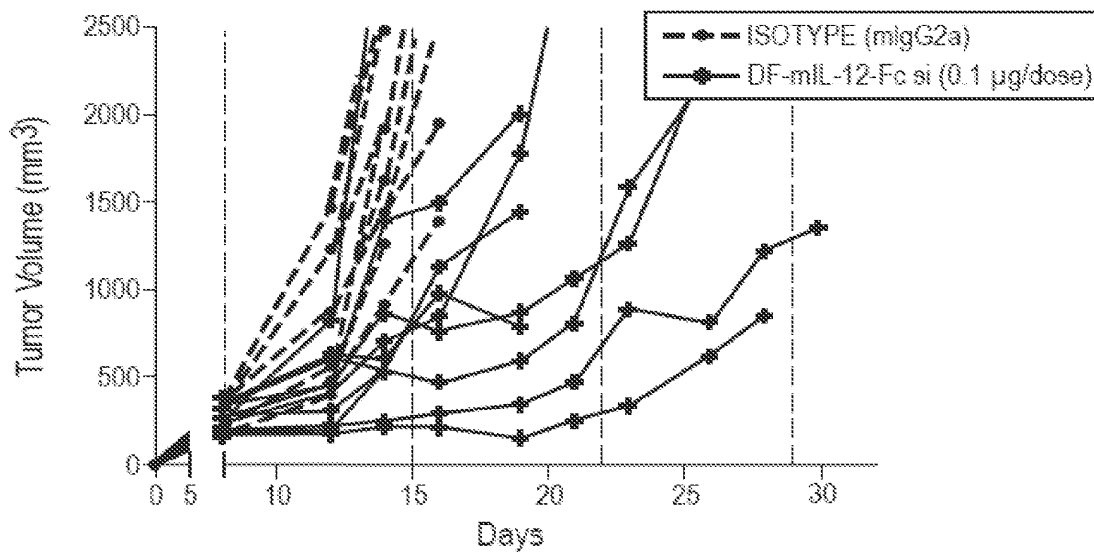
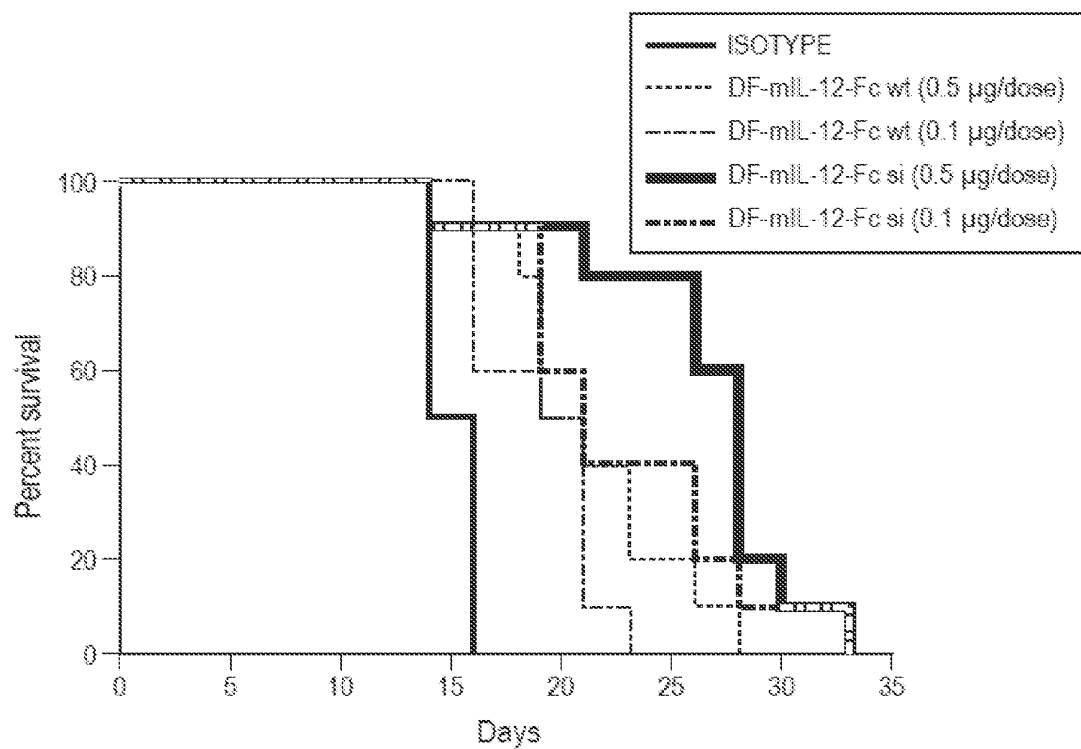


FIG. 8D



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FIG. 9



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FIG. 10A

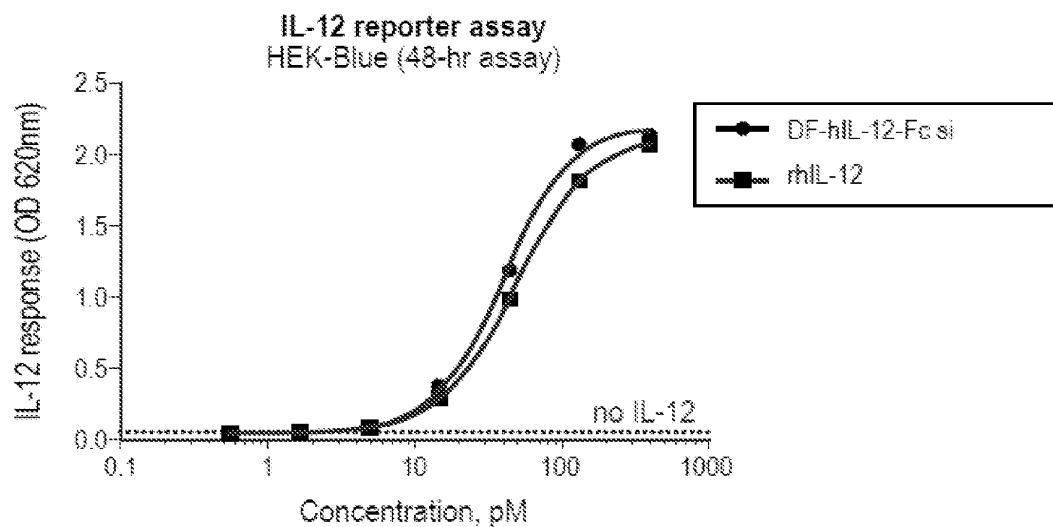


FIG. 10B

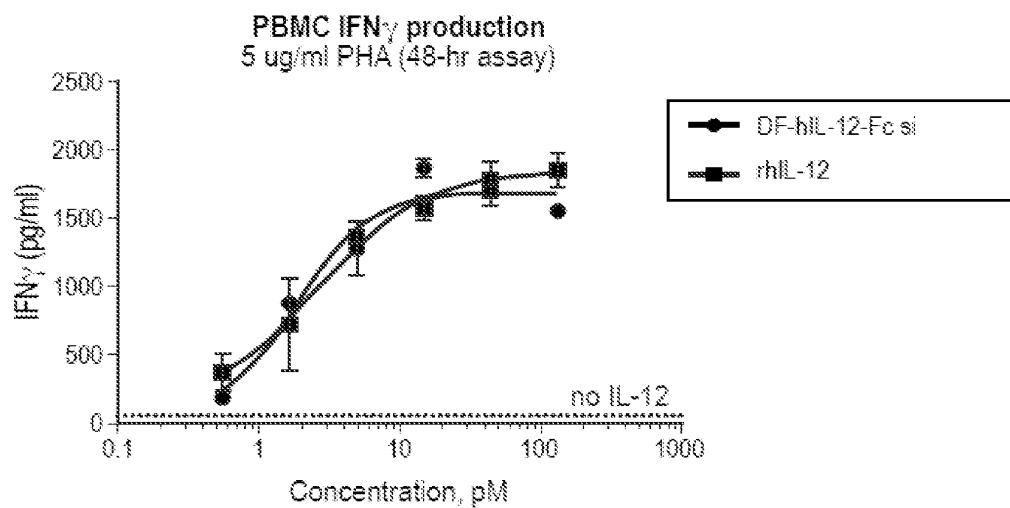
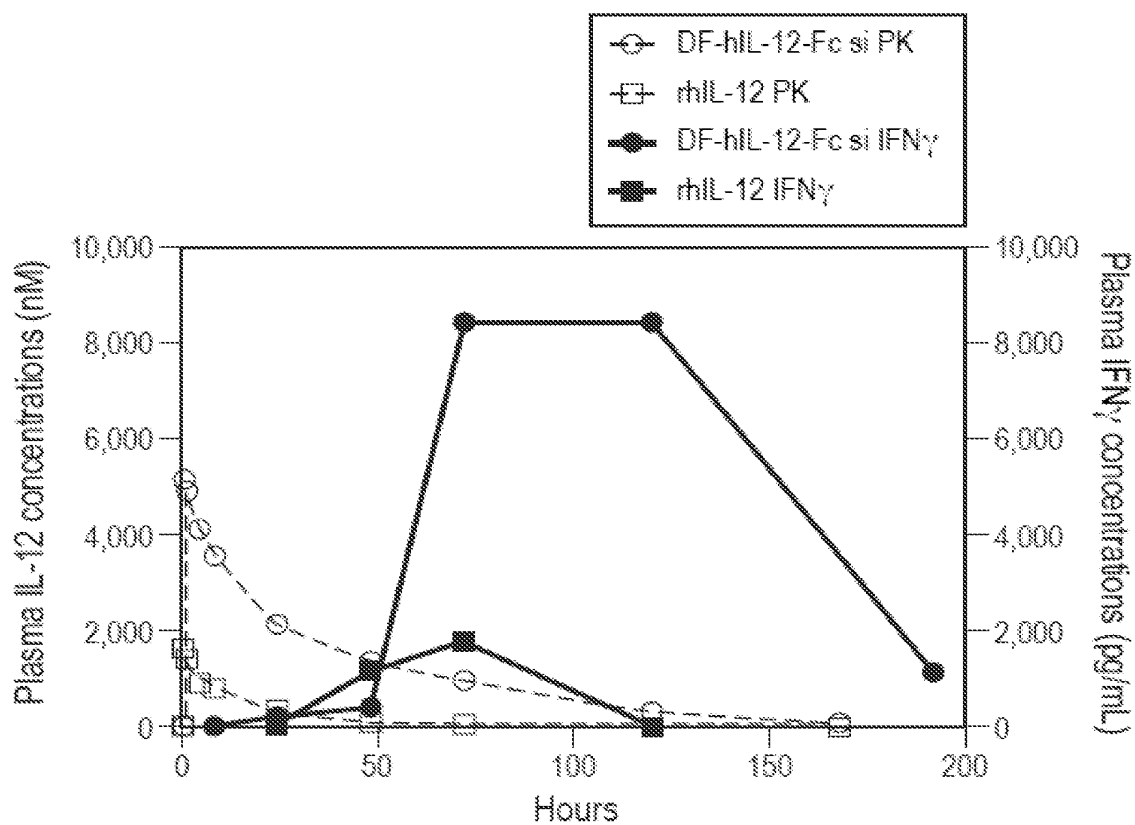


FIG. 11



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FIG. 12A

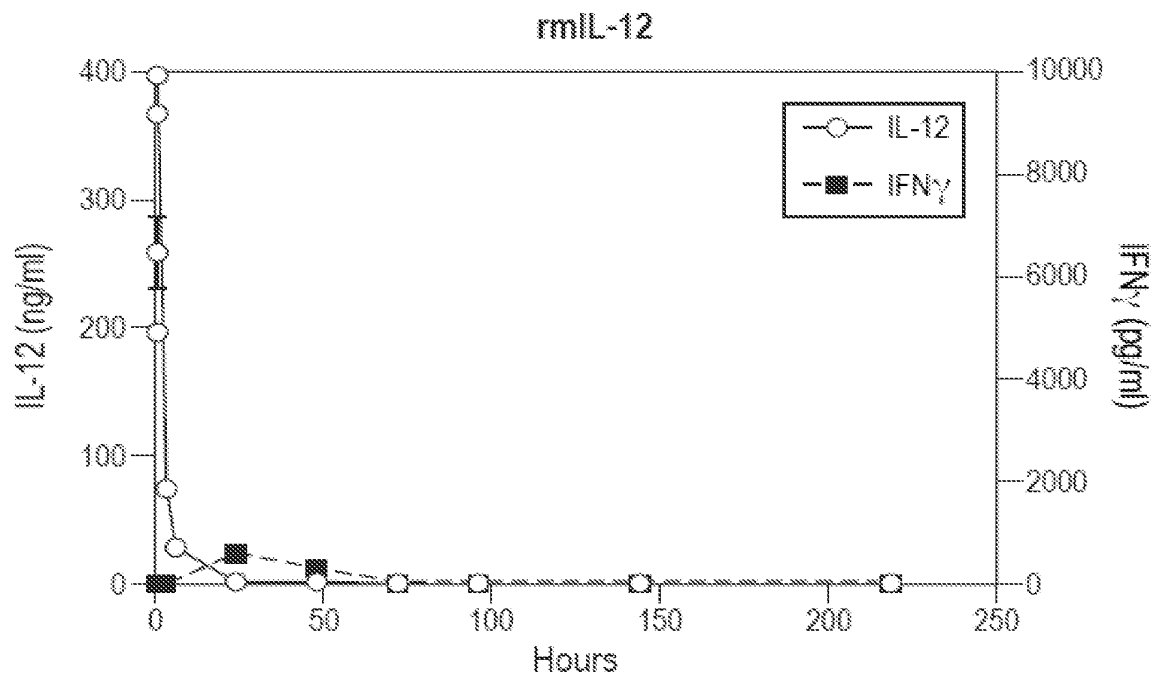
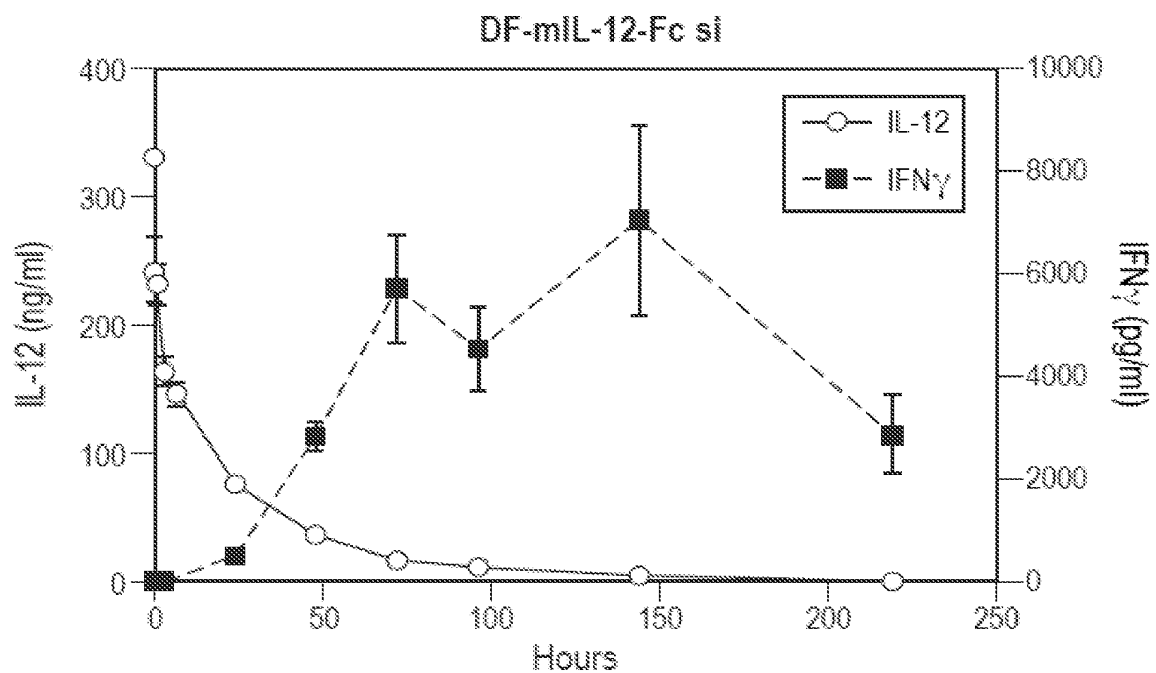


FIG. 12B



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FIG. 12C

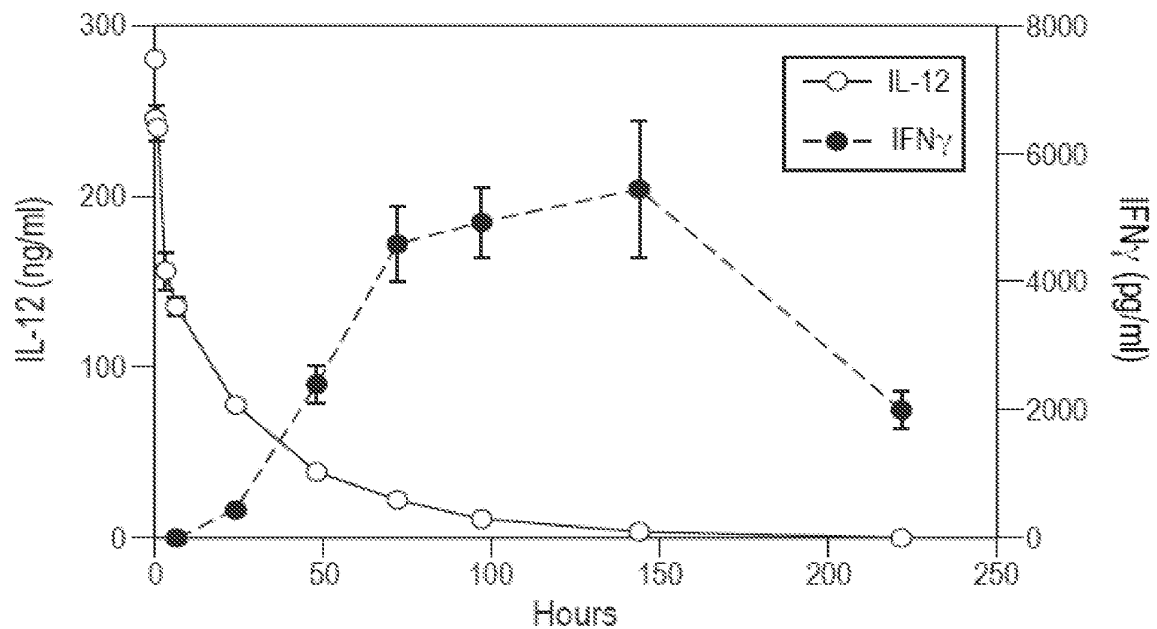
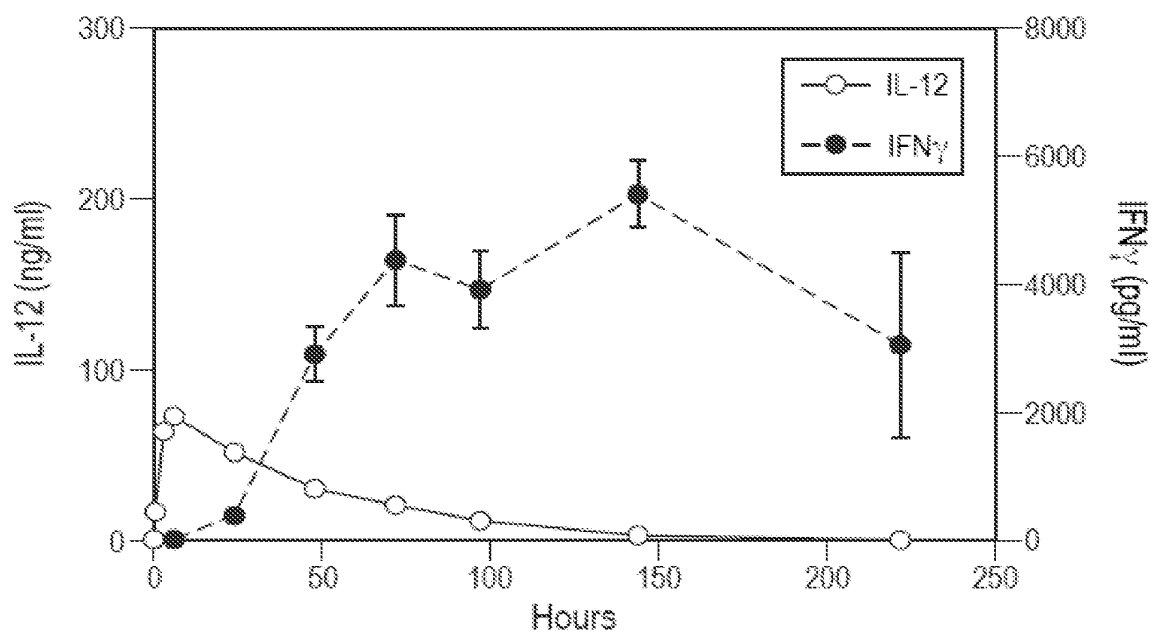
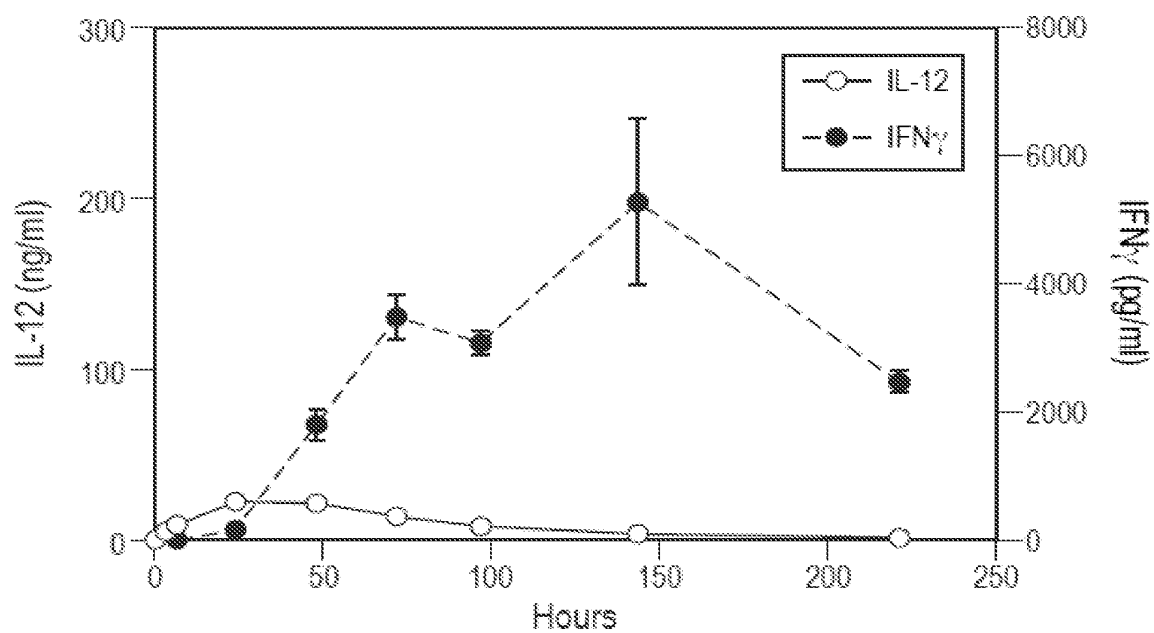


FIG. 12D



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FIG. 12E



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FIG. 13A

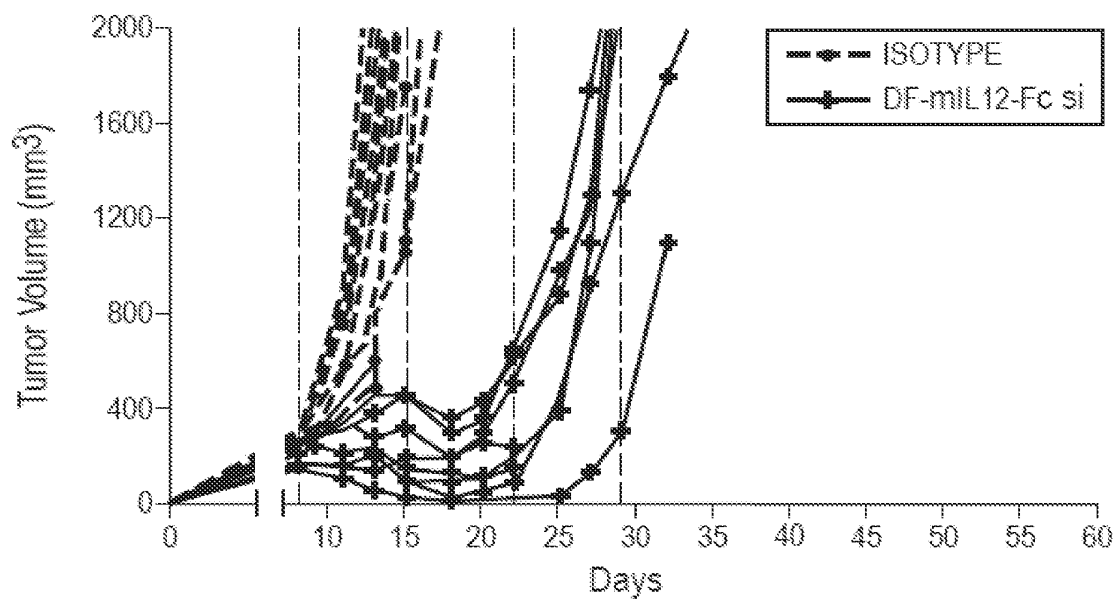
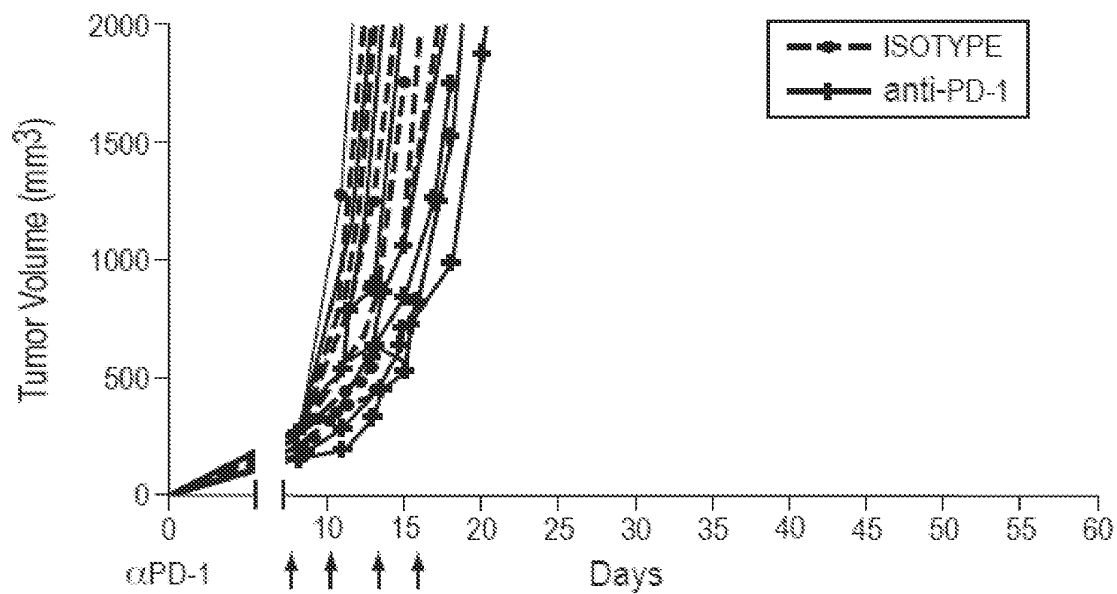
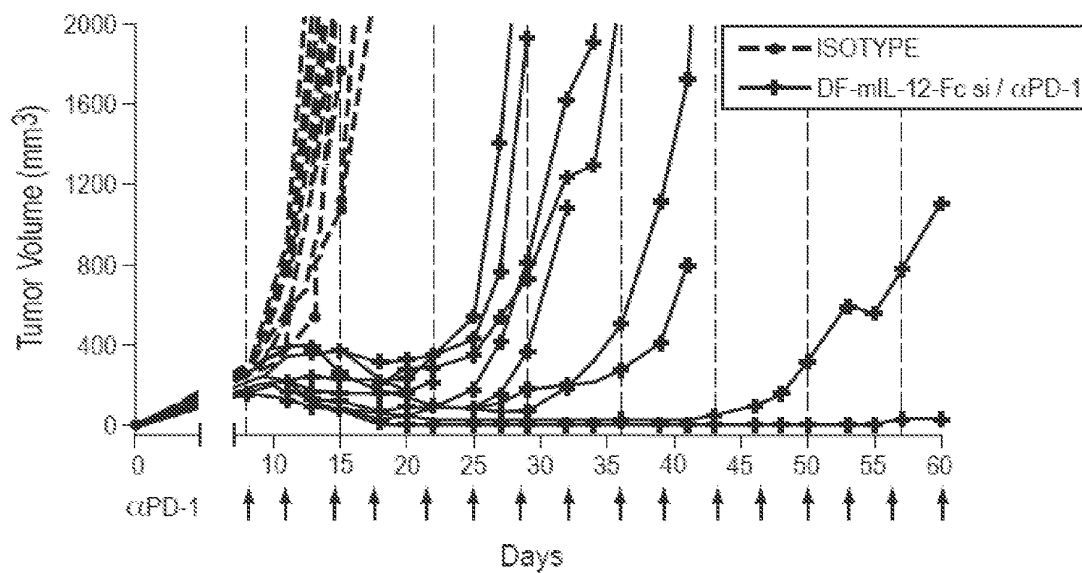


FIG. 13B



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FIG. 13C



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FIG. 14A

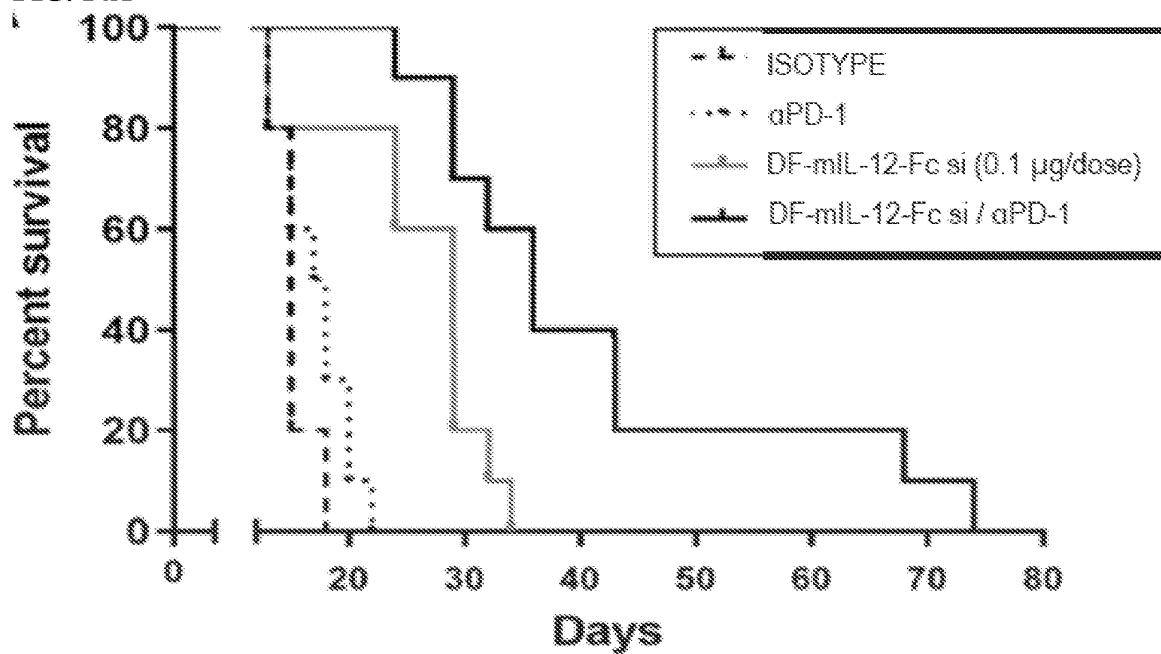
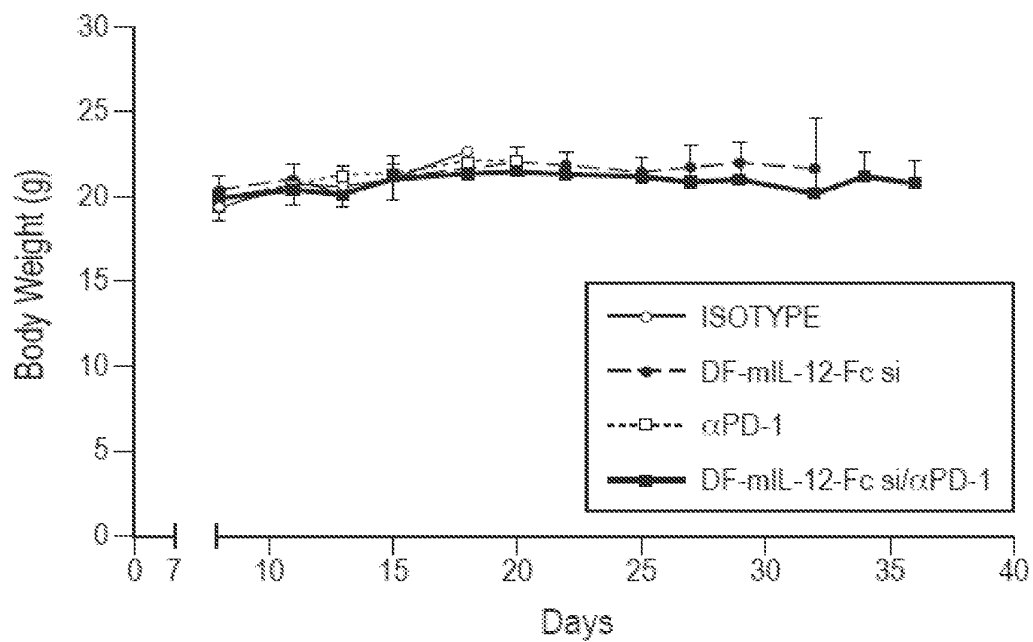


FIG. 14B



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FIG. 15A

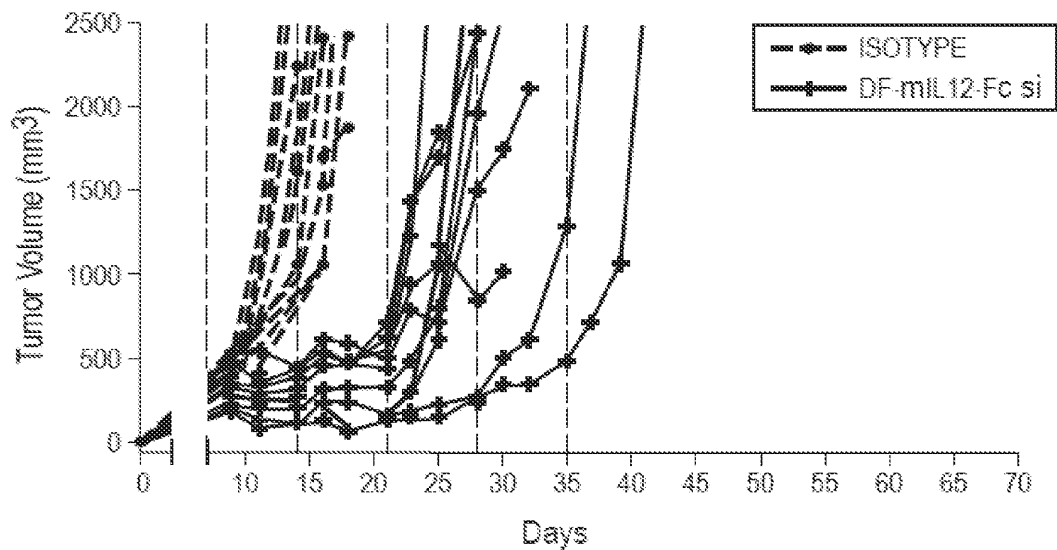
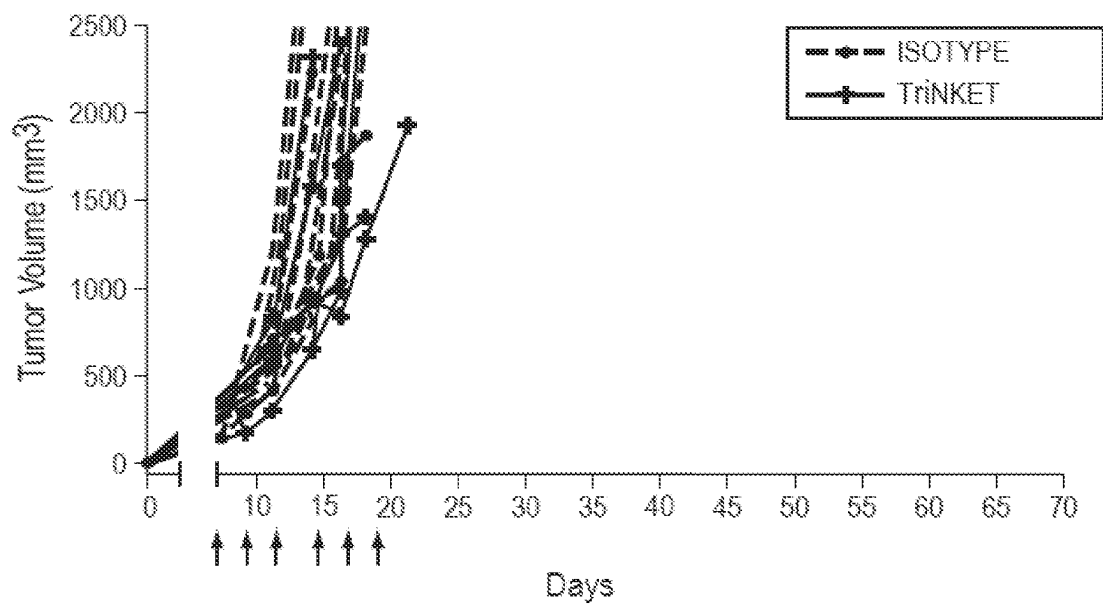
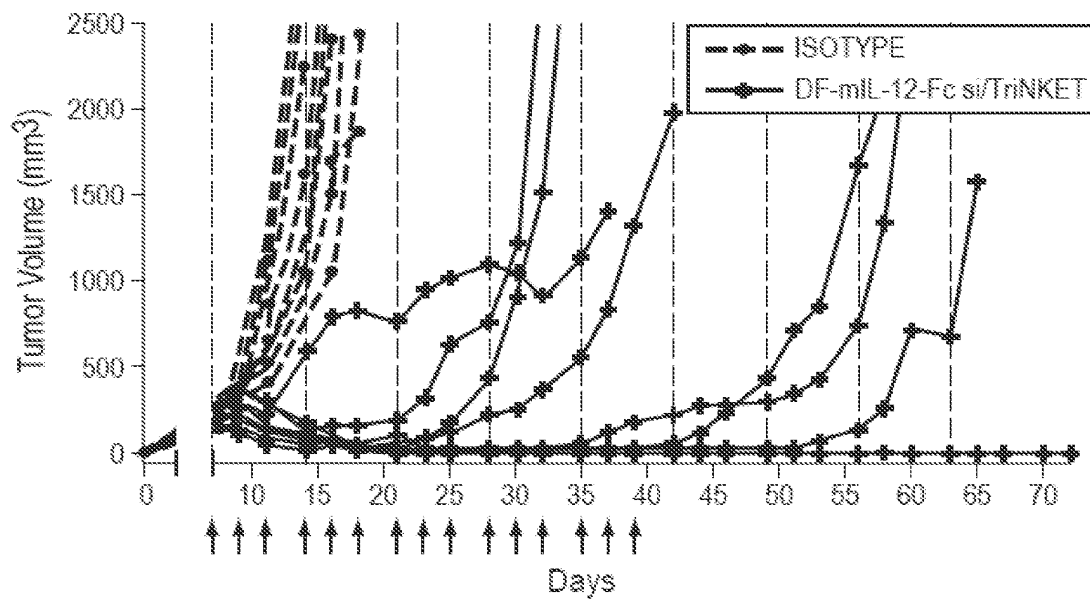


FIG. 15B



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FIG. 15C



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FIG. 16A

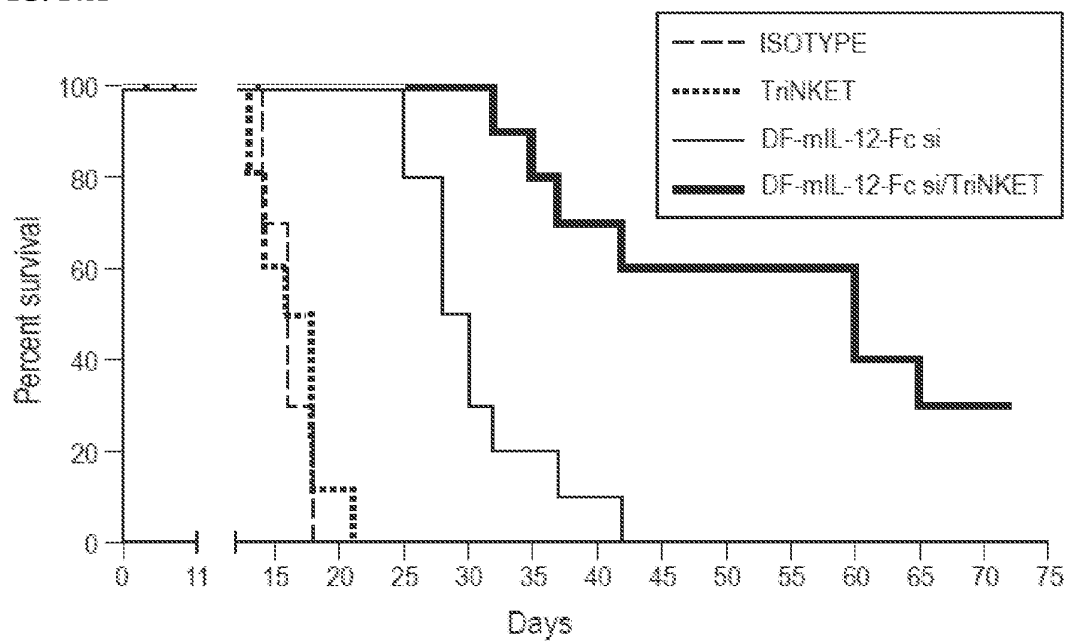
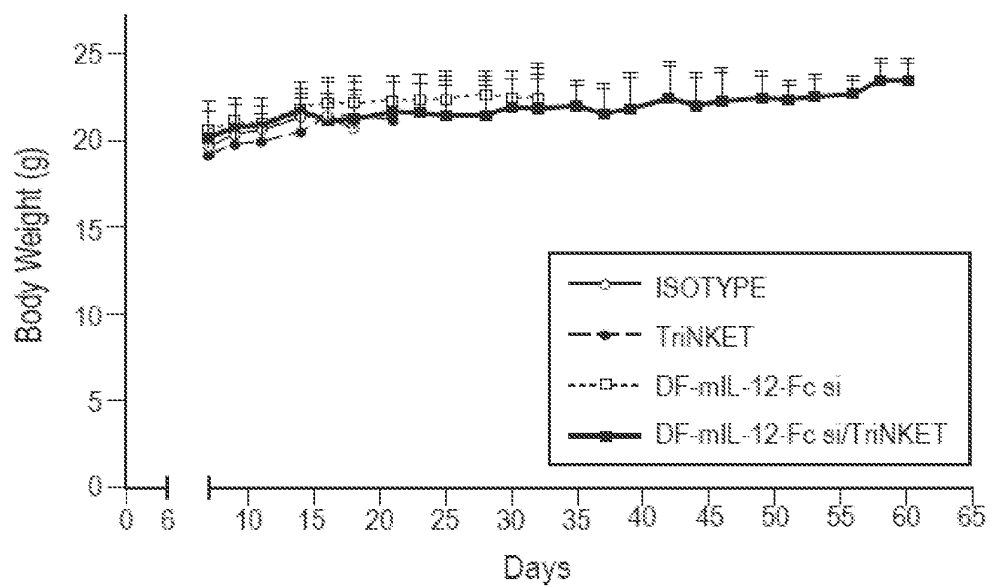
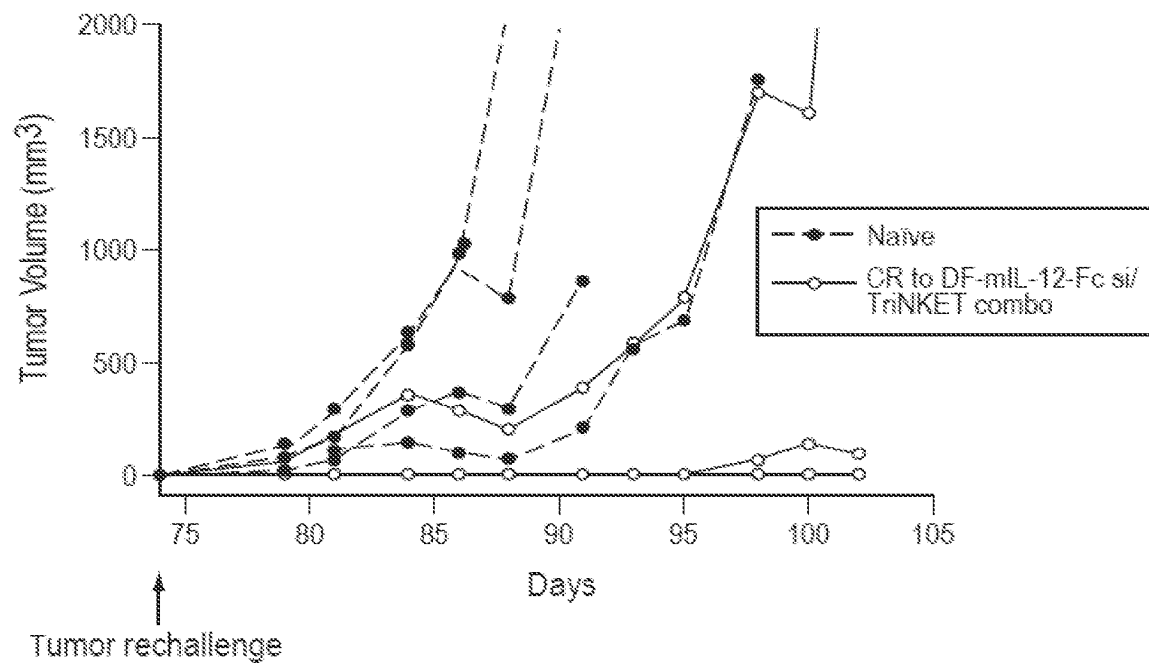


FIG. 16B



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FIG. 17



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FIG. 18A

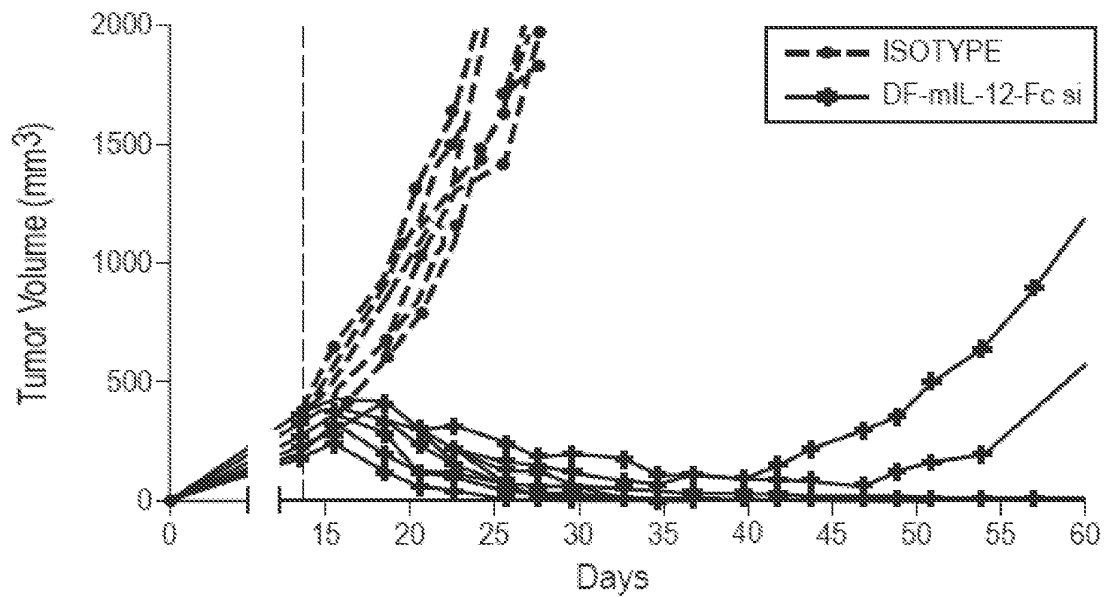
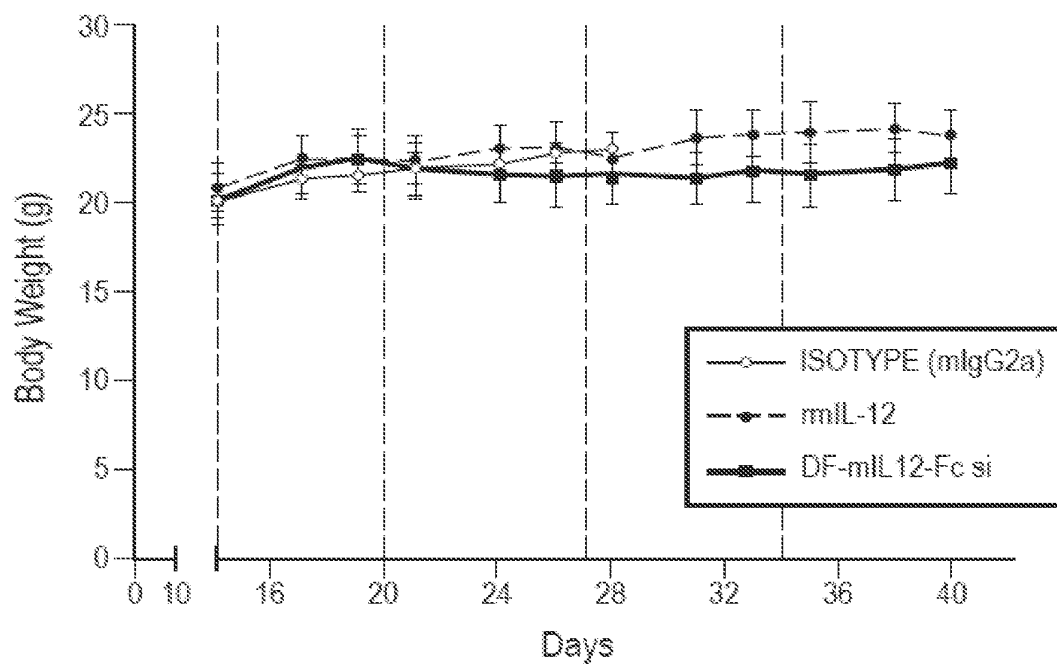
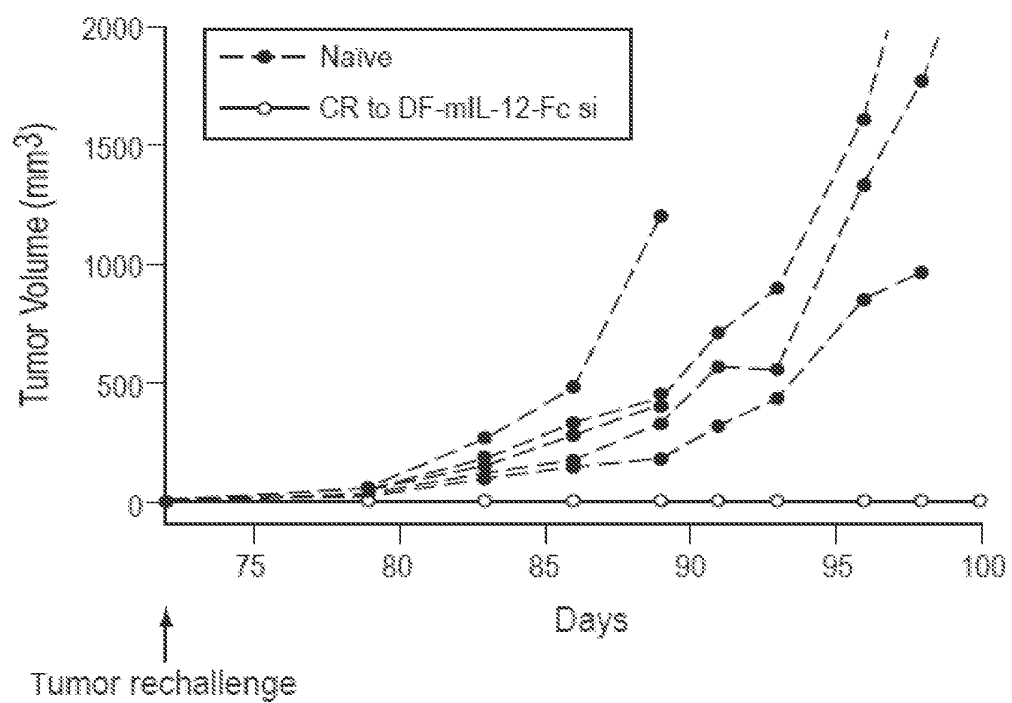


FIG. 18B



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FIG. 18C



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FIG. 19A

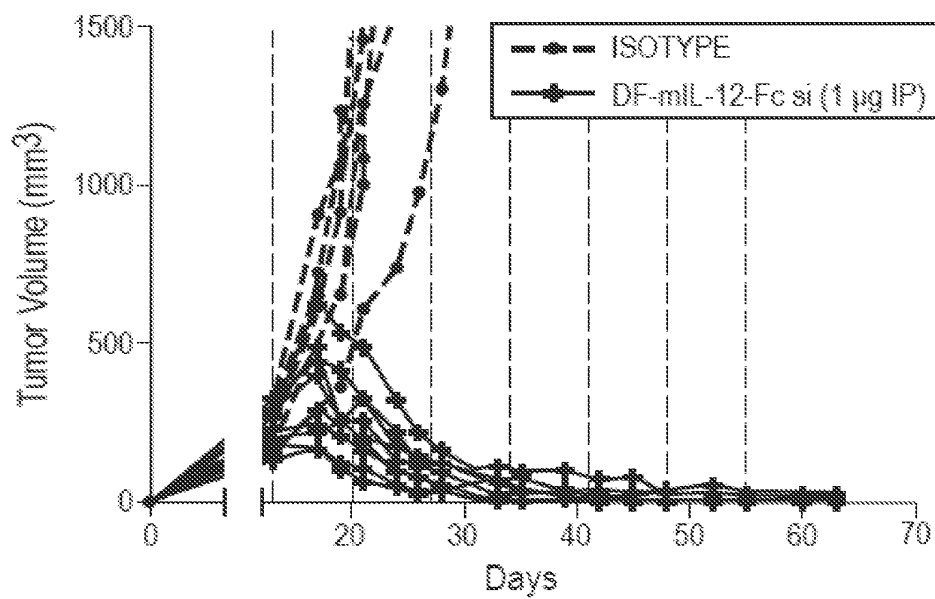
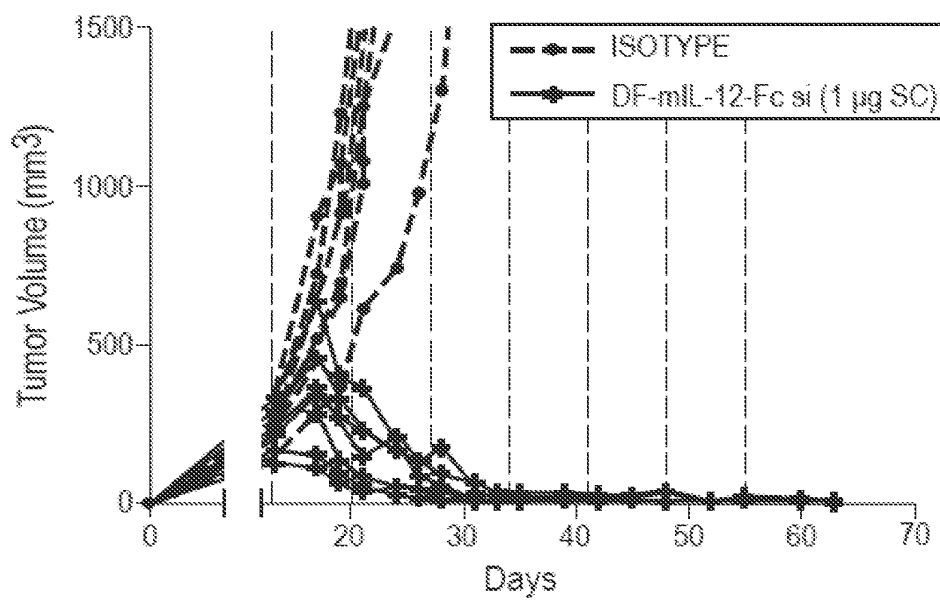
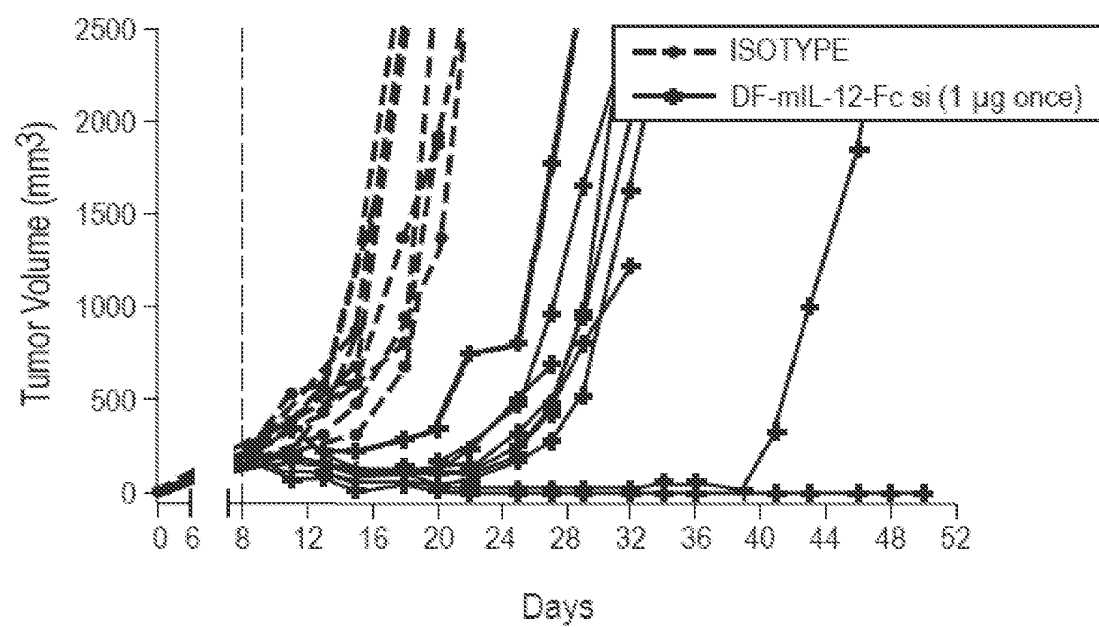


FIG. 19B



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FIG. 20



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FIG. 21A

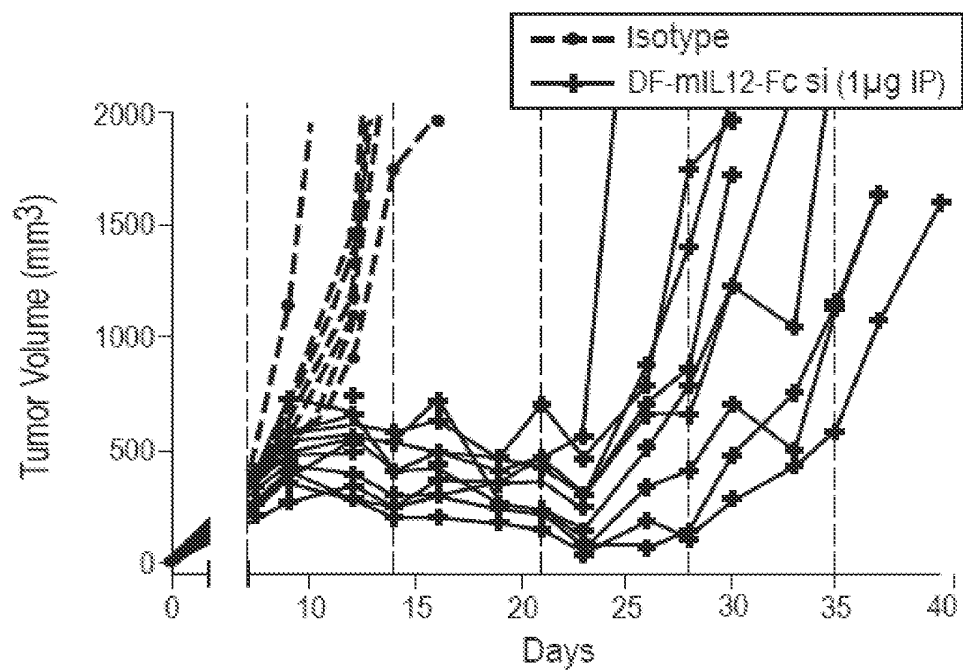
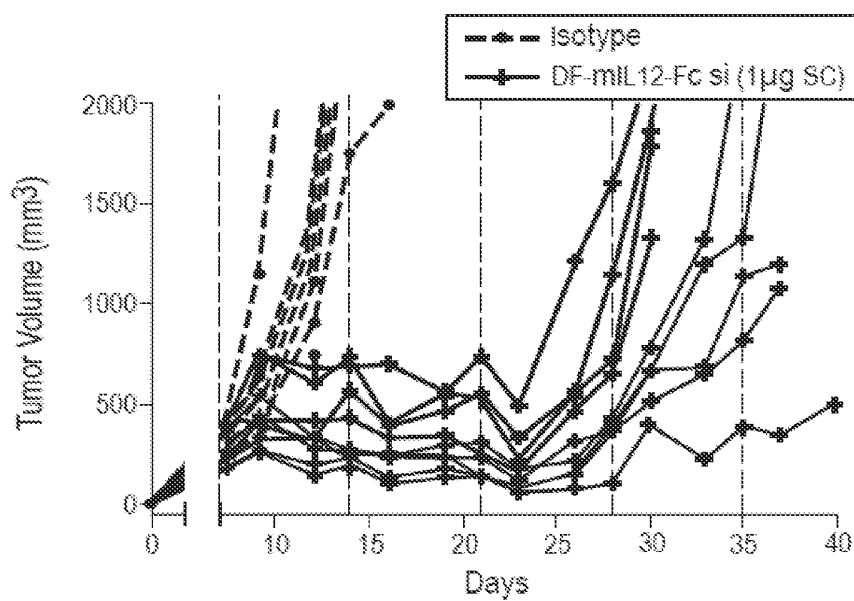


FIG. 21B



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FIG. 22A

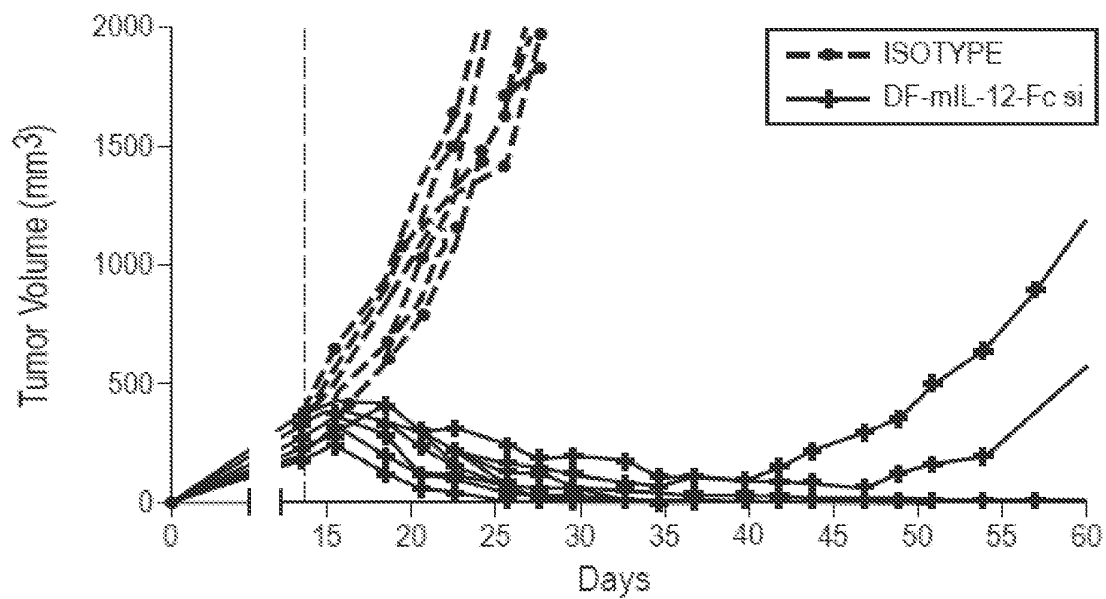
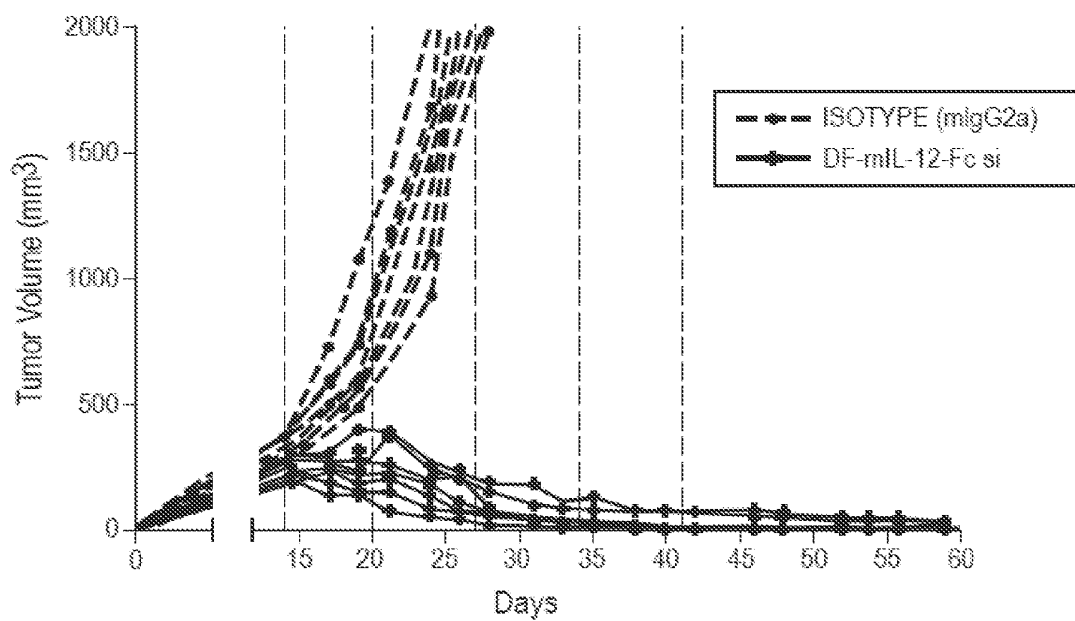


FIG. 22B



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FIG. 23A

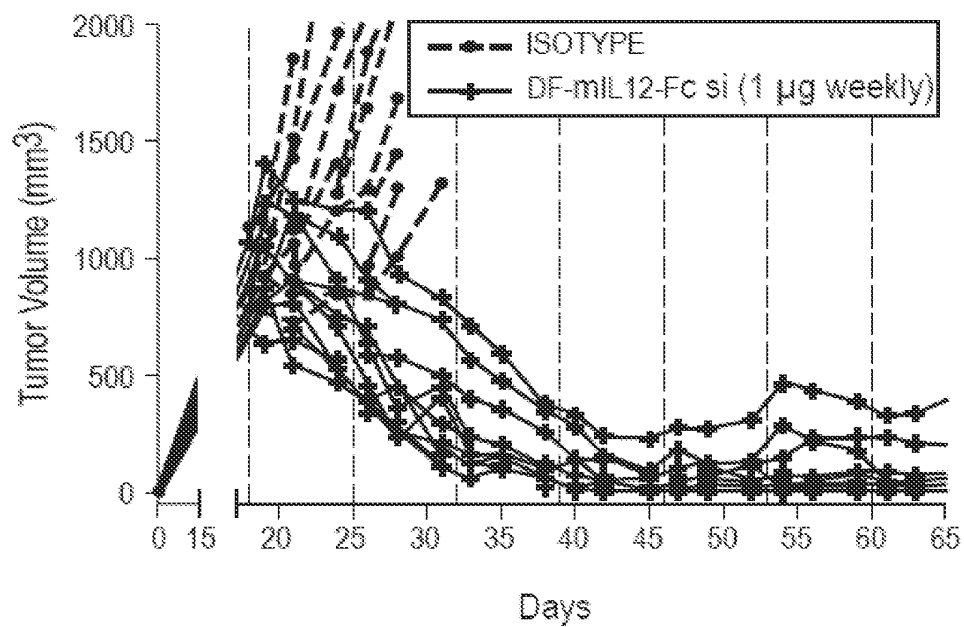
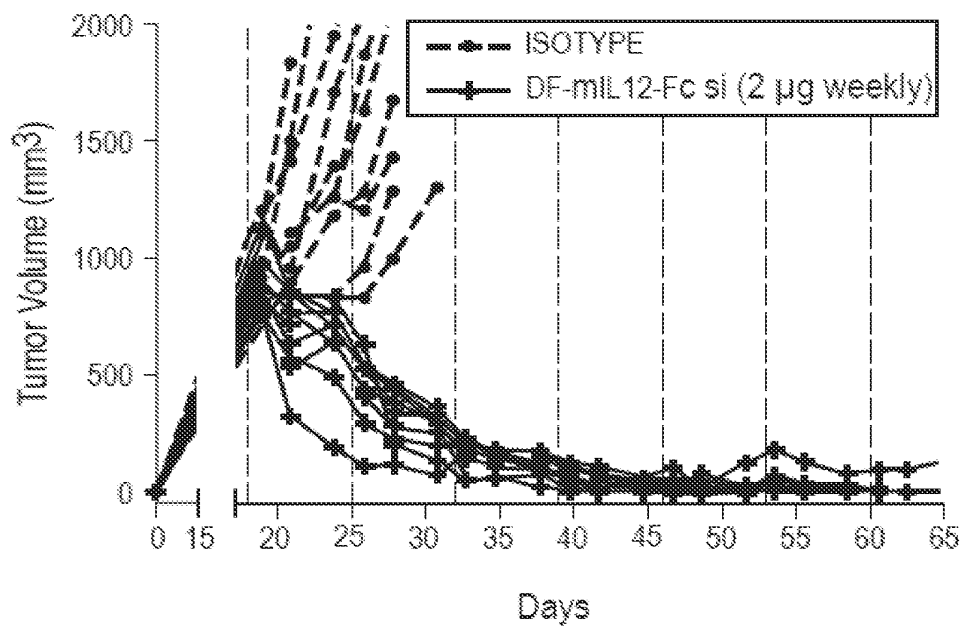


FIG. 23B



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FIG. 24A

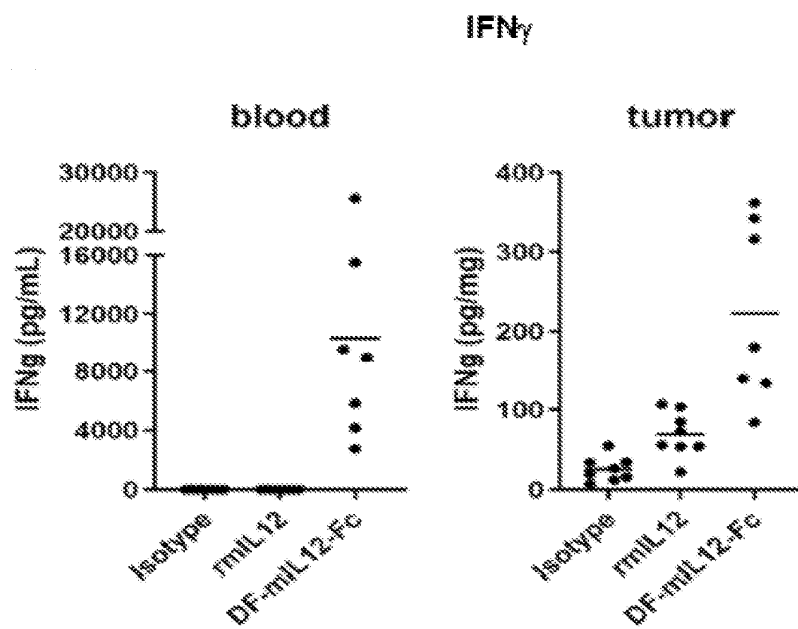
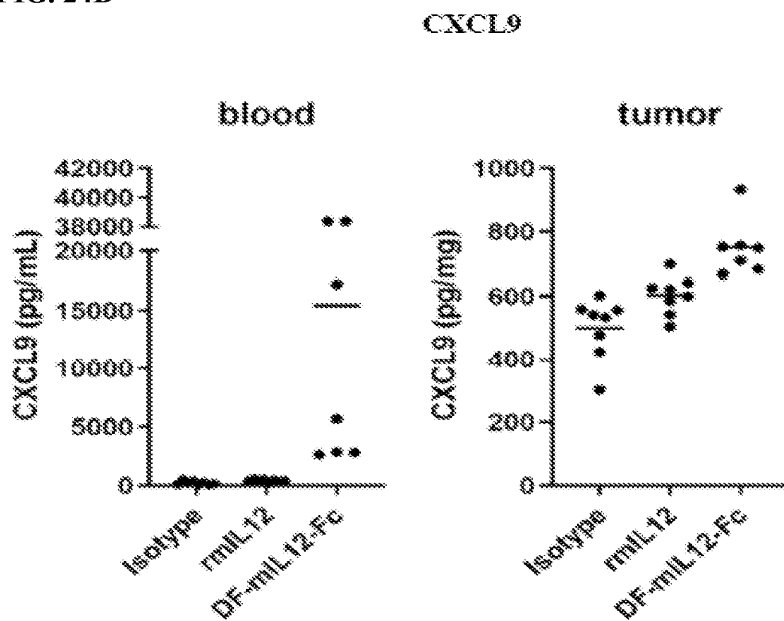


FIG. 24B



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FIG. 24C

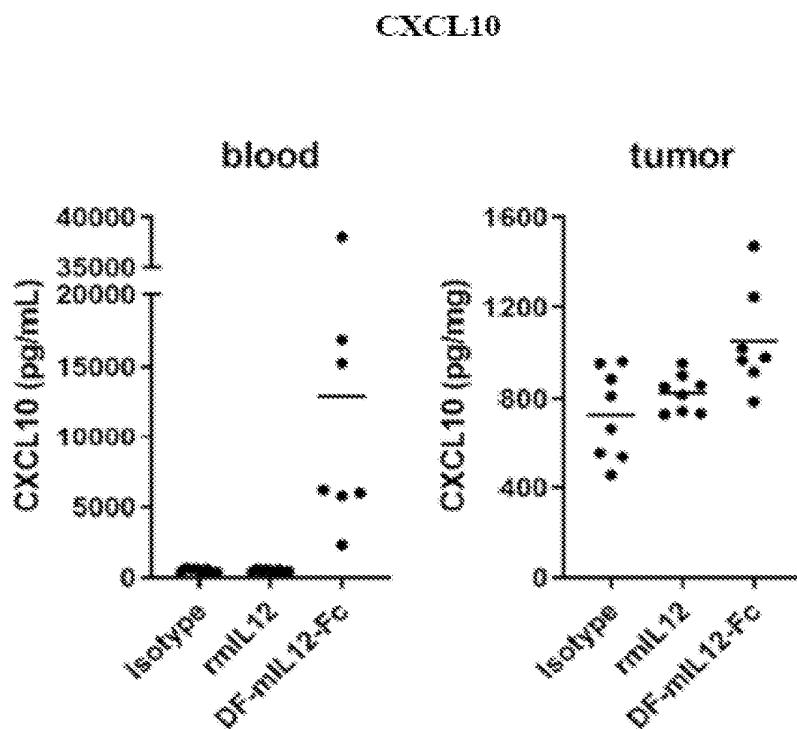
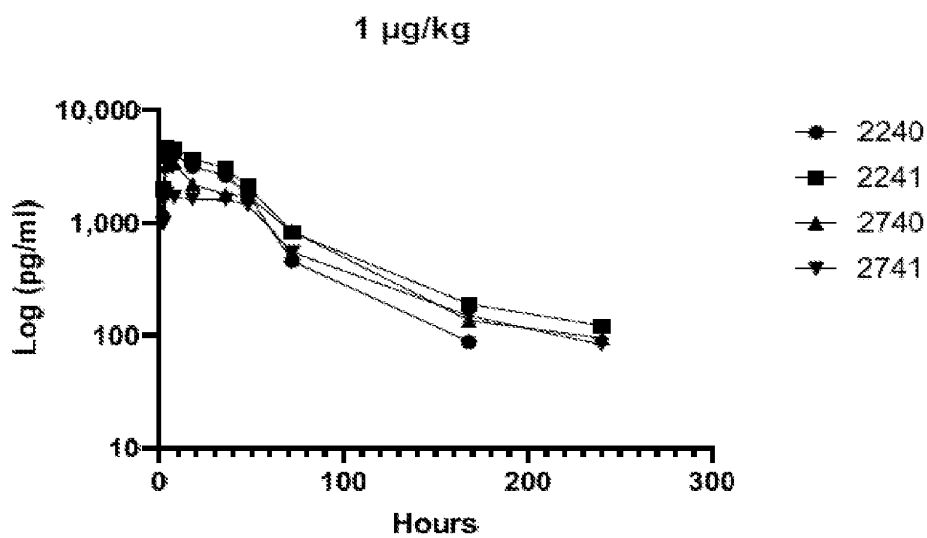


FIG. 25A



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FIG. 25B

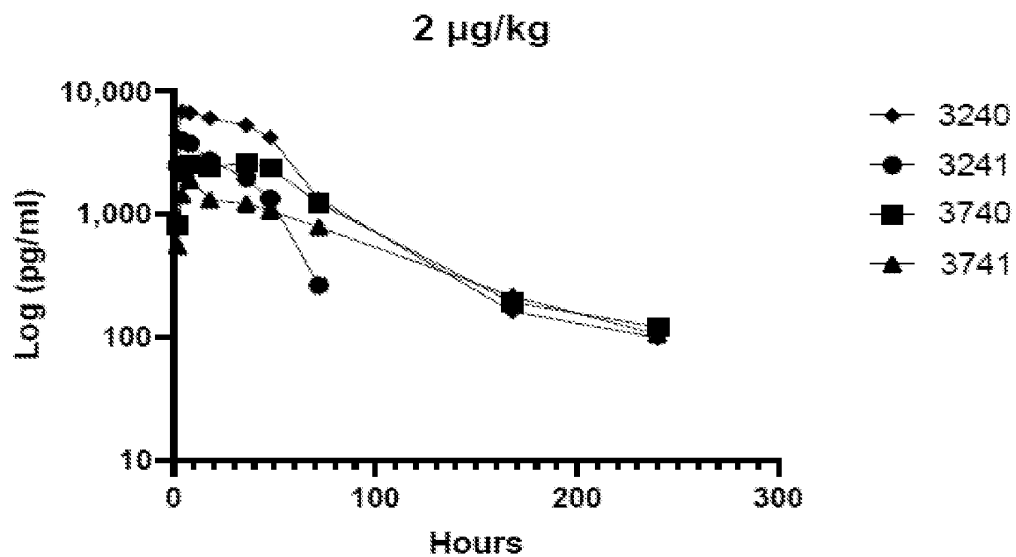
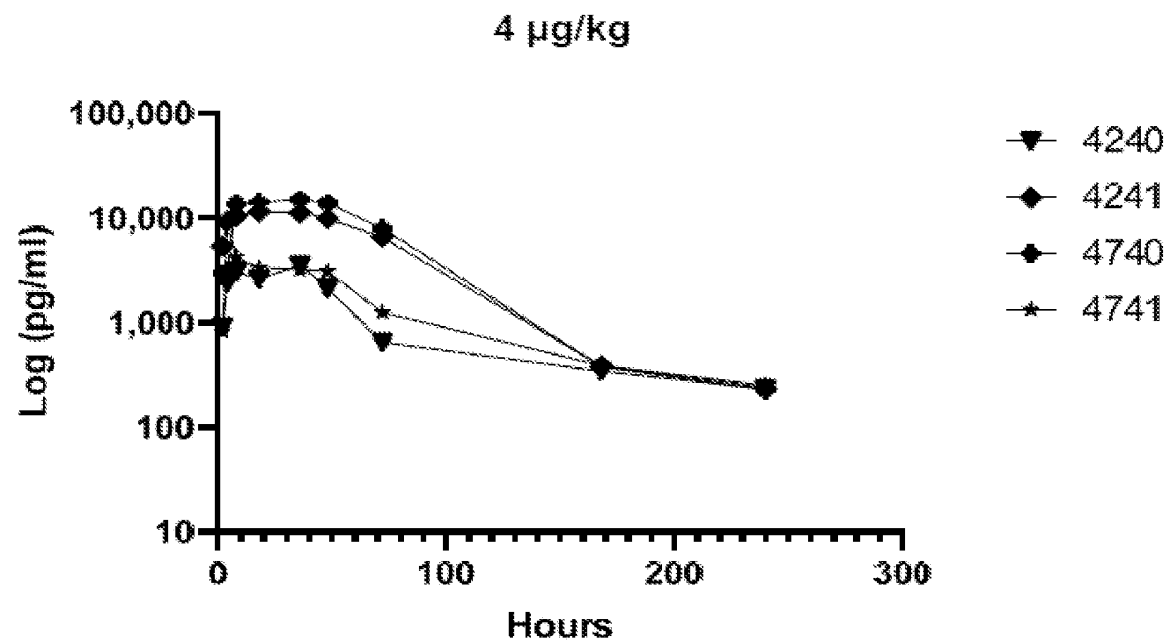


FIG. 25C



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FIG. 26A

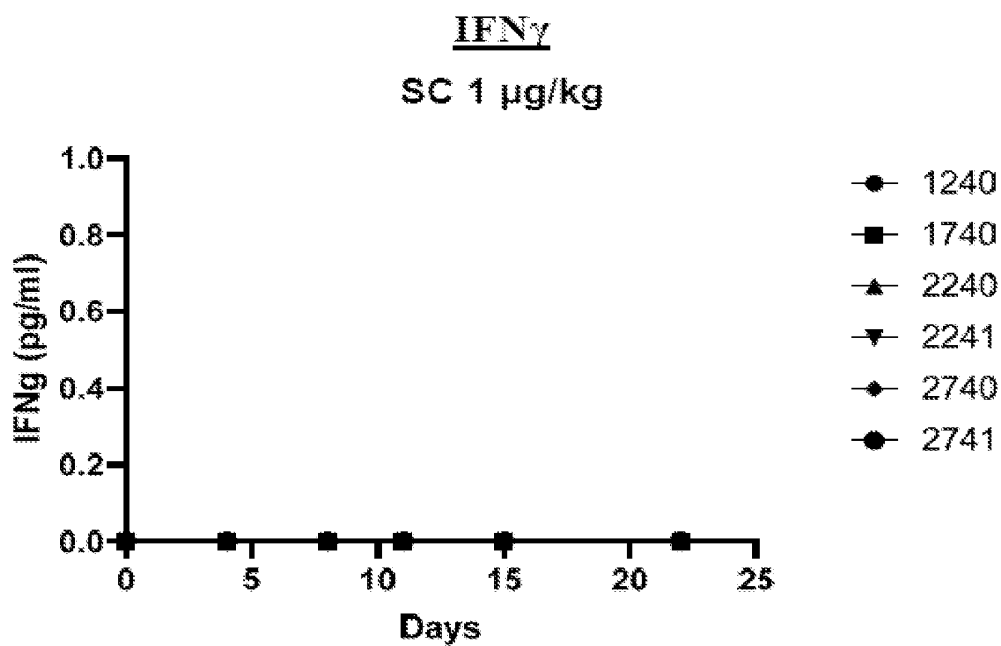
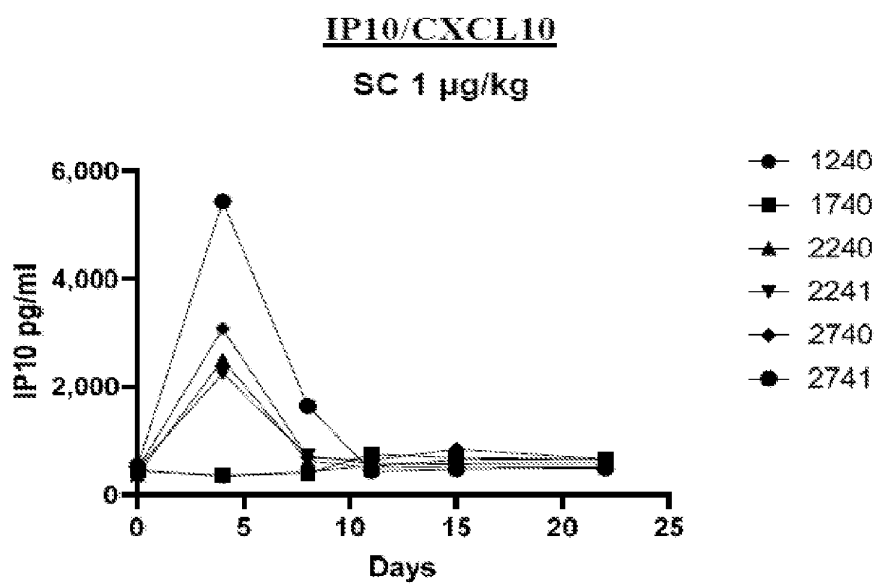


FIG. 26B



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FIG. 26C

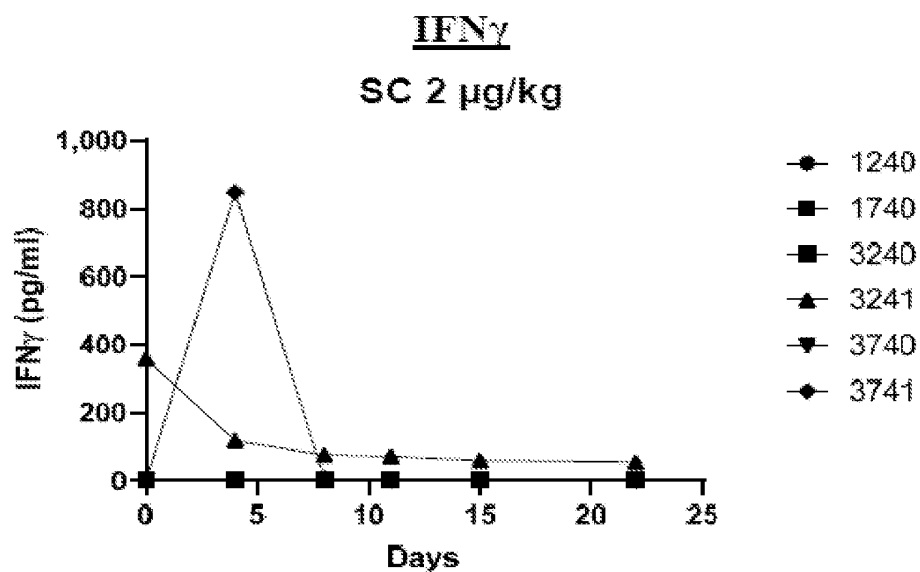
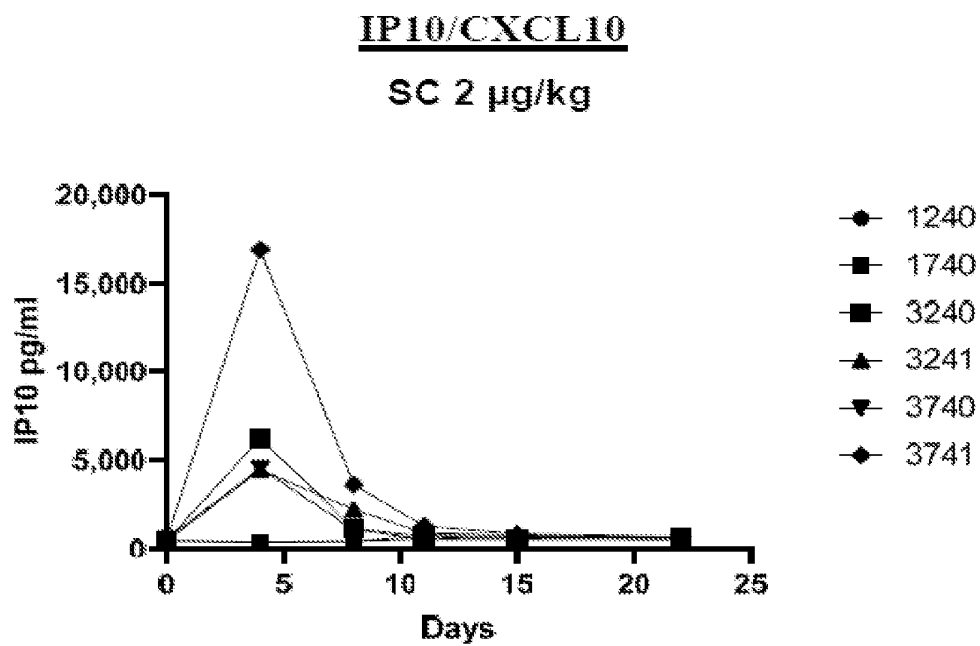


FIG. 26D



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FIG. 26E

IFN γ
SC 4 μ g/kg

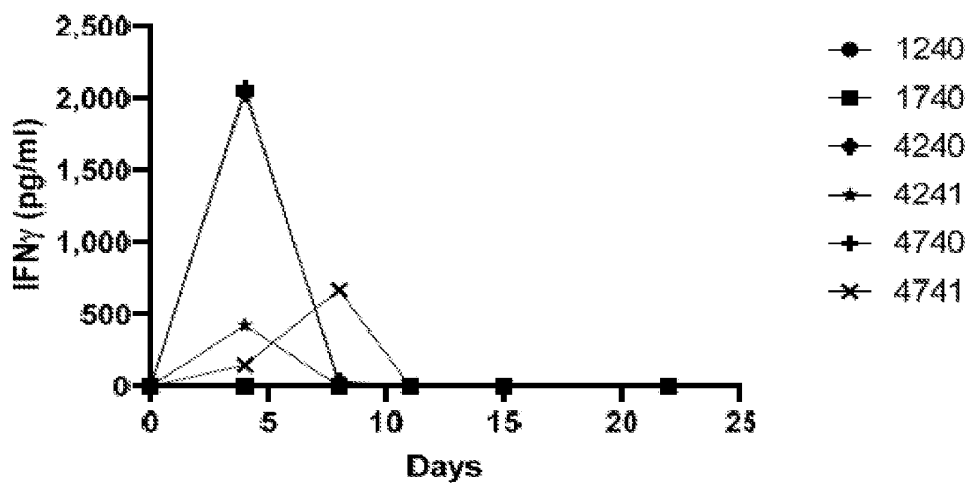
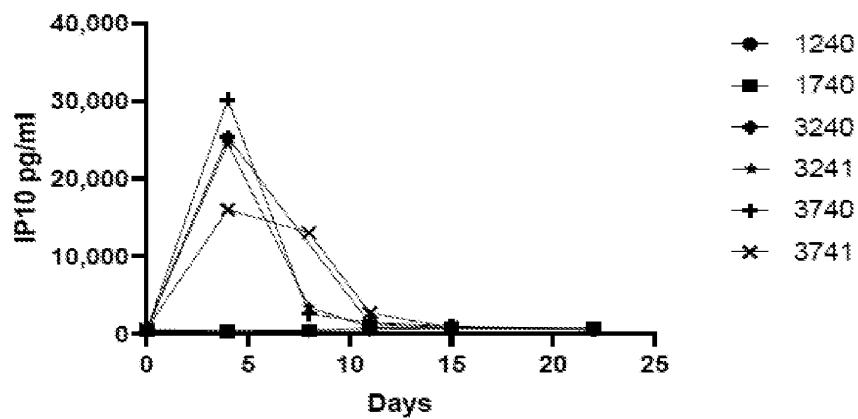


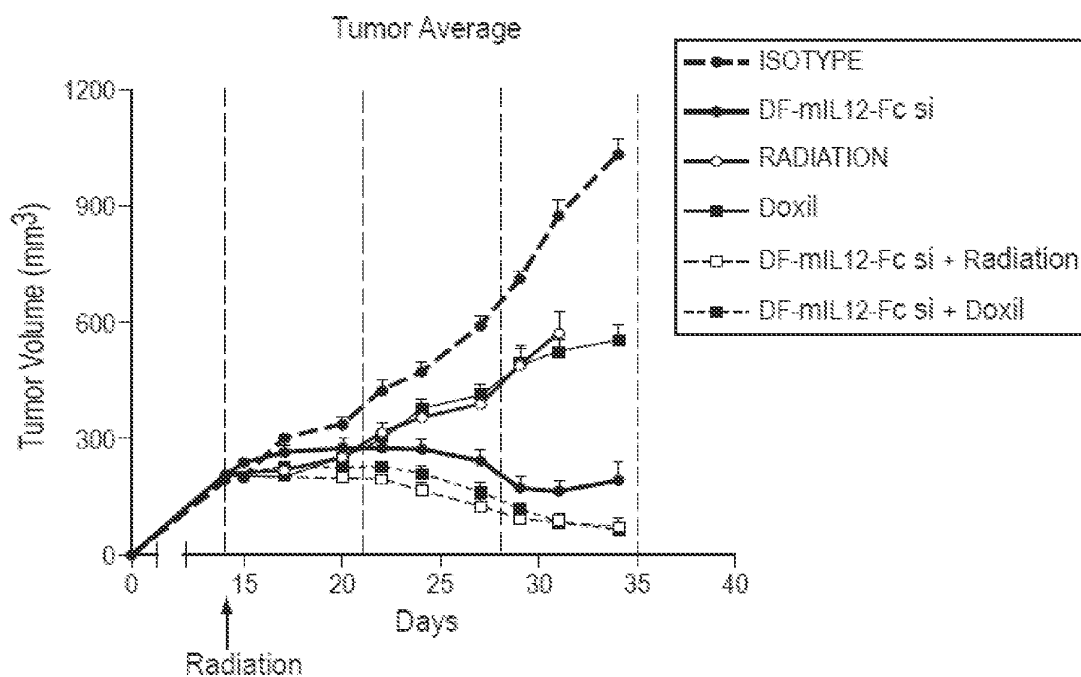
FIG. 26F

IP10/CXCL10
SC 4 μ g/kg



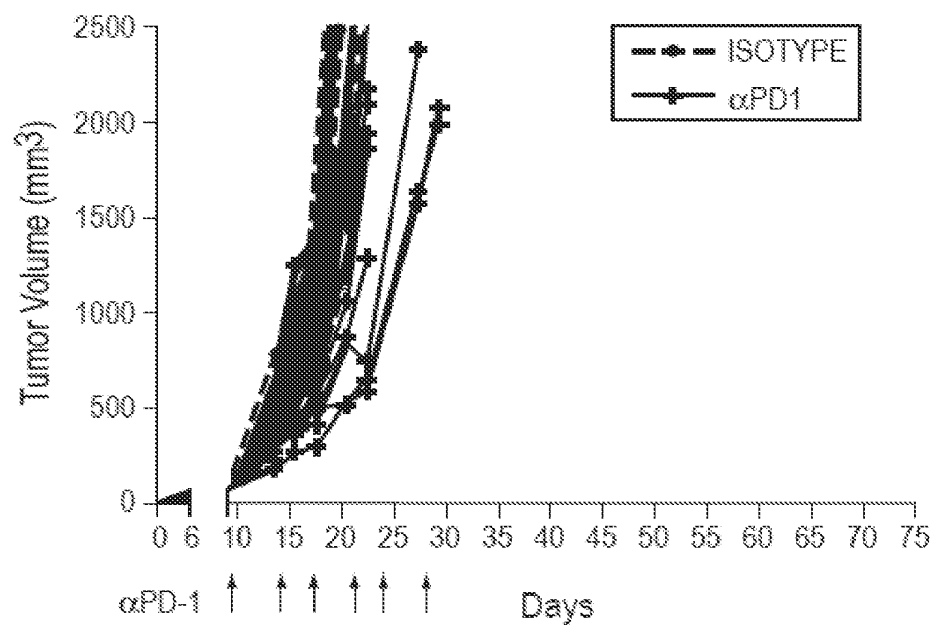
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FIG. 27



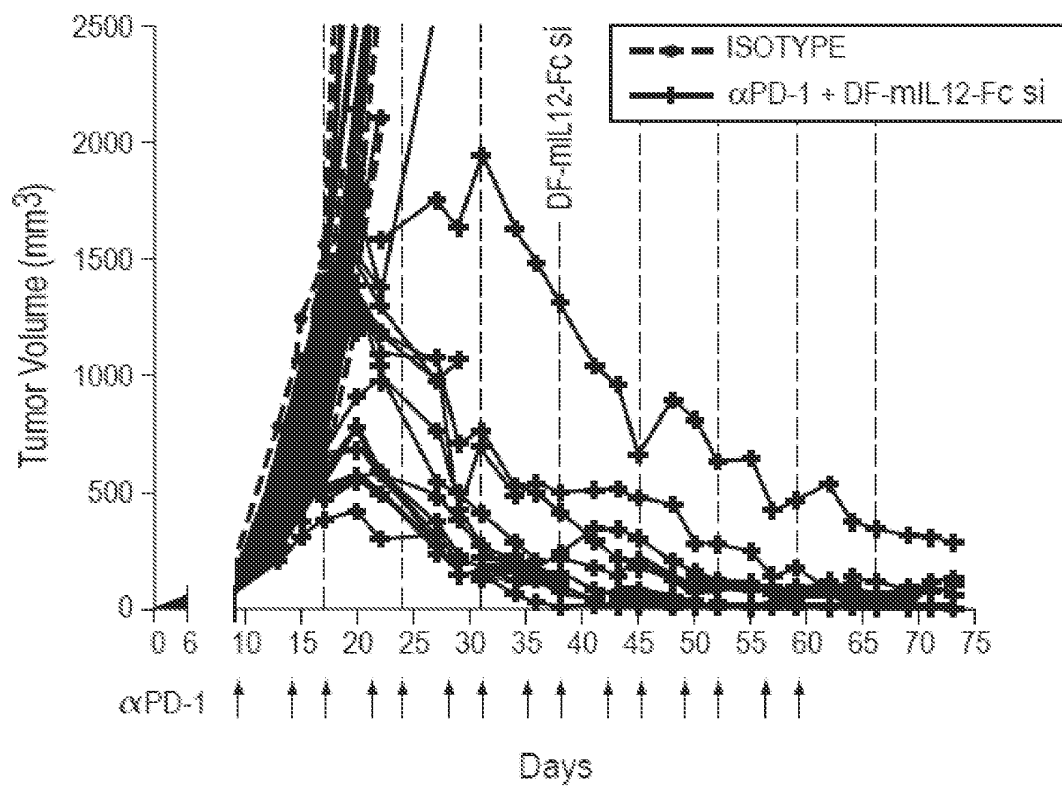
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FIG. 28A



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FIG. 28B



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FIG. 29A

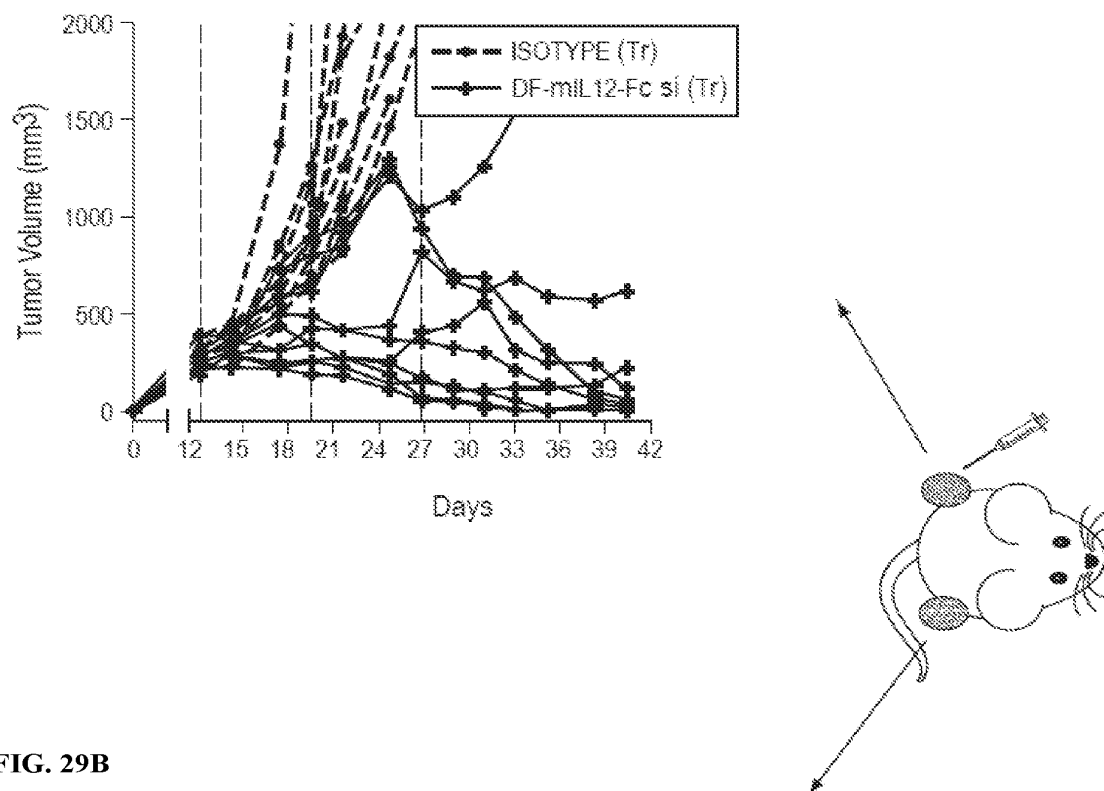
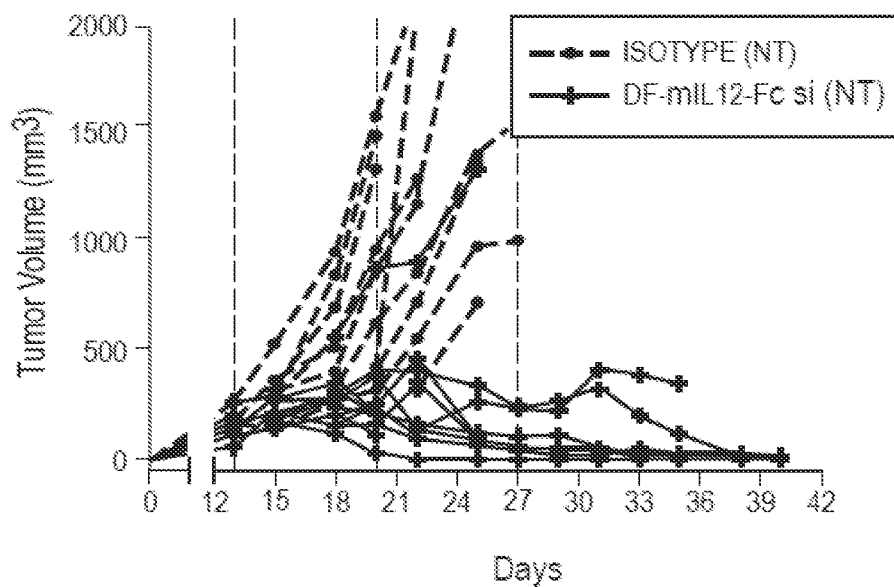


FIG. 29B



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FIG. 30A

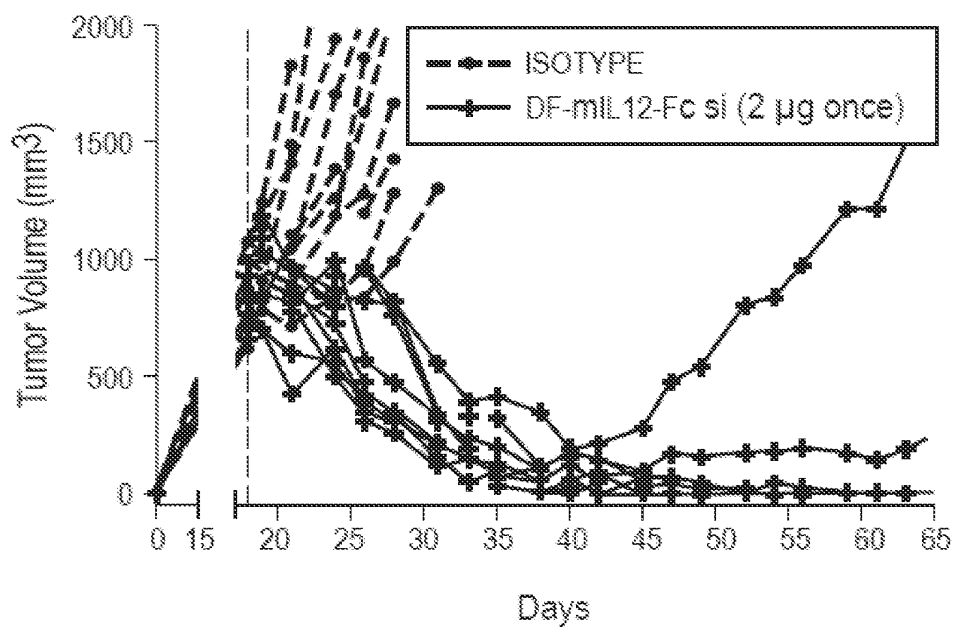
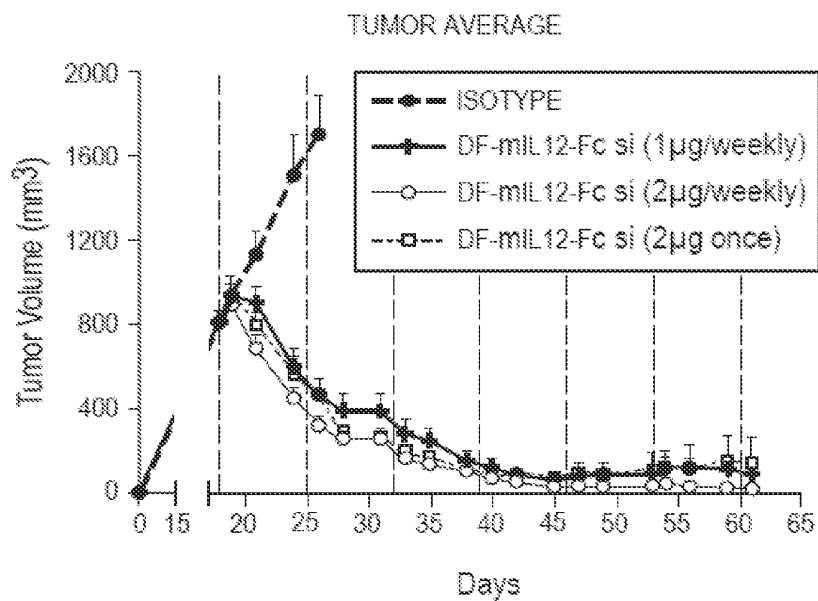


FIG. 30B



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FIG. 31A

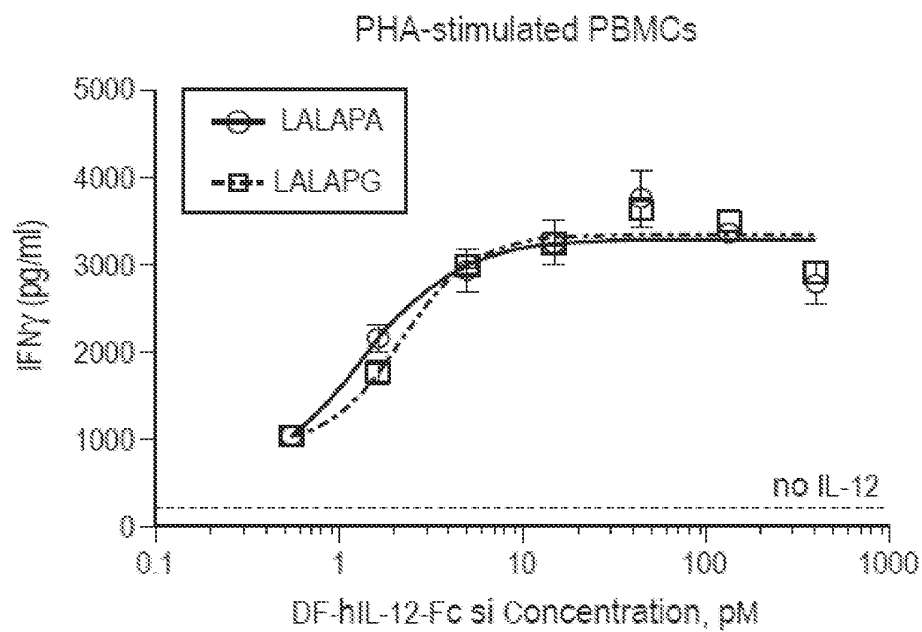


FIG. 31B

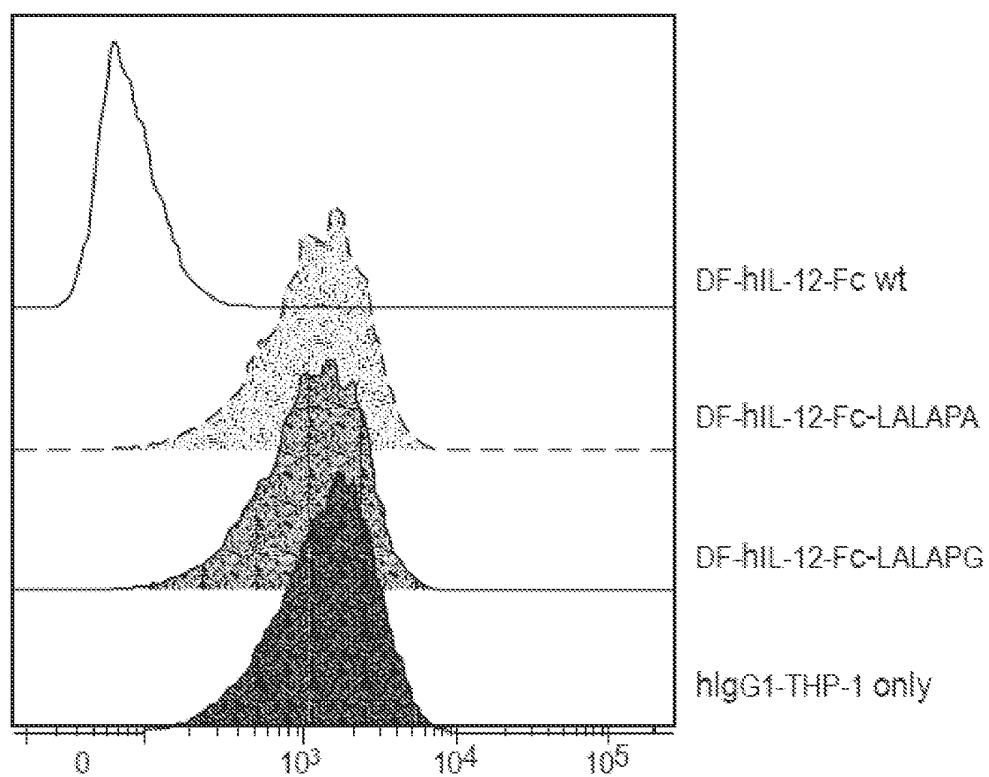
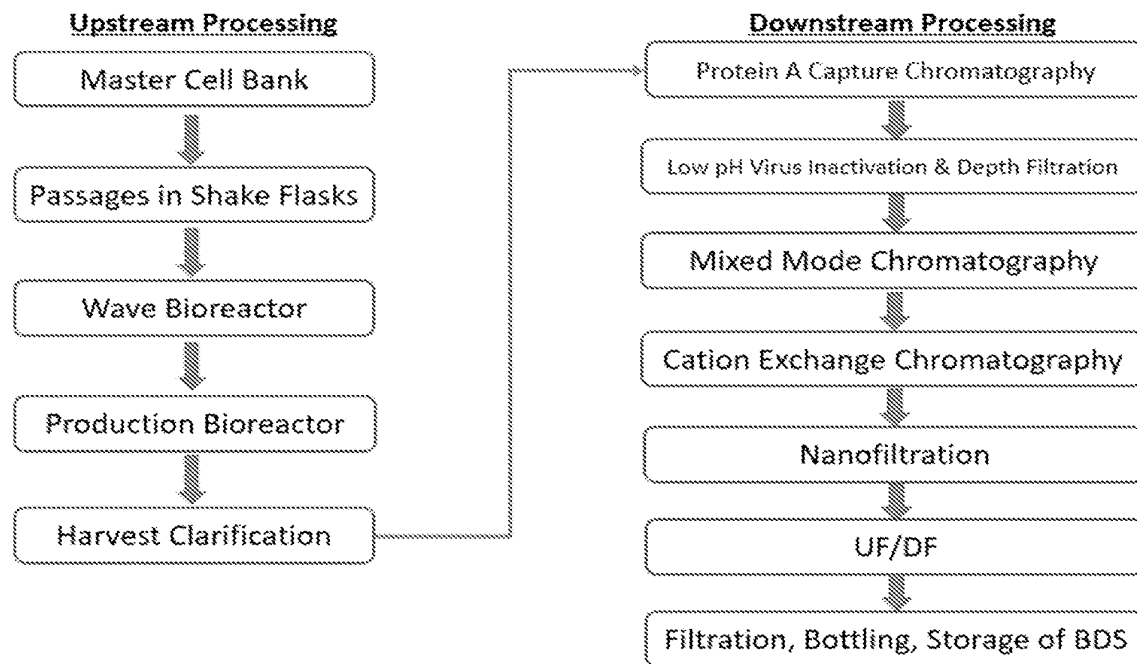
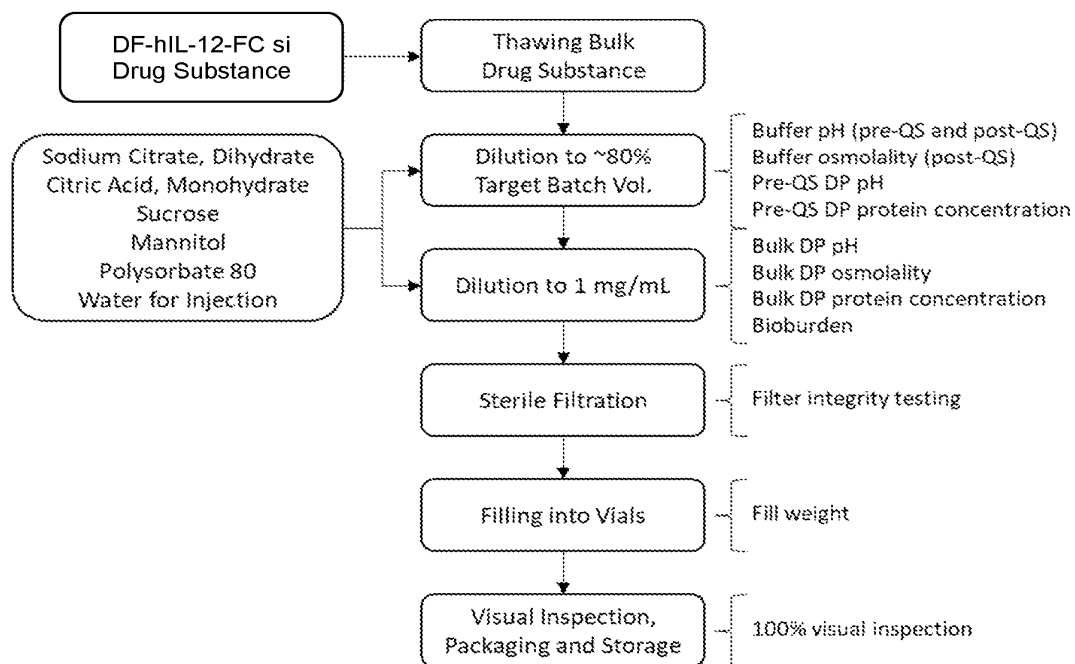


FIG. 32



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FIG. 33



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FIG. 34A

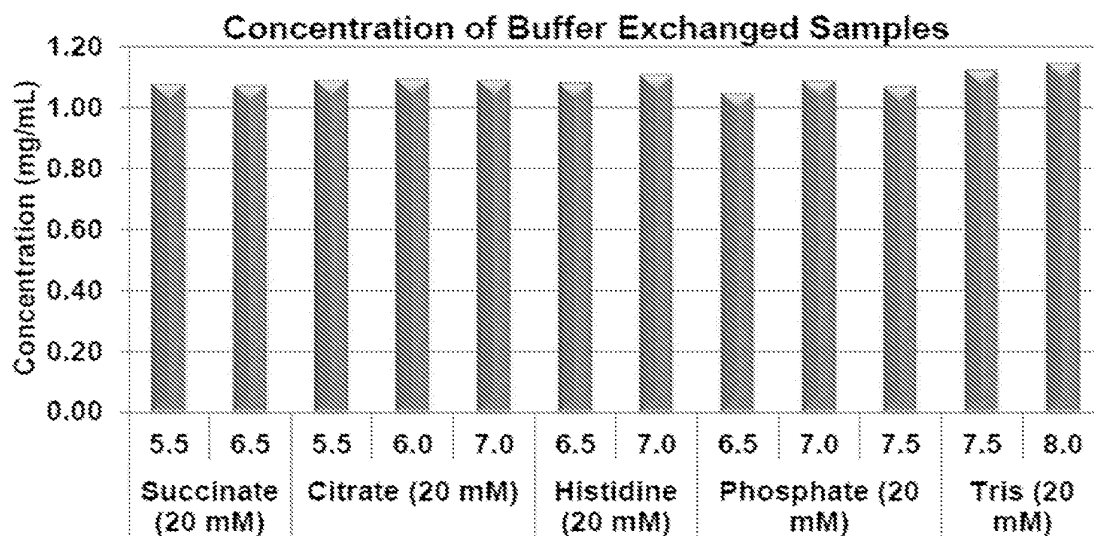


FIG. 34B

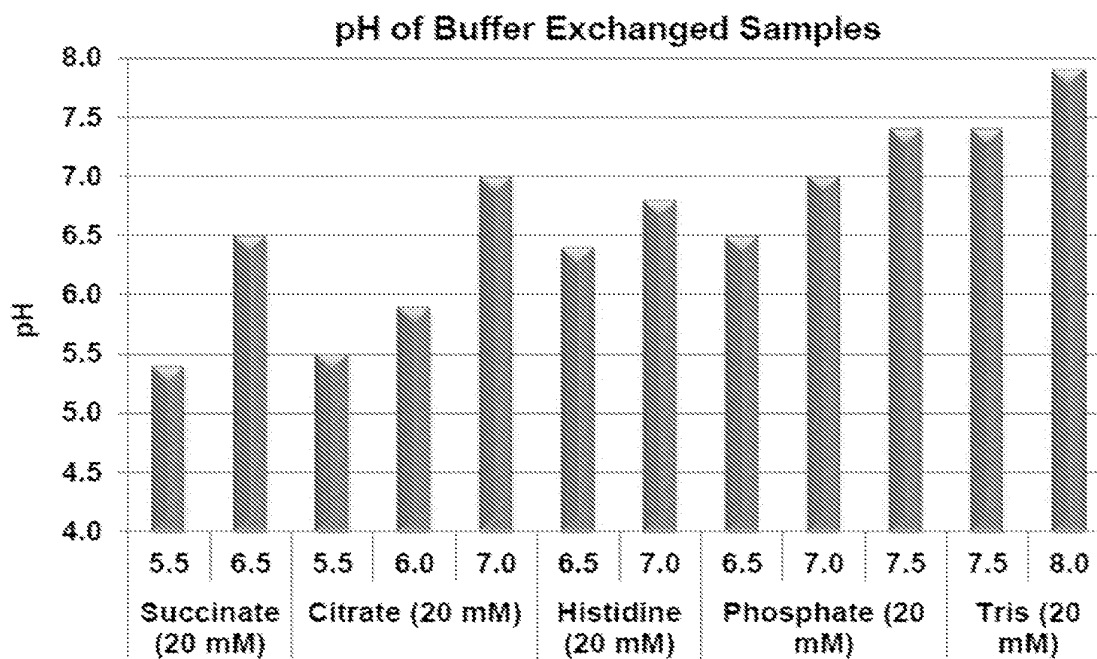


FIG. 35A

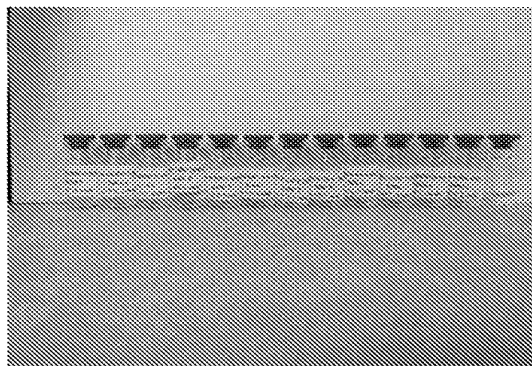
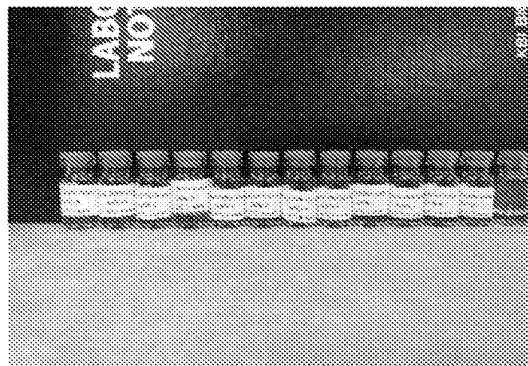
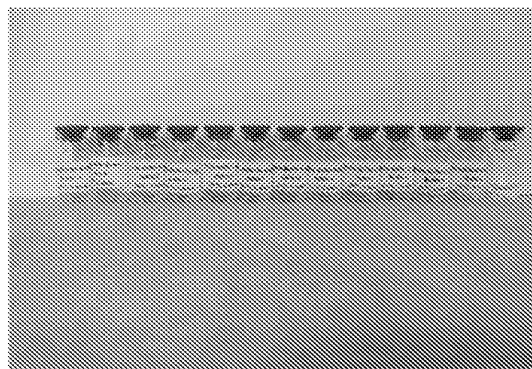
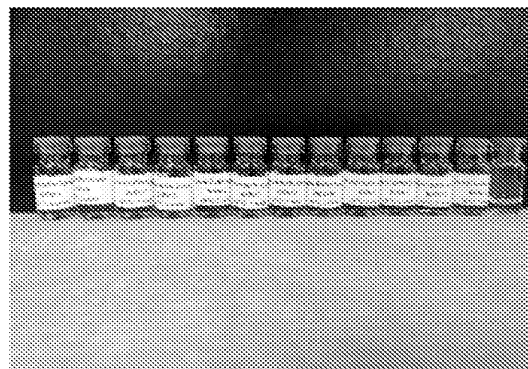


FIG. 35B



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FIG. 36A

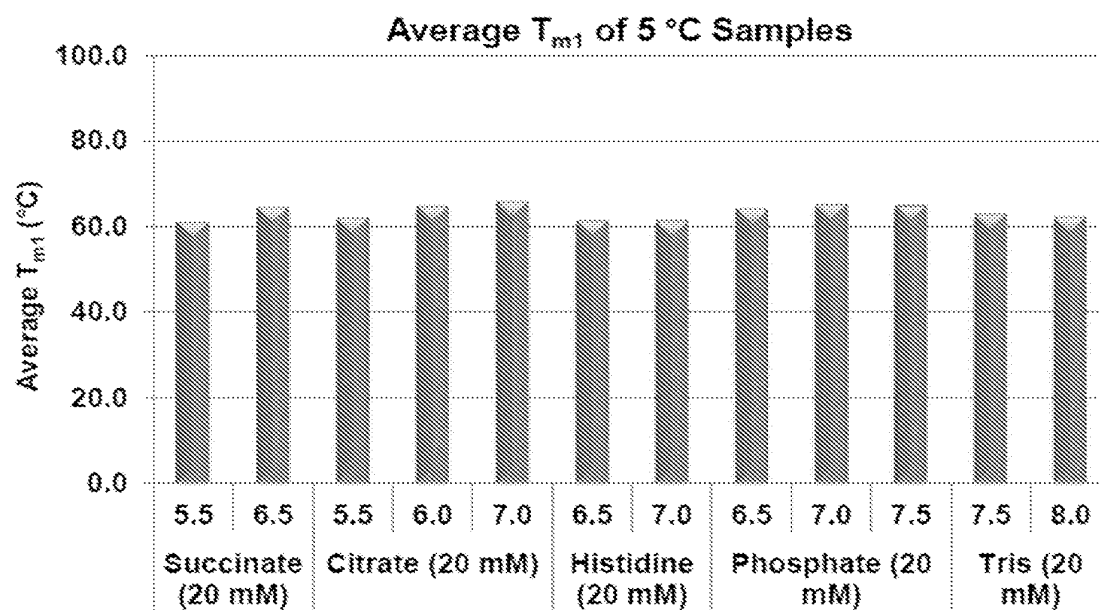
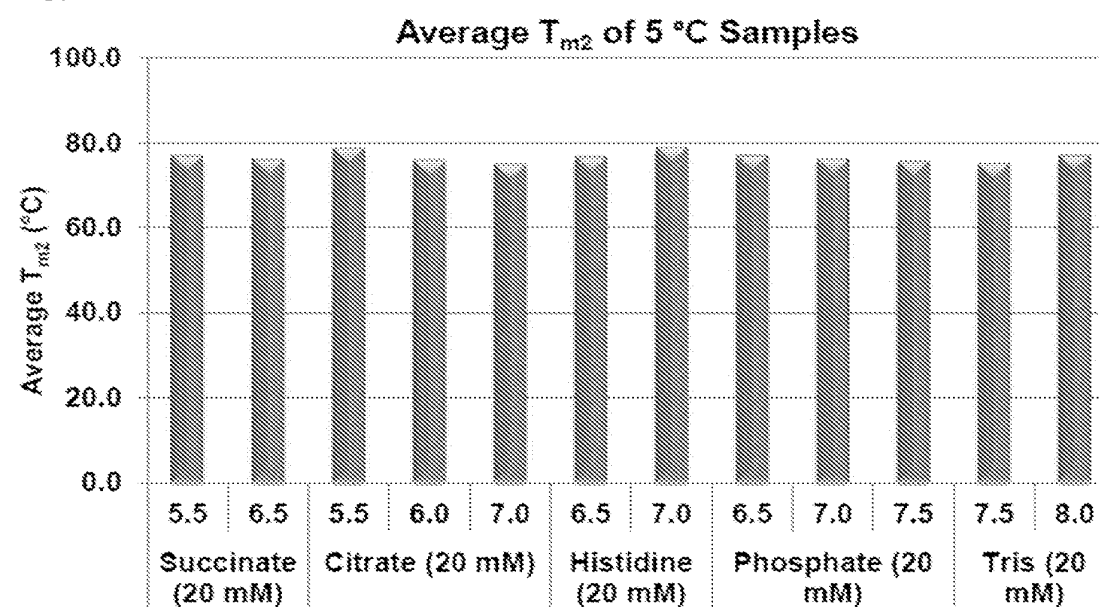


FIG. 36B



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FIG. 36C

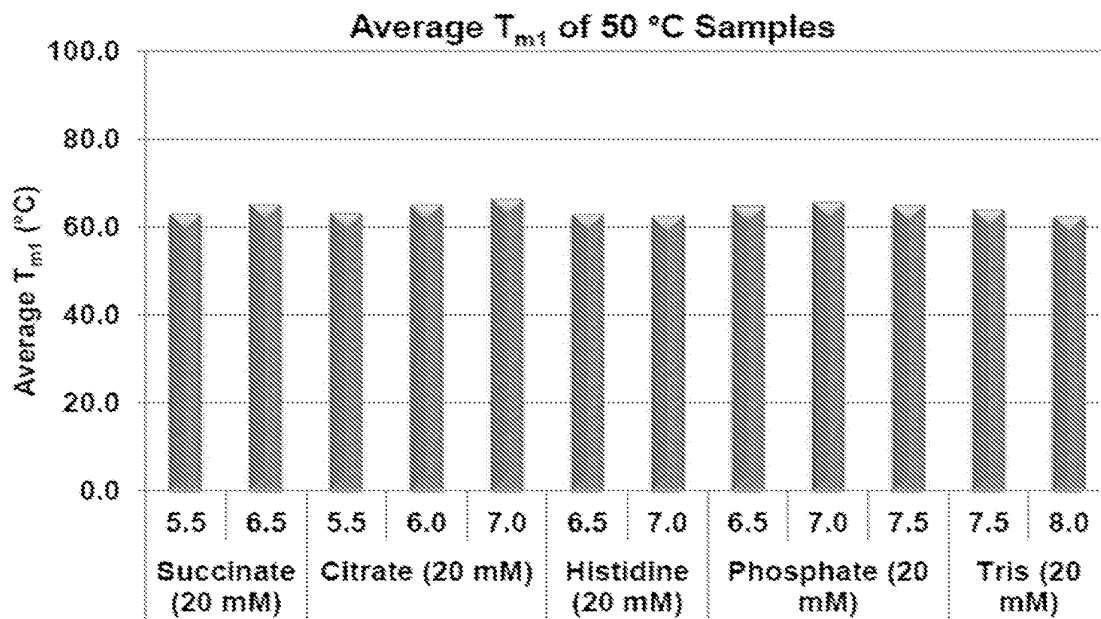
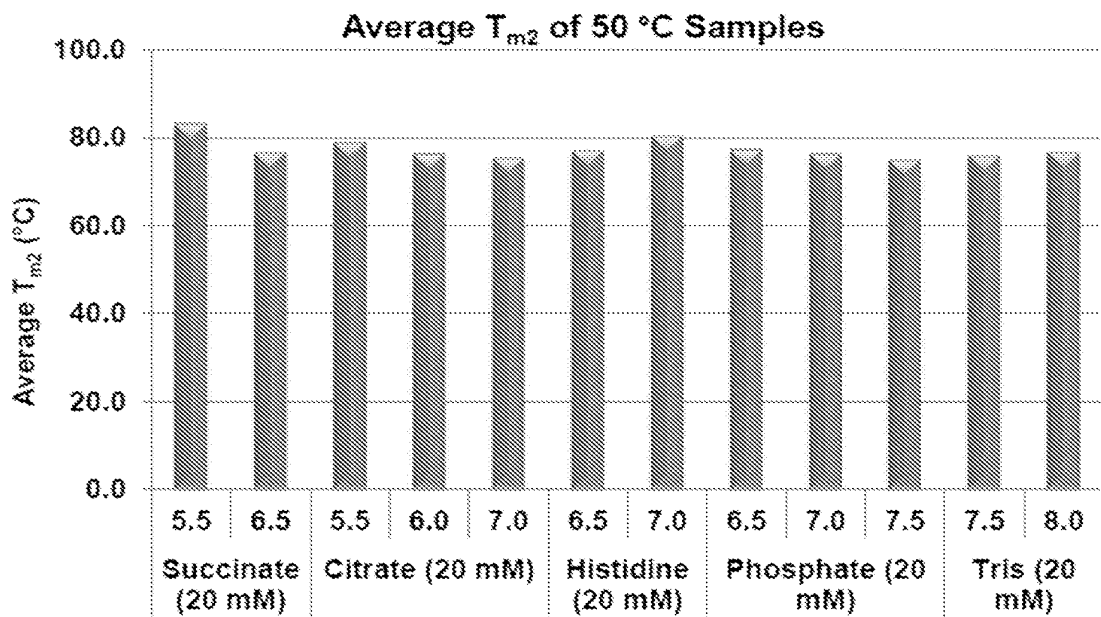


FIG. 36D



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FIG. 36E

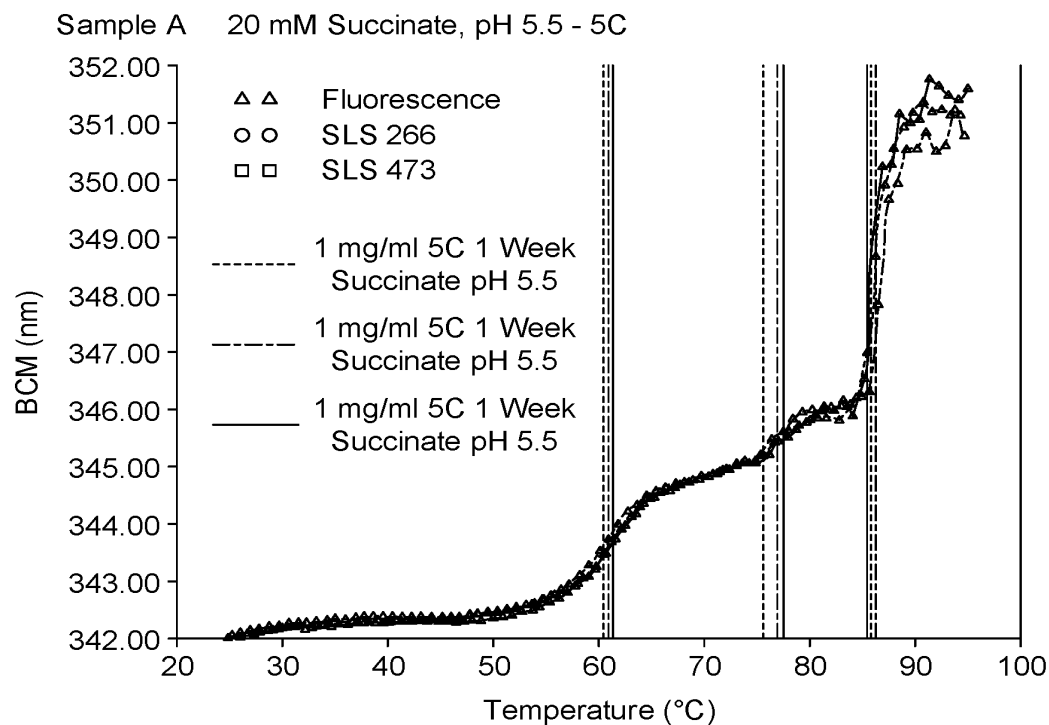
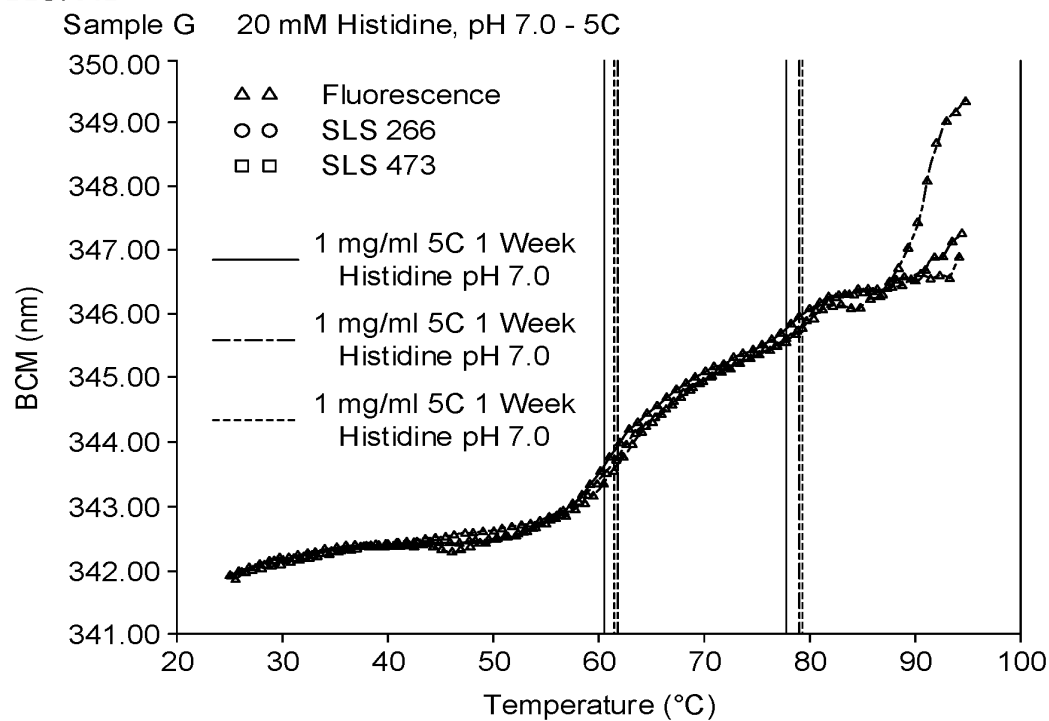
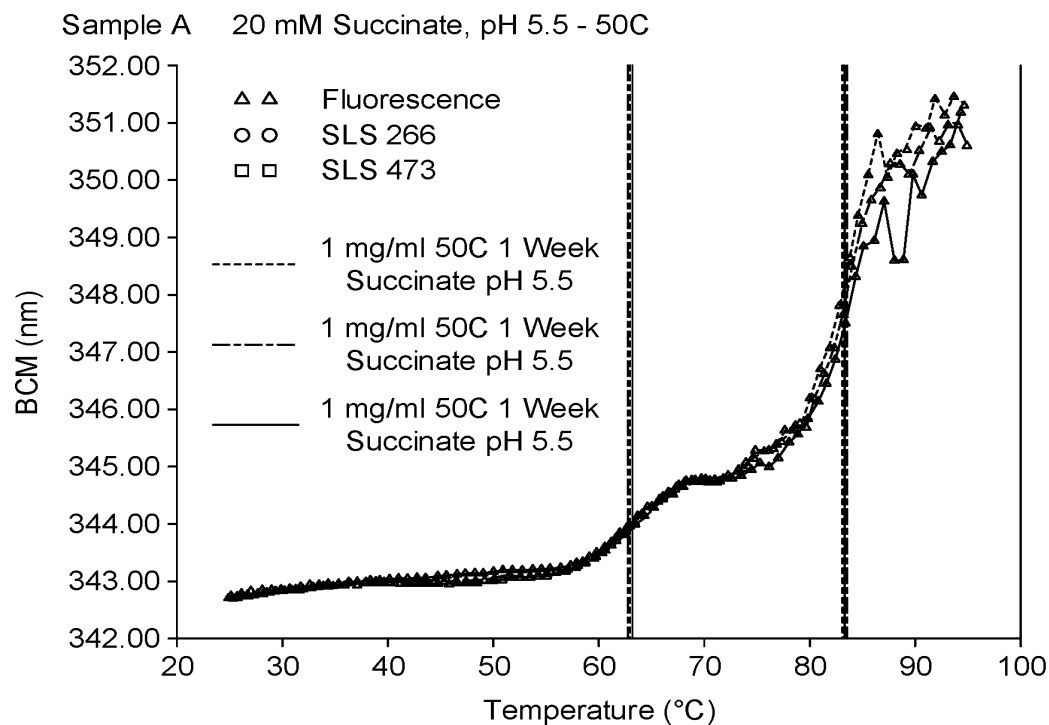
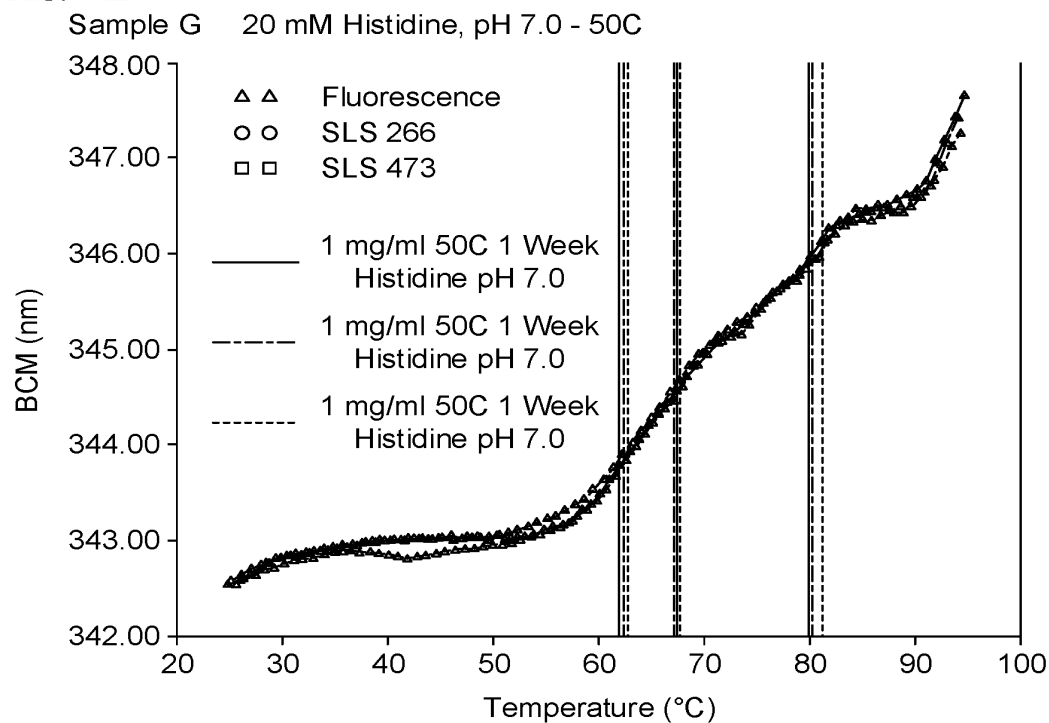


FIG. 36F



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FIG. 36G**FIG. 36H**

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FIG. 37A

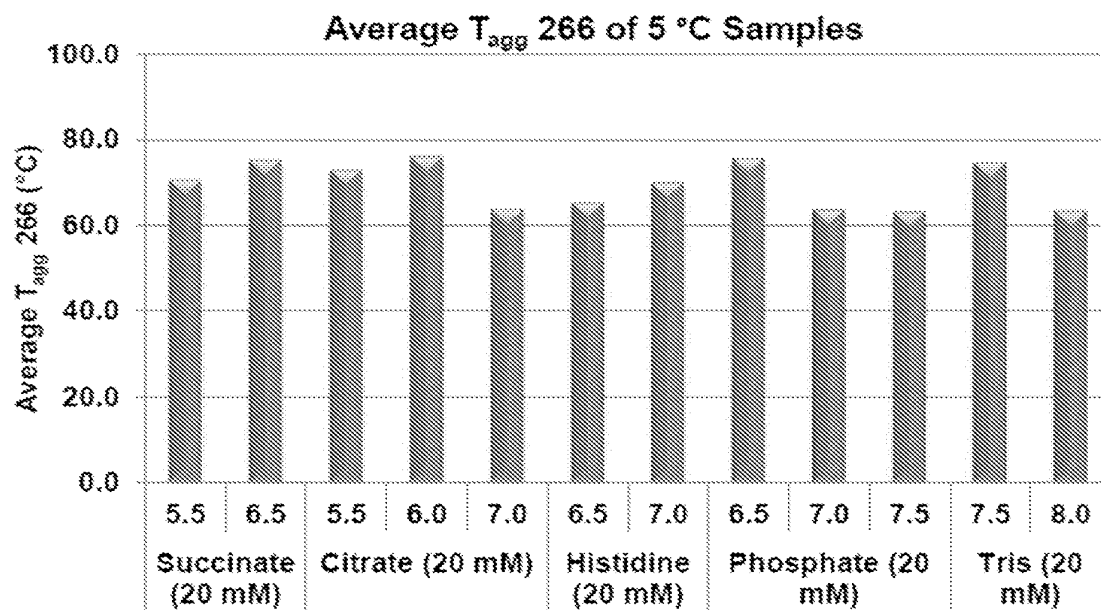
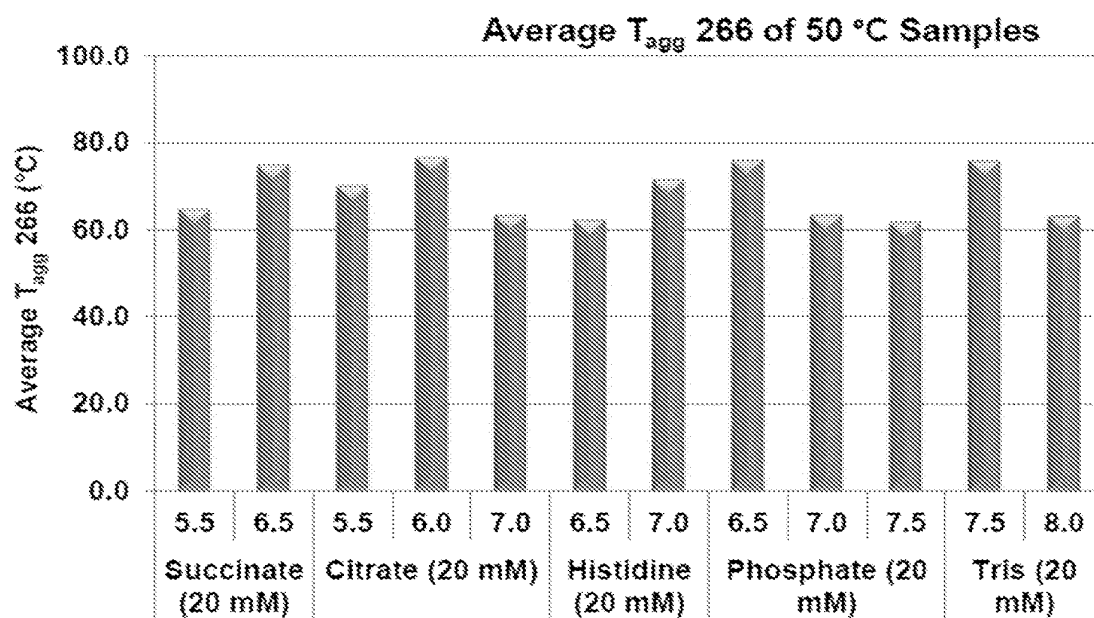


FIG. 37B



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FIG. 37C

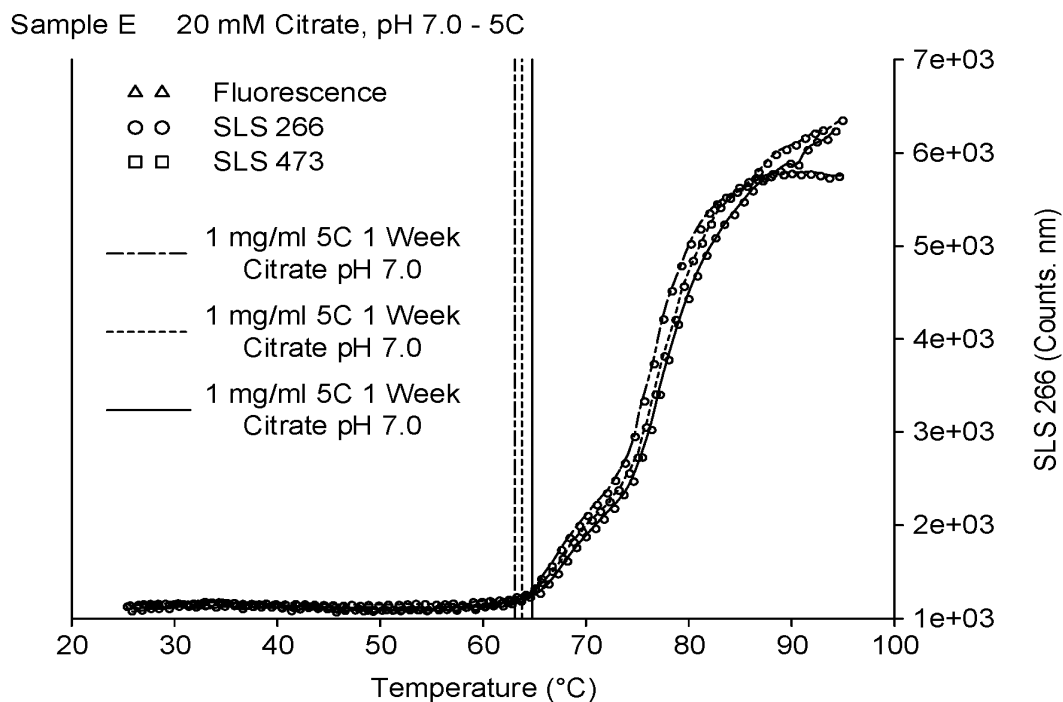
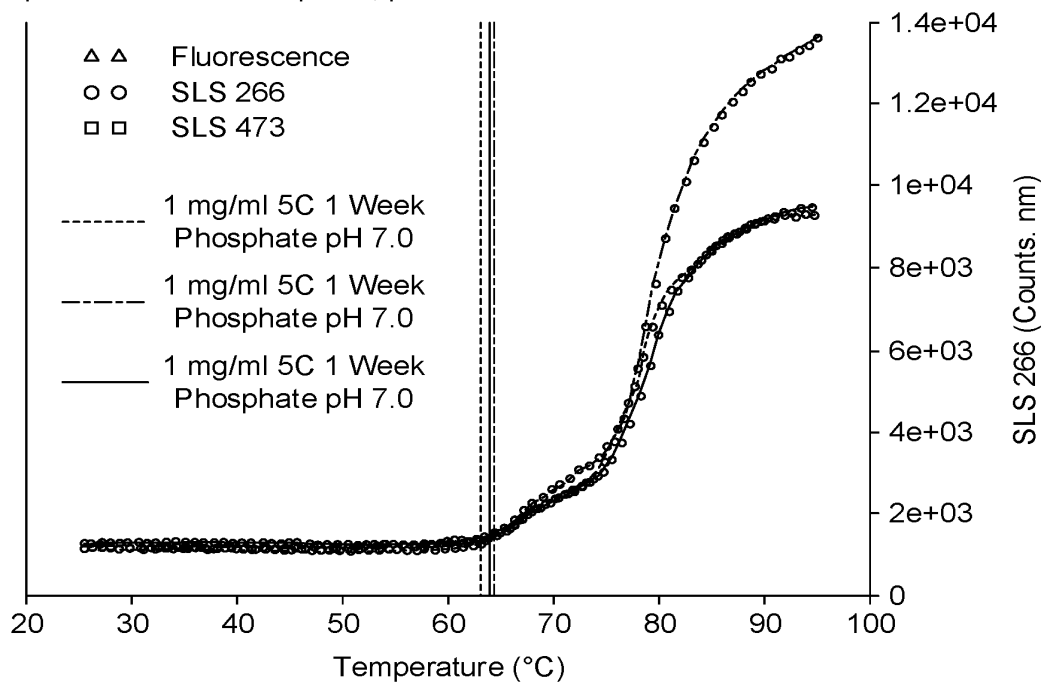


FIG. 37D

Sample J 20 mM Phosphate, pH 7.0 - 5C



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FIG. 37E

Sample F 20 mM Histidine, pH 6.5 - 50C

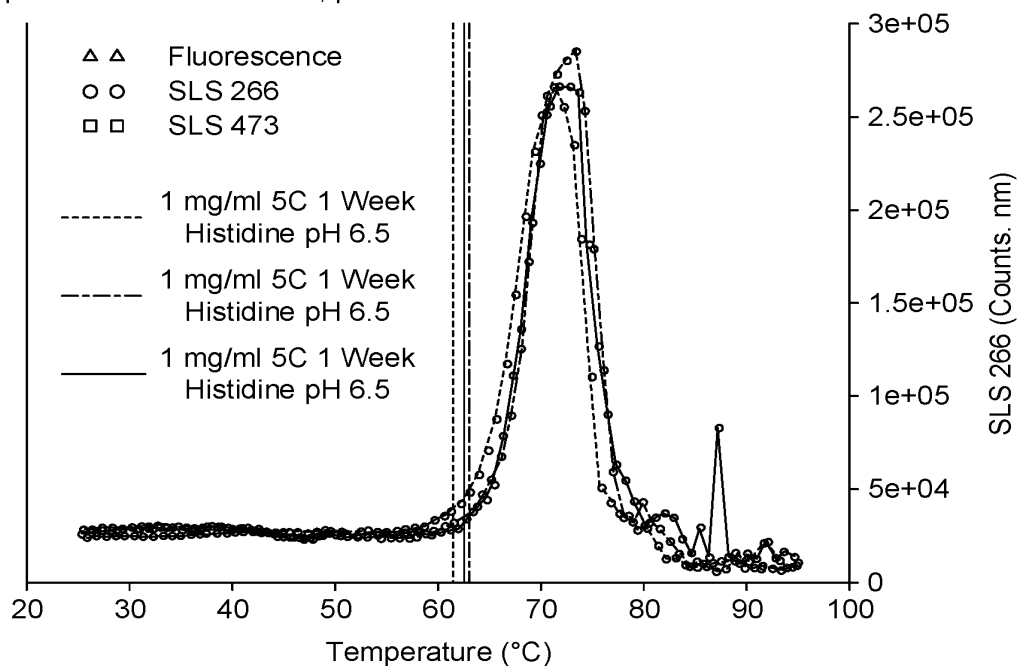
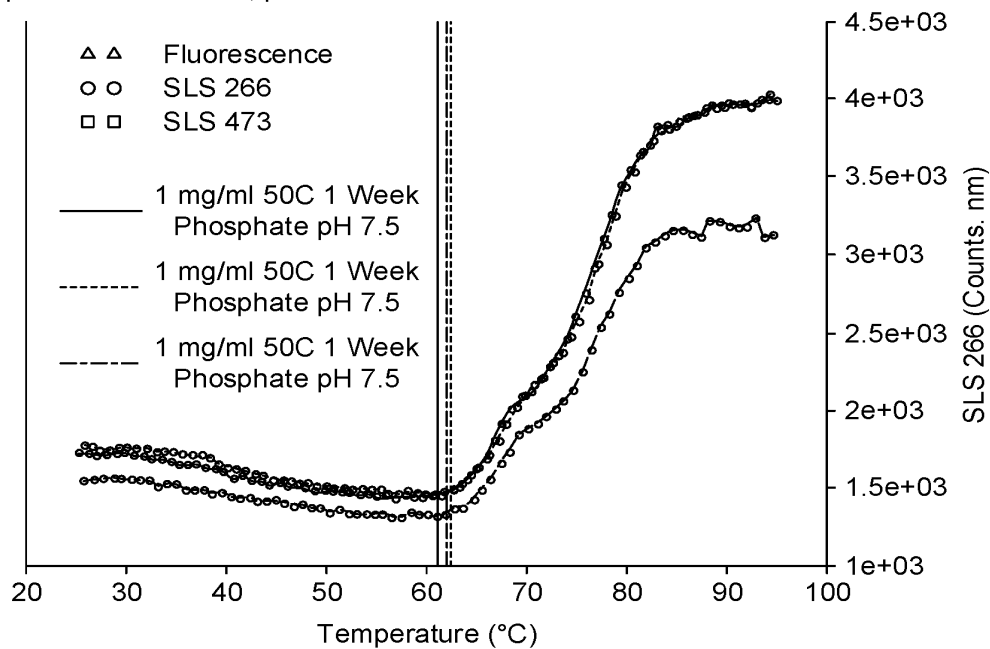


FIG. 37F

Sample L 20 mM Tris, pH 8.0 - 50C



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FIG. 38A

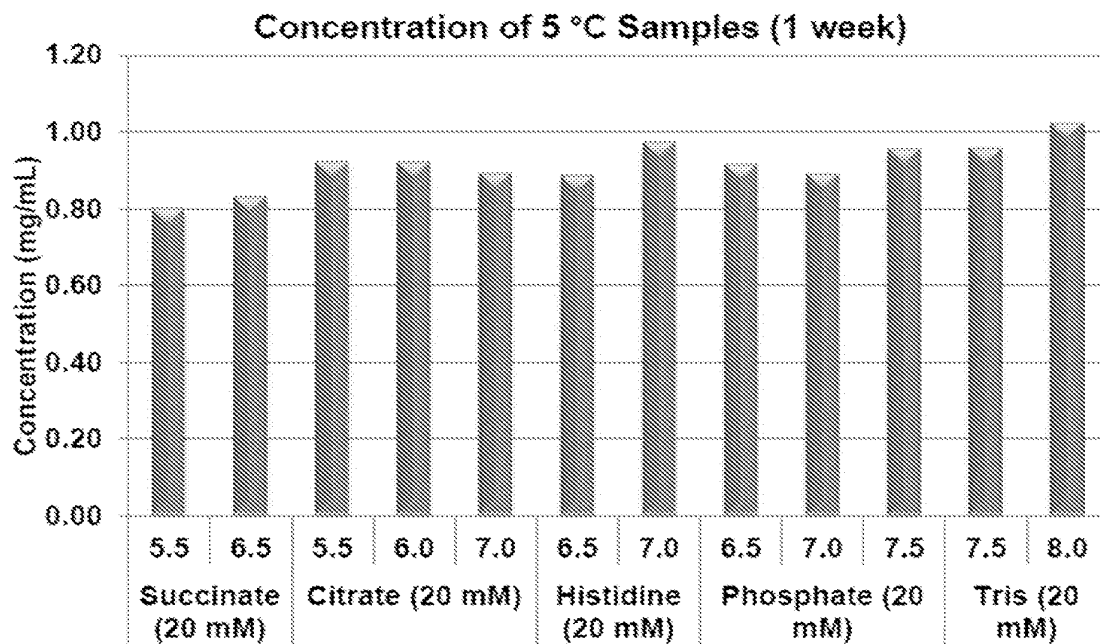
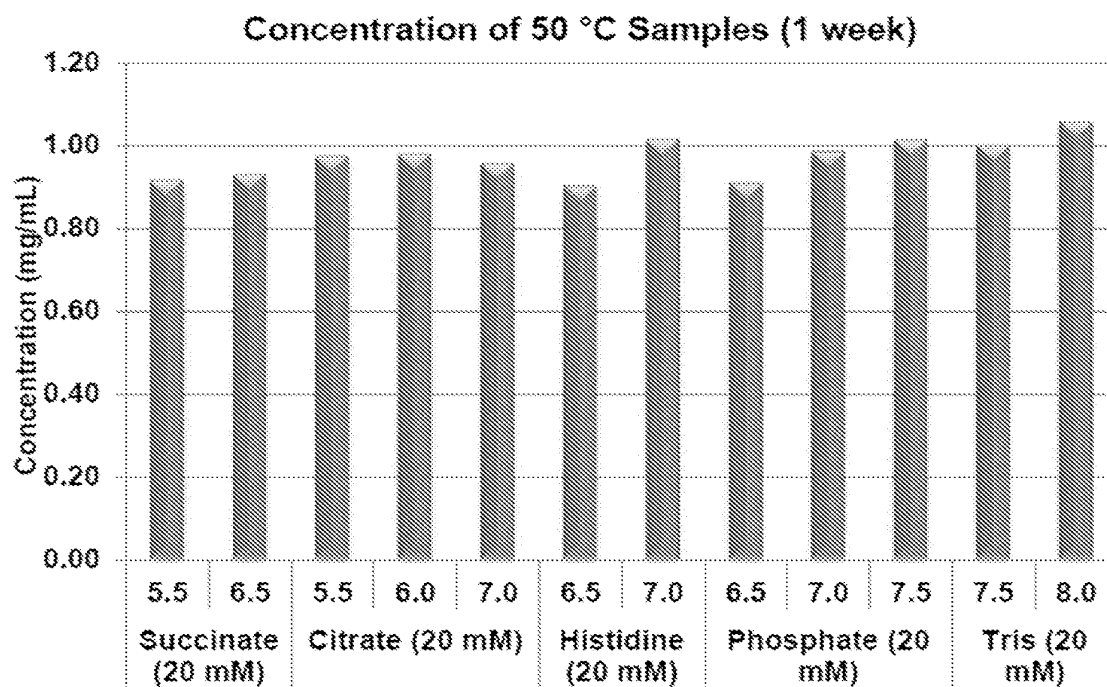


FIG. 38B



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FIG. 39A

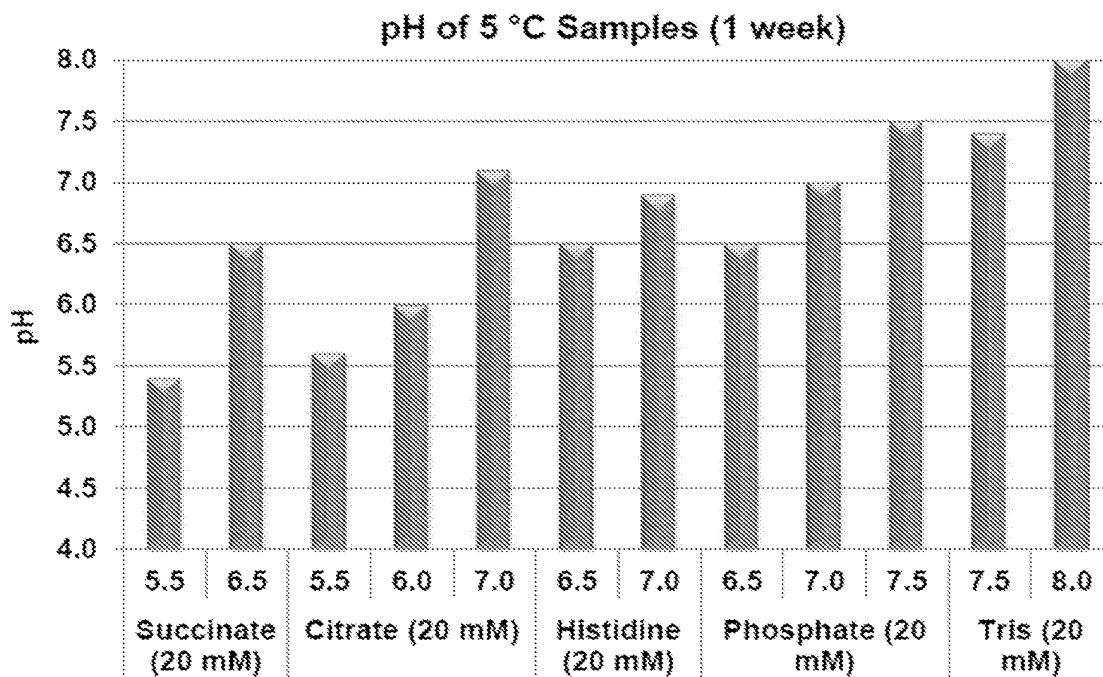
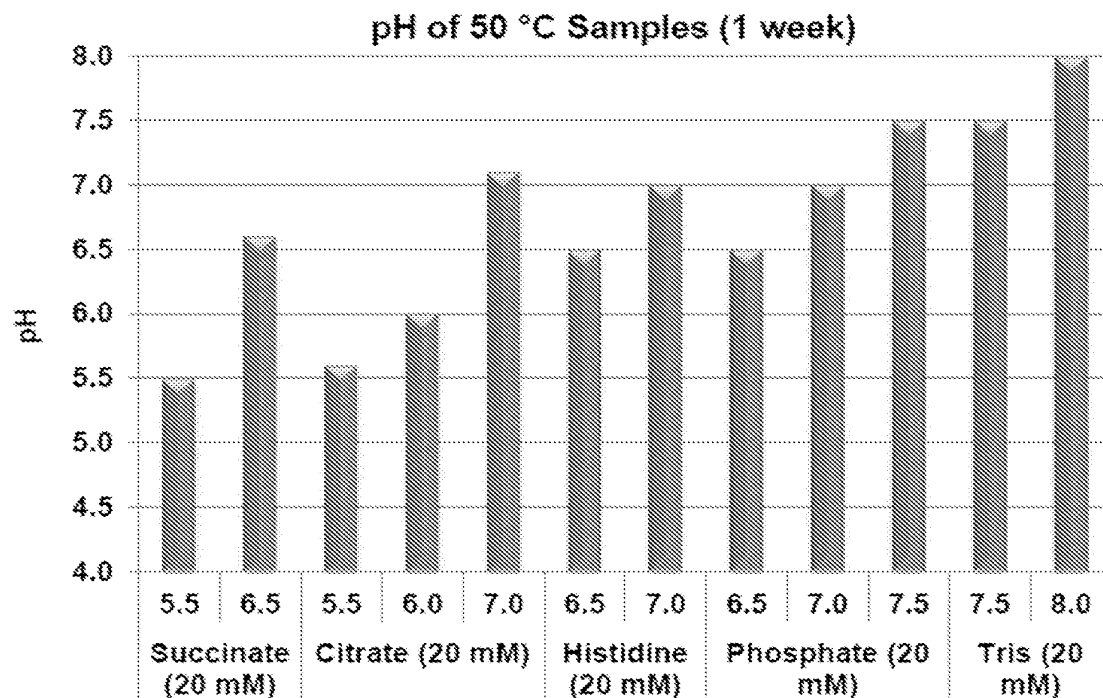


FIG. 39B



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FIG. 40A

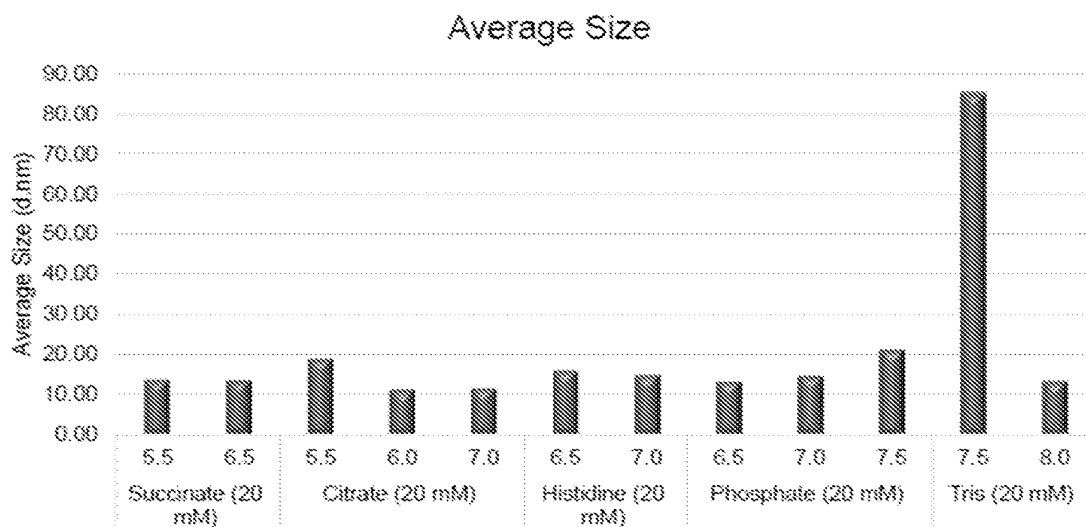
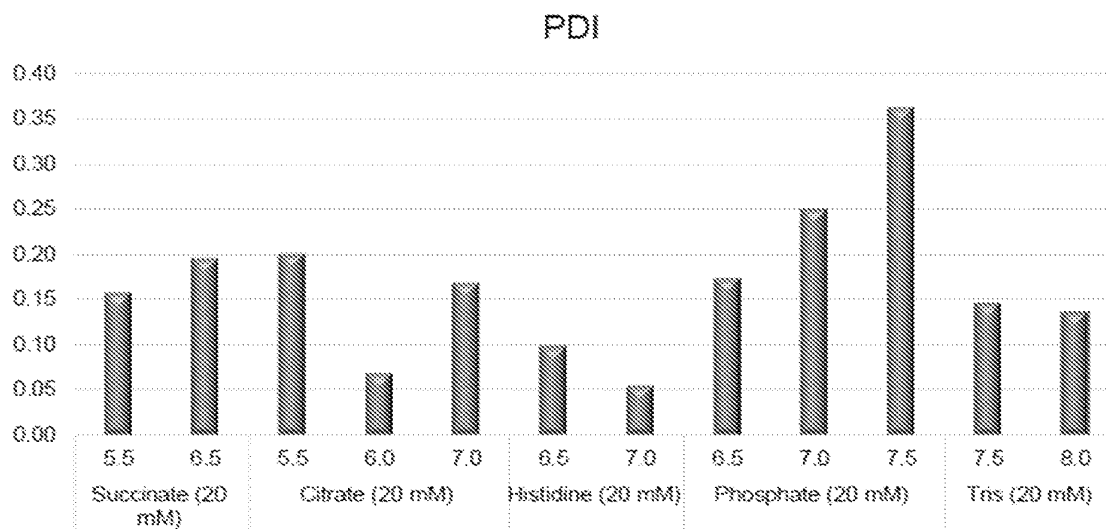


FIG. 40B



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FIG. 40C

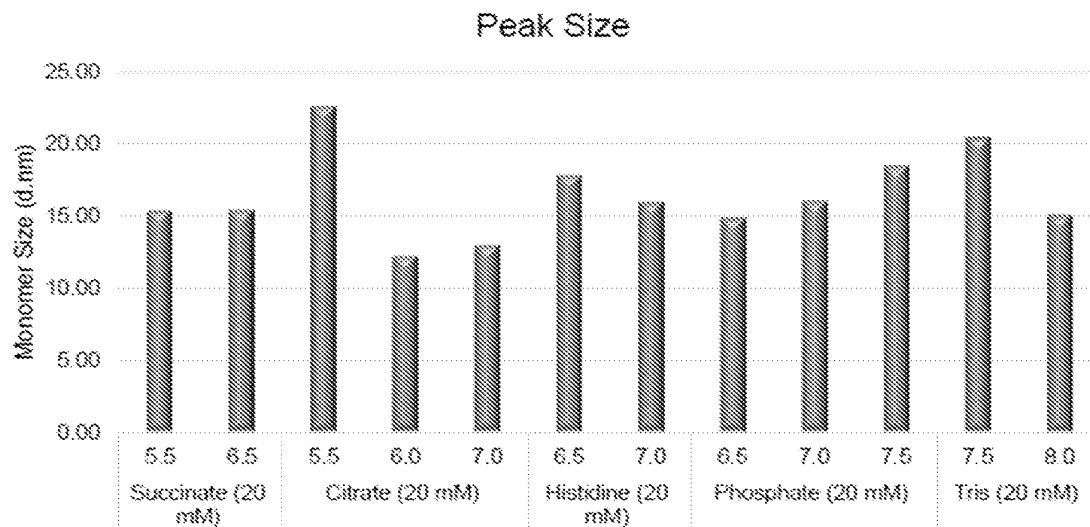
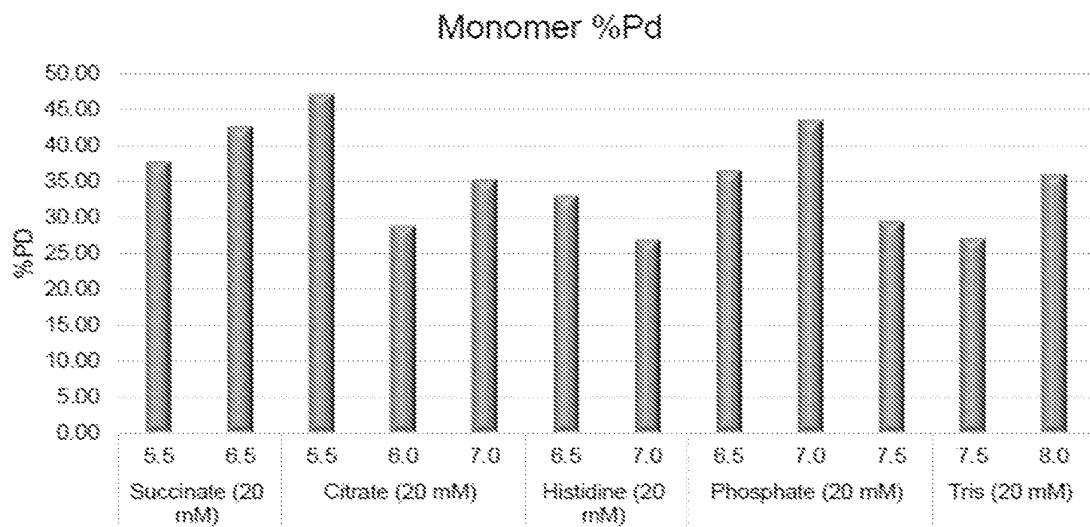


FIG. 40D



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FIG. 40E

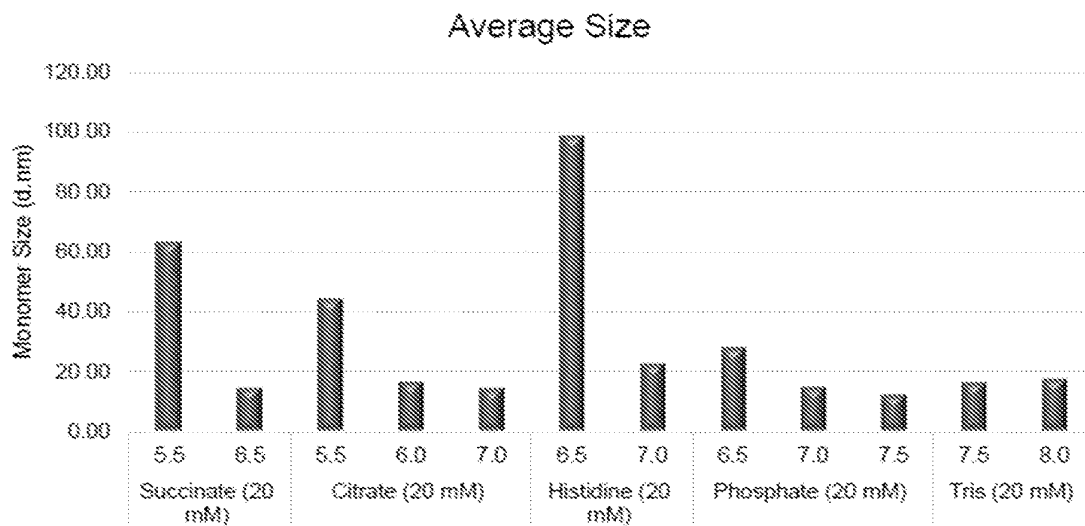
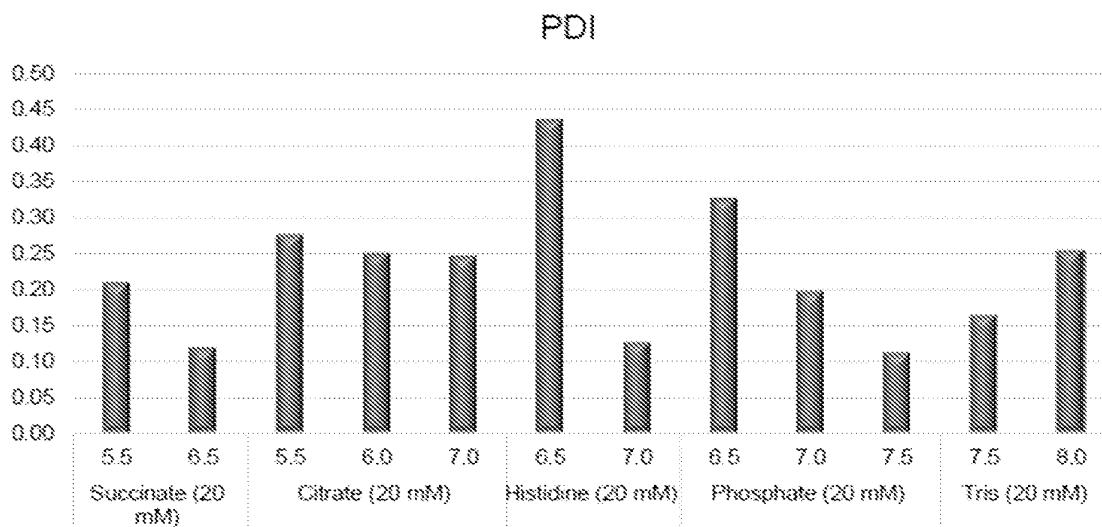


FIG. 40F



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FIG. 40G

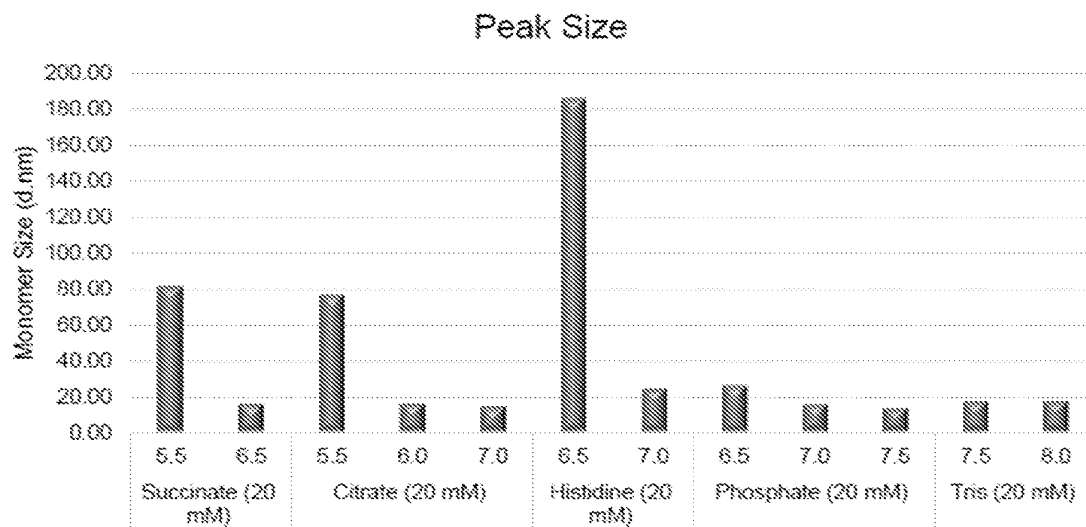
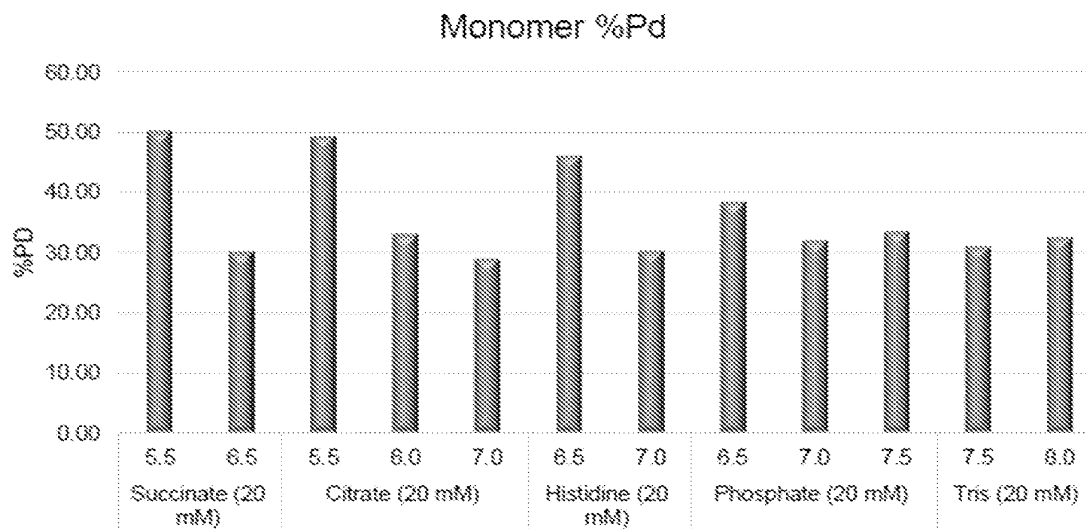


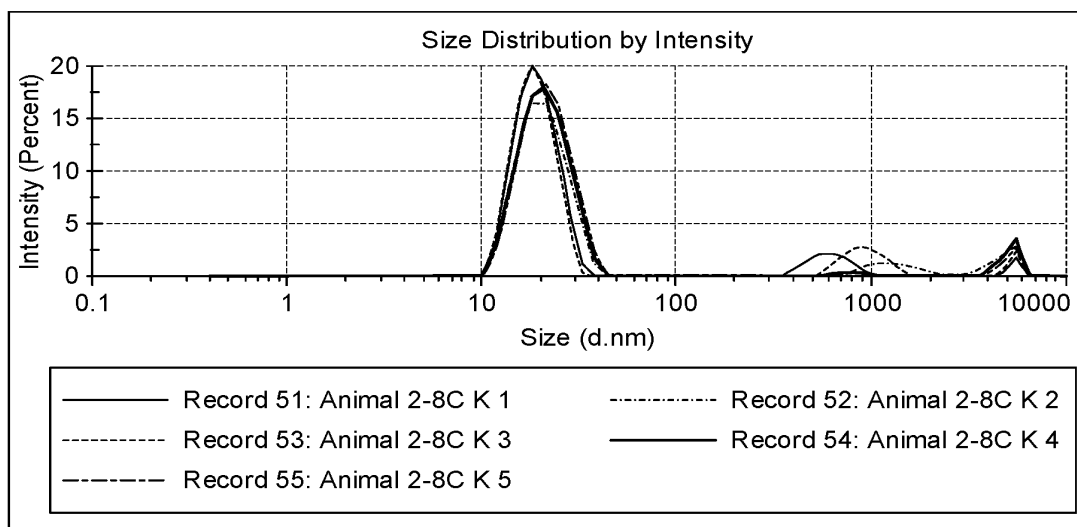
FIG. 40H



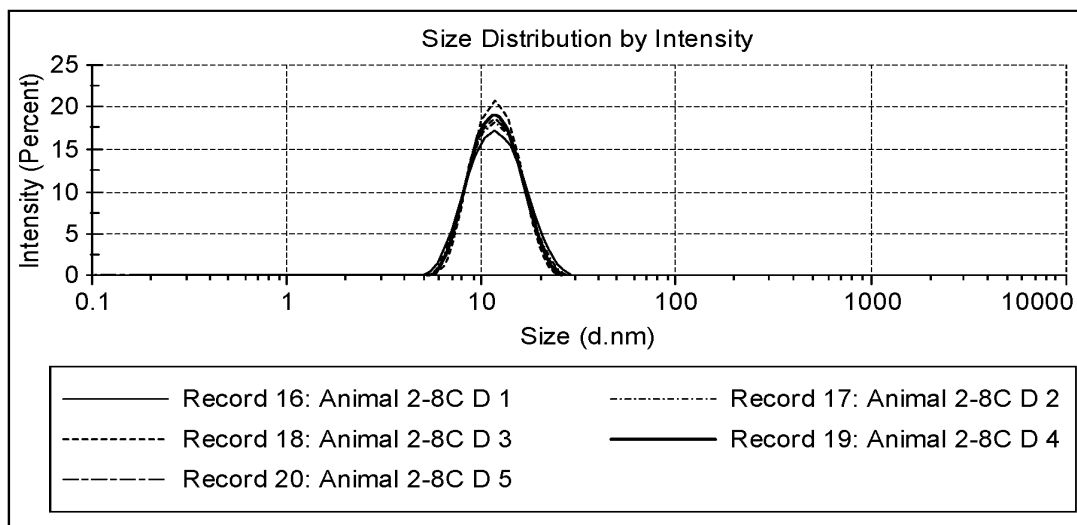
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FIG. 40I

Sample K 20 mM Tris, pH 7.5 - 5C

**FIG. 40J**

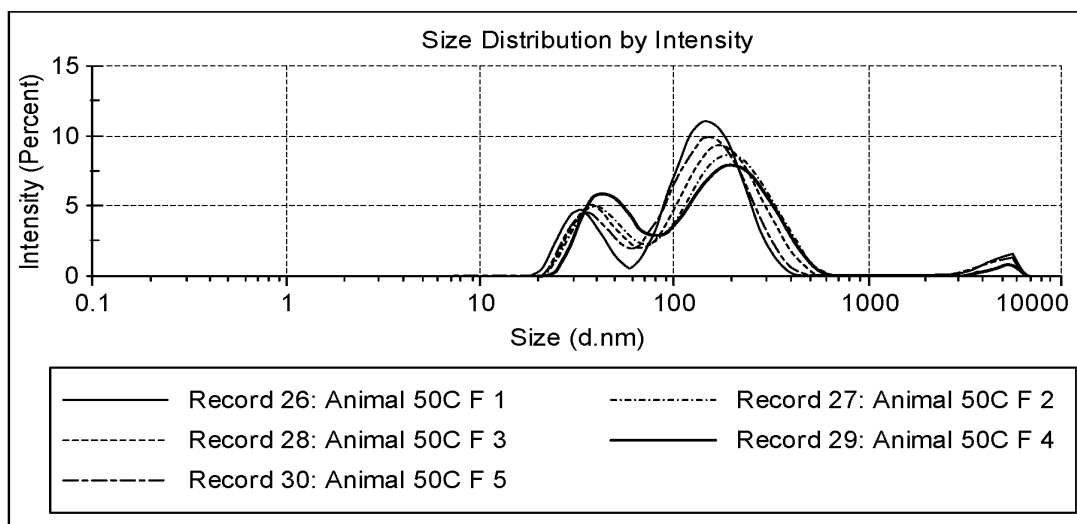
Sample H 20 mM Citrate, pH 6.0 - 5C



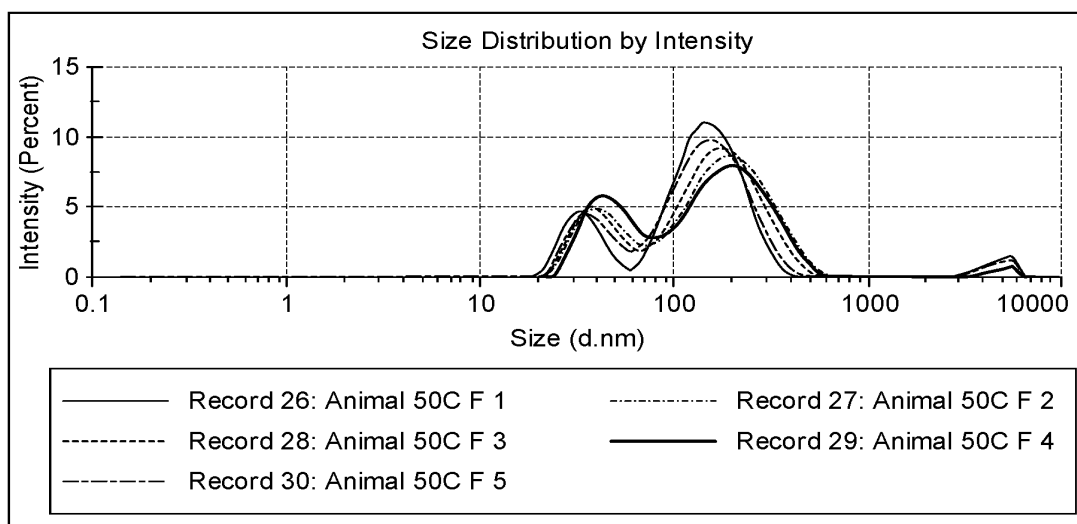
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FIG. 40K

Sample F 20 mM Histidine, pH 6.0 - 50C

**FIG. 40L**

Sample J 20 mM Phosphate, pH 7.4 - 50C



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FIG. 41

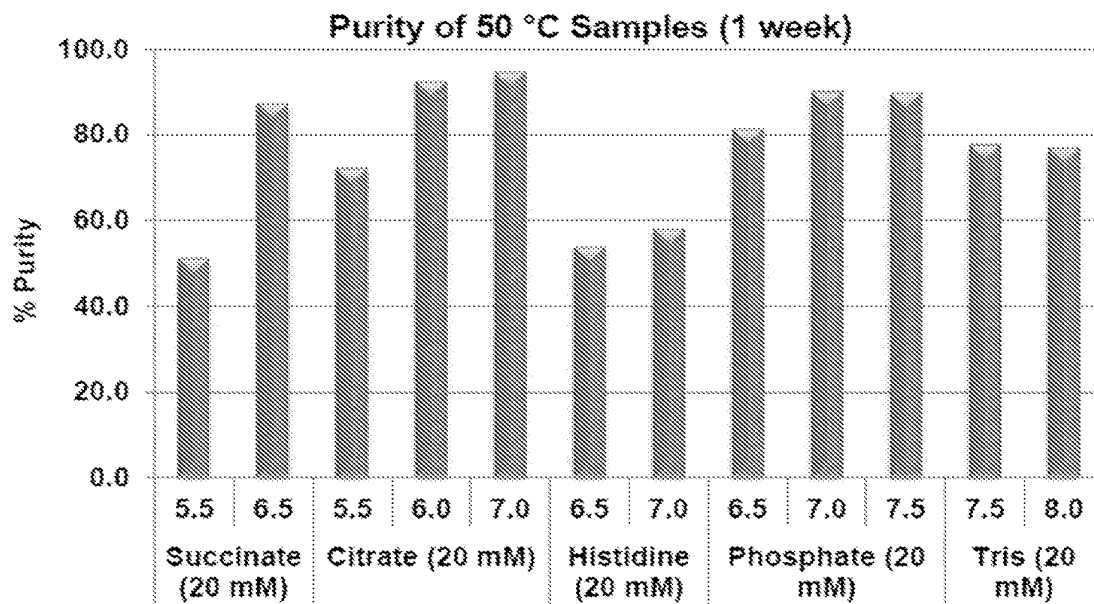


FIG. 42

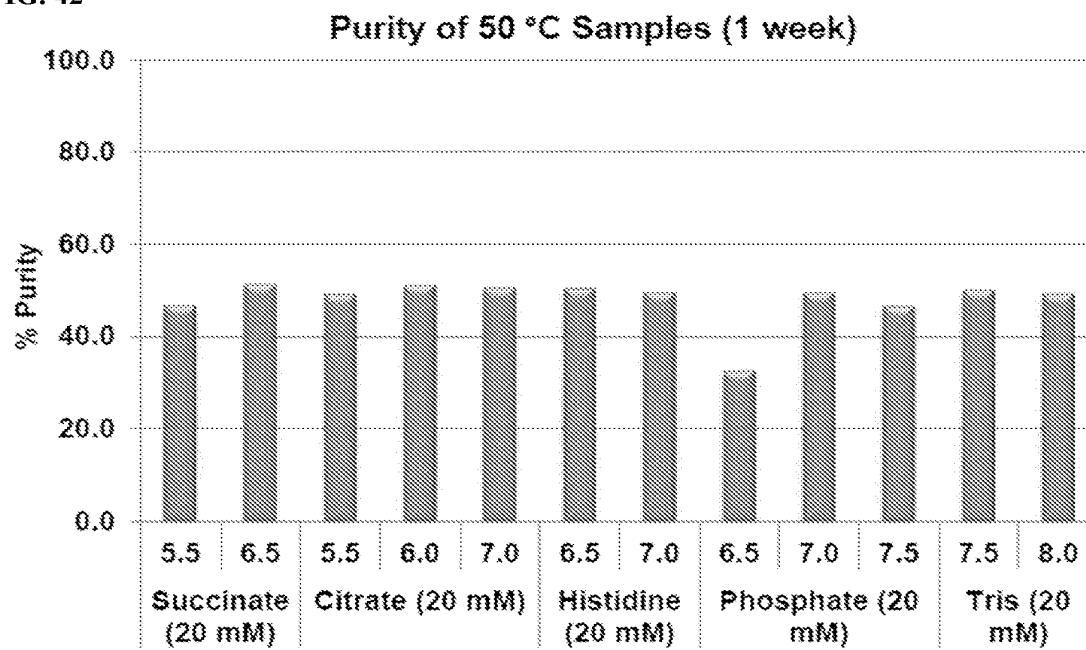


FIG. 43A

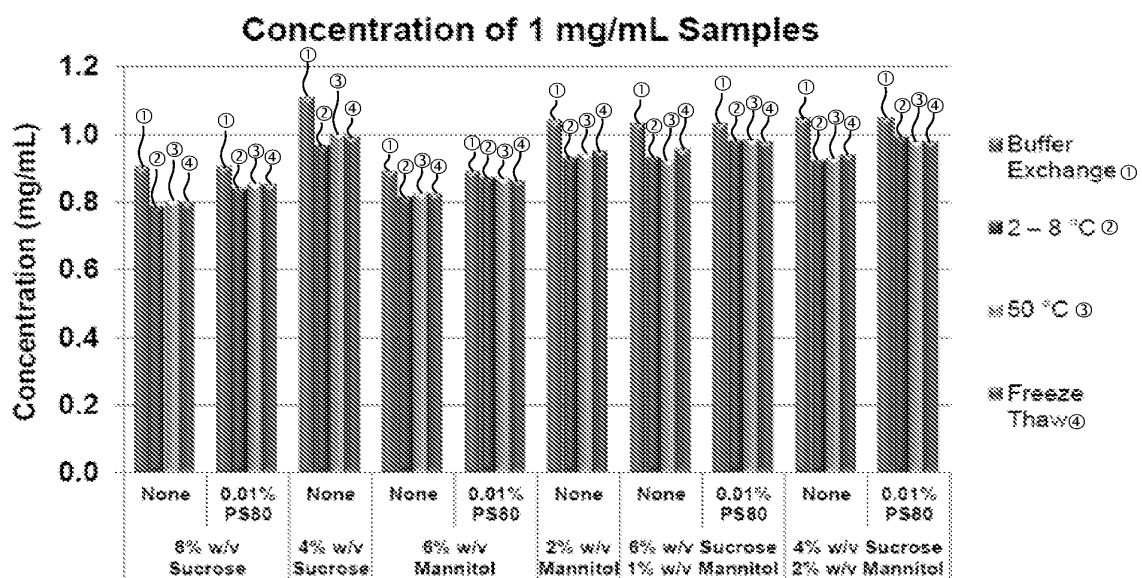
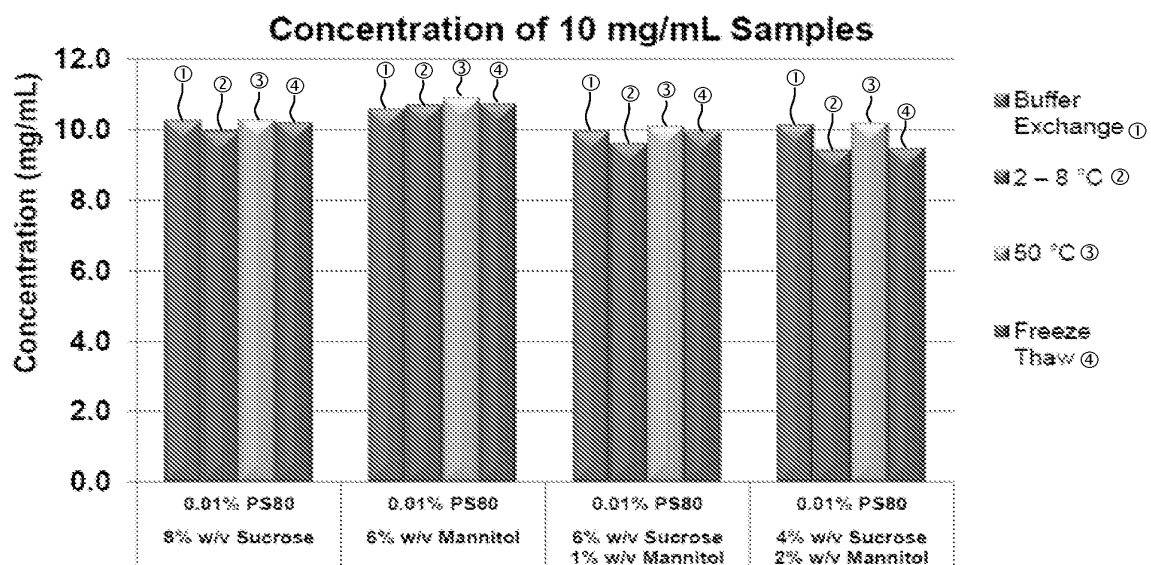


FIG. 43B



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FIG. 44A

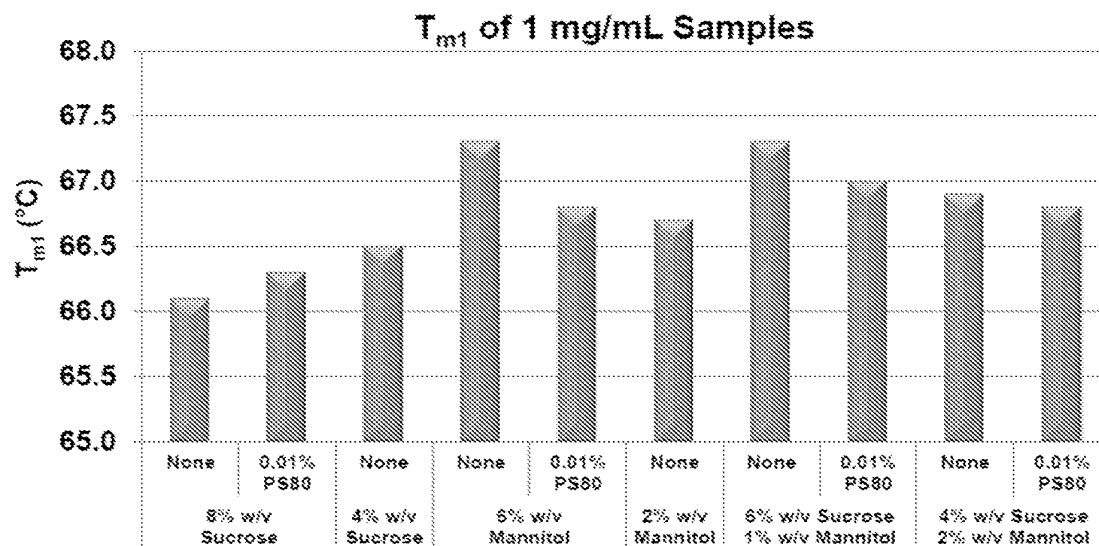
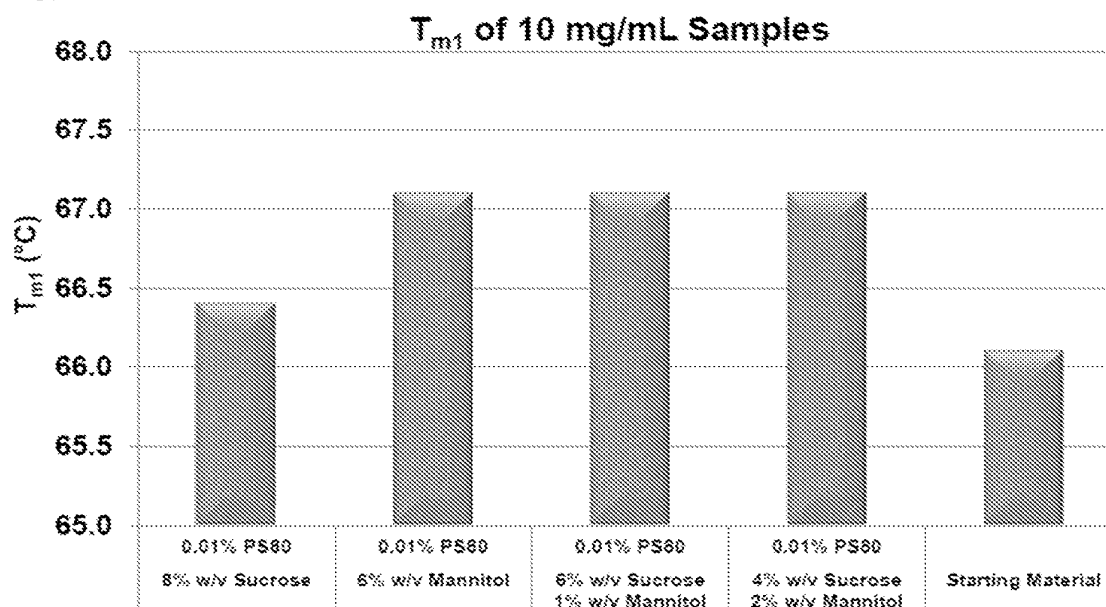
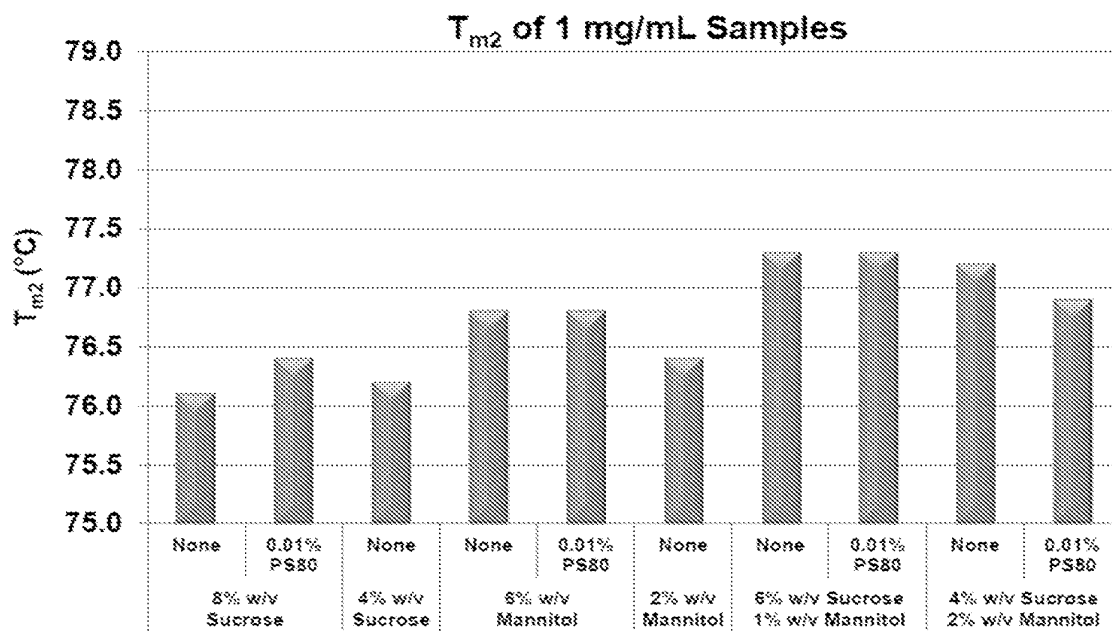


FIG. 44B



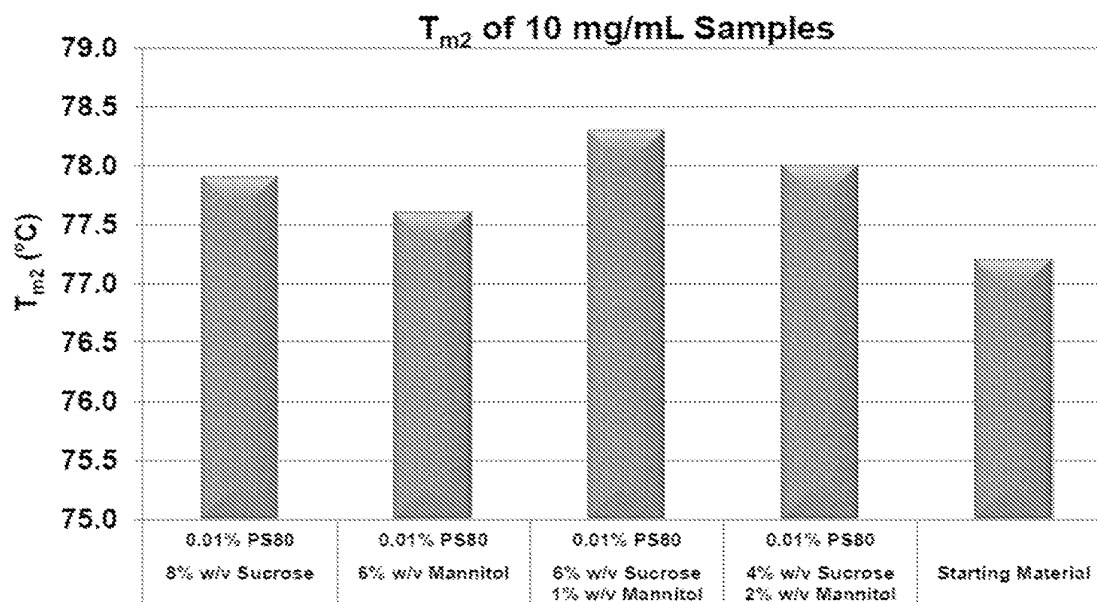
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FIG. 44C



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FIG. 44D



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FIG. 45A

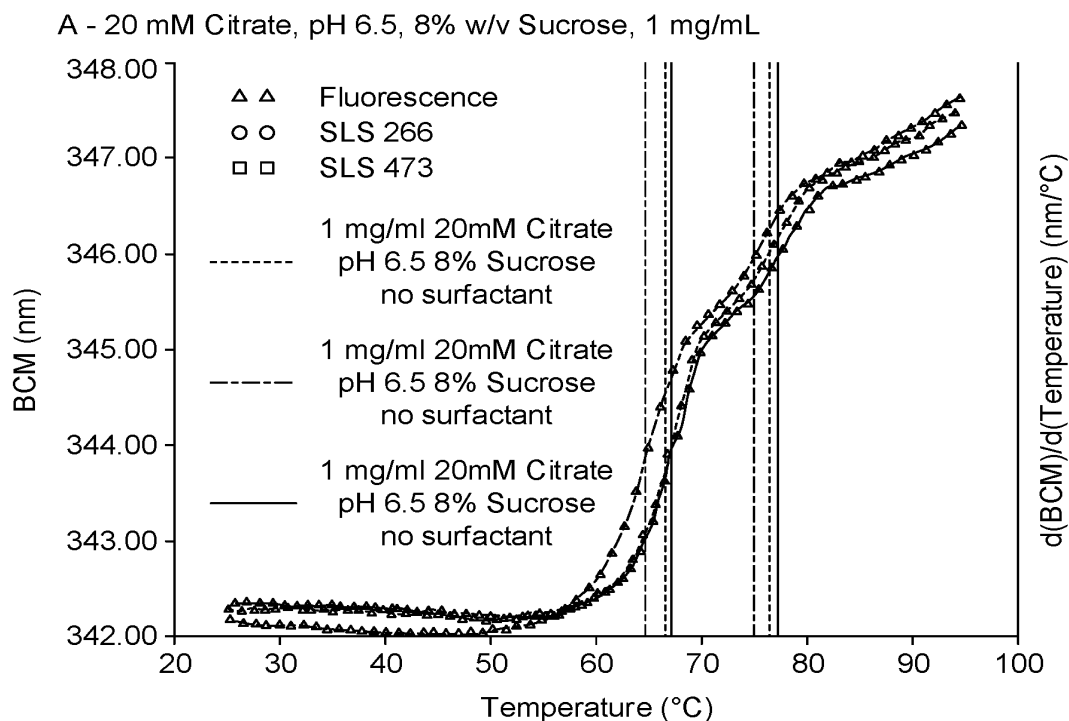
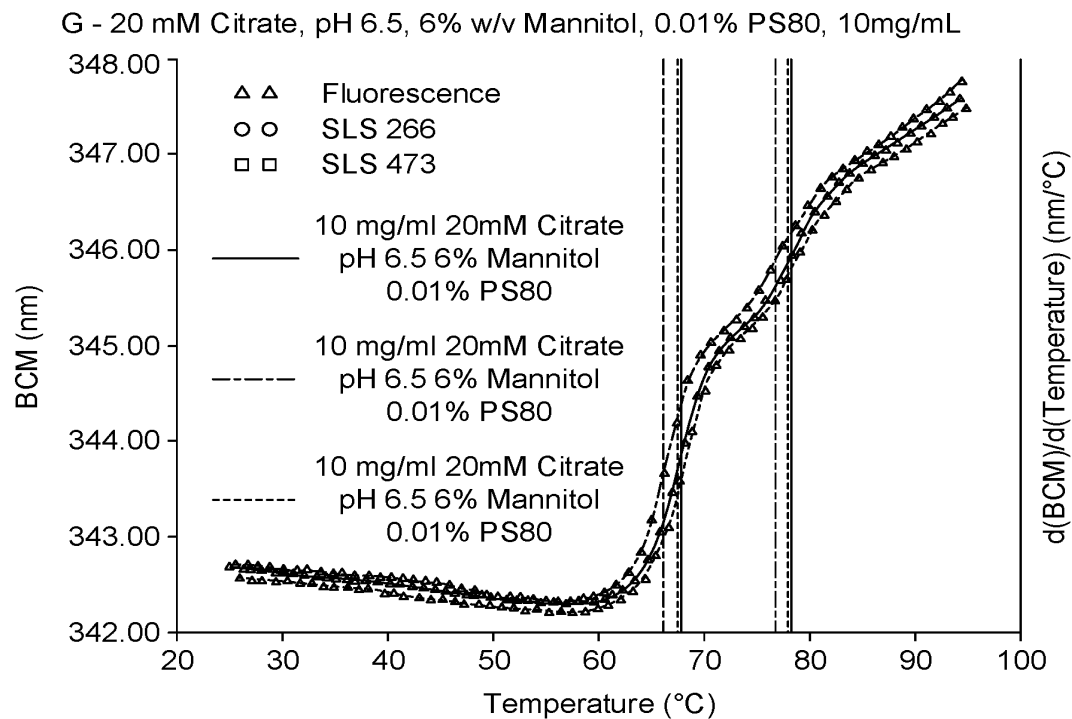


FIG. 45B



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FIG. 45C

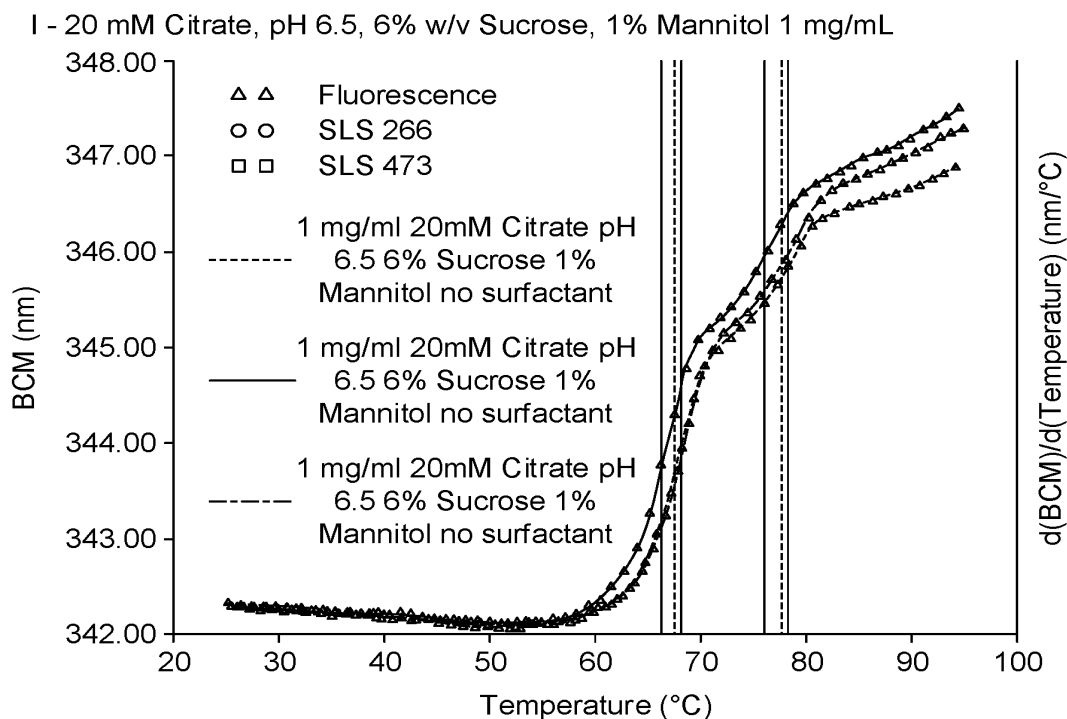
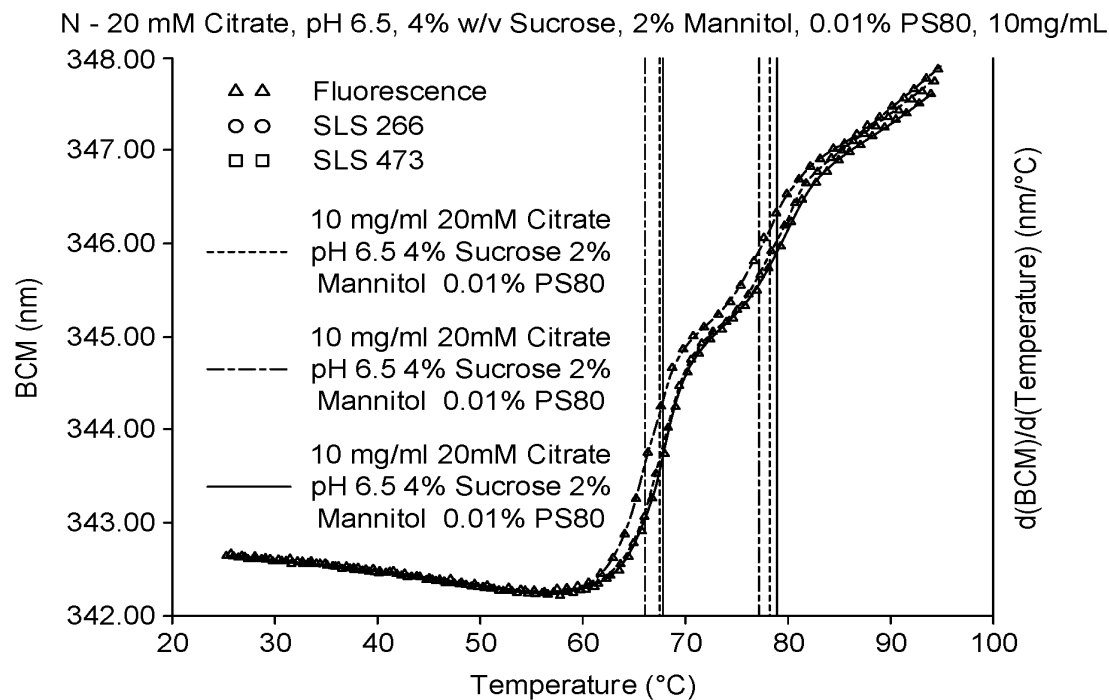


FIG. 45D



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FIG. 46A

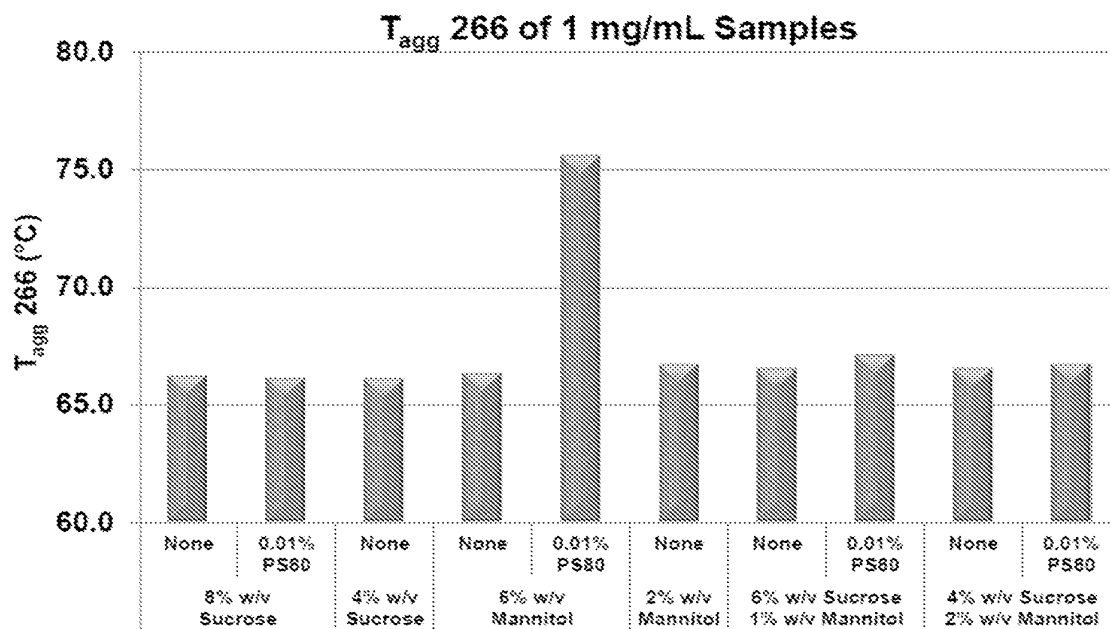
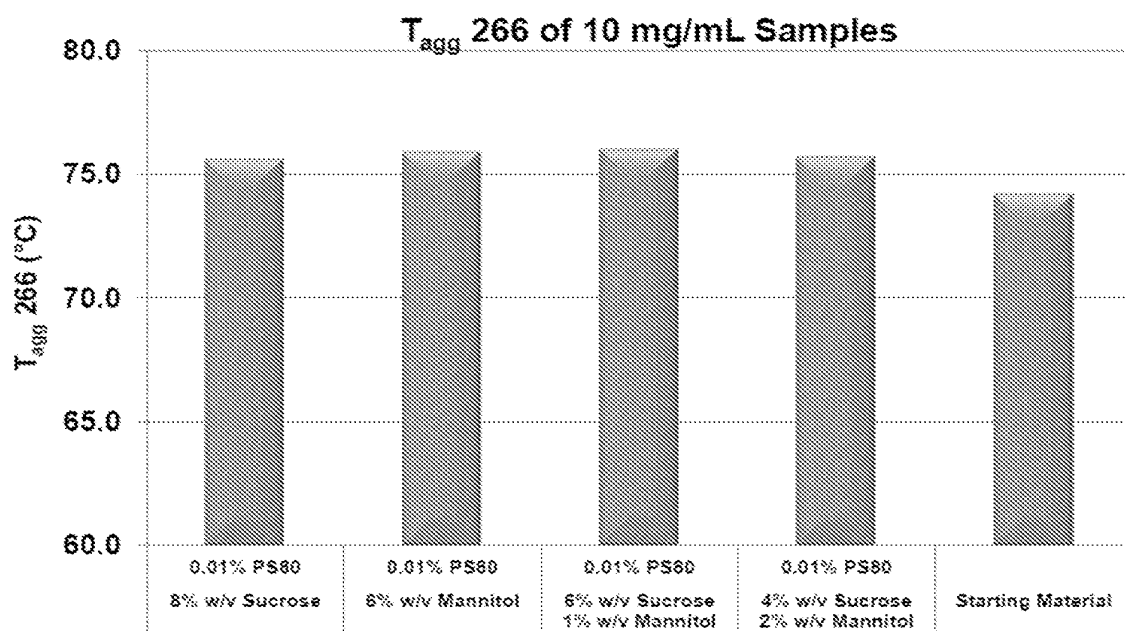


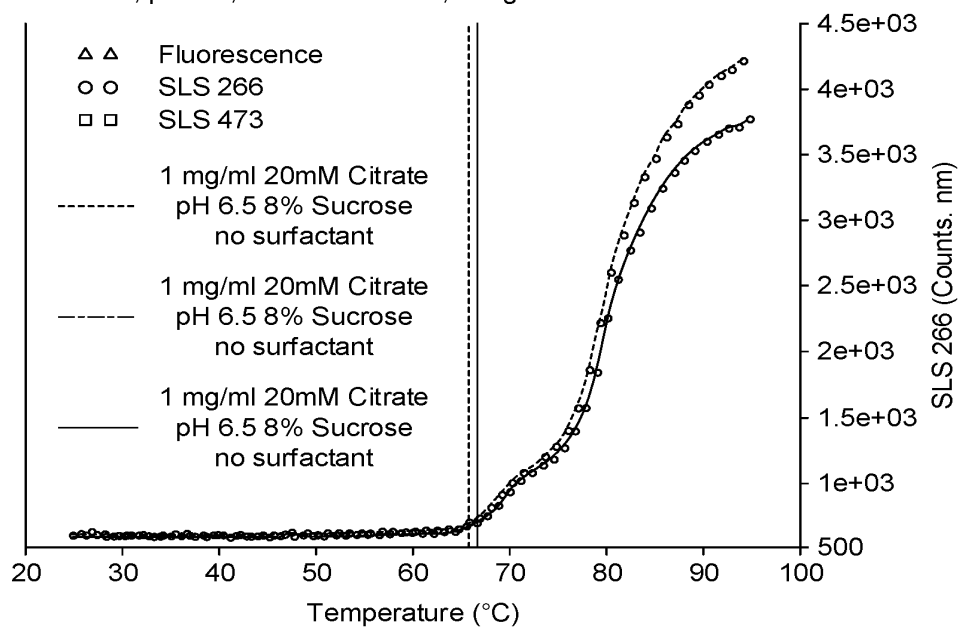
FIG. 46B



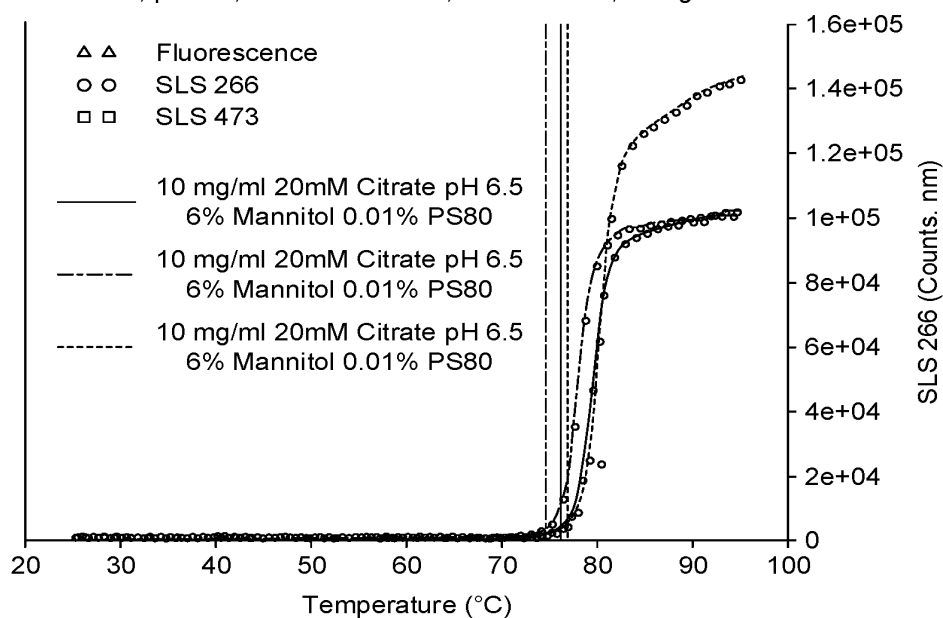
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FIG. 46C

A - 20 mM Citrate, pH 6.5, 8% w/v Sucrose, 1 mg/mL

**FIG. 46D**

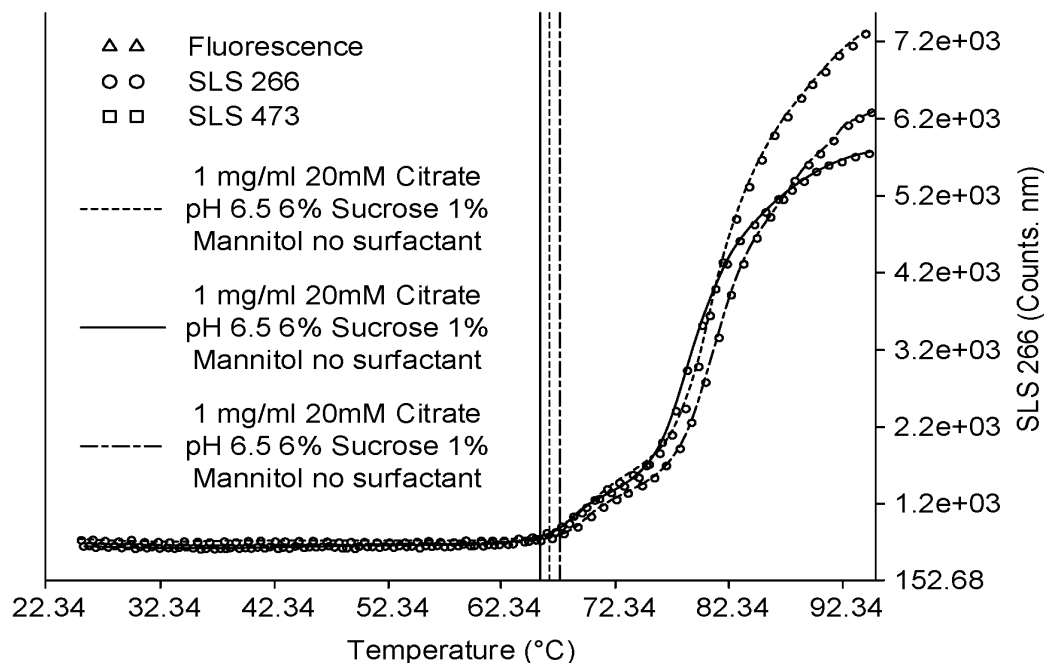
G - 20 mM Citrate, pH 6.5, 6% w/v Mannitol, 0.01% PS80, 10mg/mL



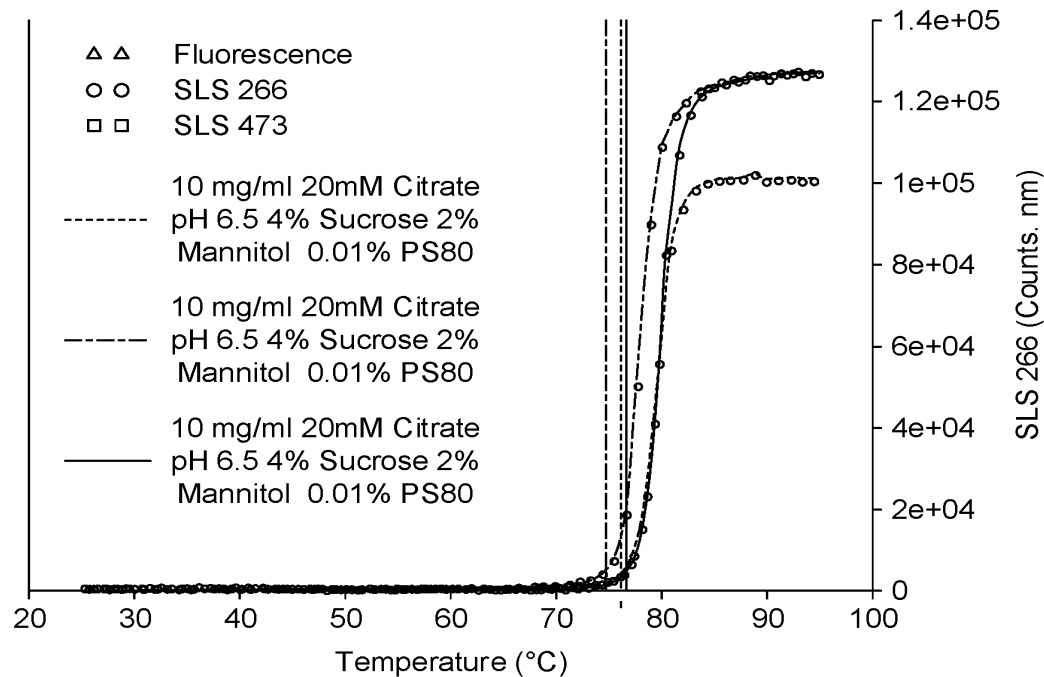
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FIG. 46E

I - 20 mM Citrate, pH 6.5, 6% w/v Sucrose, 1% Mannitol, 1 mg/mL

**FIG. 46F**

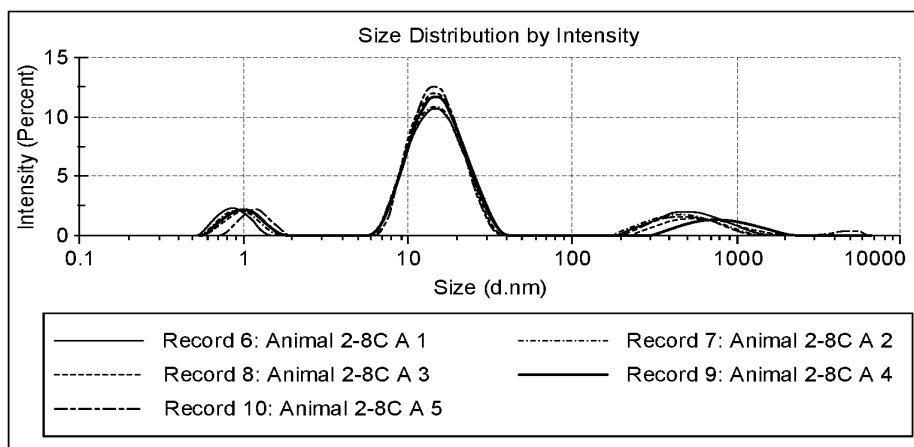
N - 20 mM Citrate, pH 6.5, 4% w/v Sucrose, 2% Mannitol, 0.01% PS80, 10mg/mL



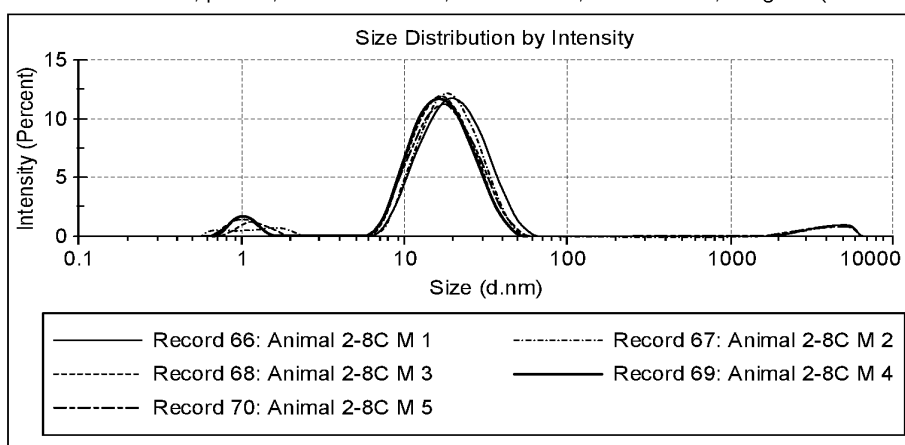
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FIG. 47A

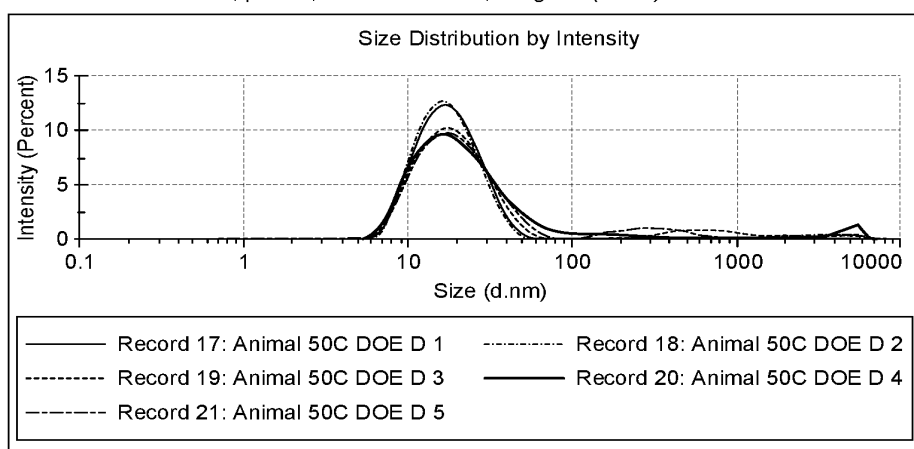
A - 20 mM Citrate, pH 6.5, 8% w/v Sucrose, 1 mg/mL (2 – 8 °C)

**FIG. 47B**

M - 20 mM Citrate, pH 6.5, 4% w/v Sucrose, 2% Mannitol, 0.01% PS80, 1 mg/mL (2 – 8 °C)

**FIG. 47C**

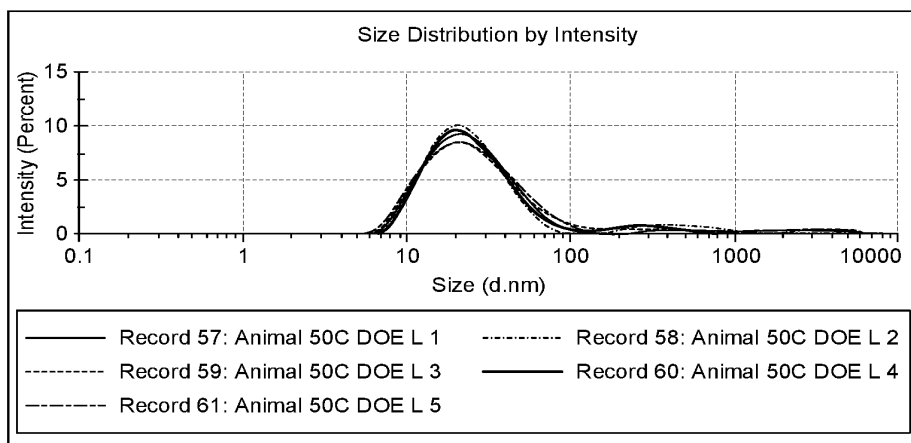
D - 20 mM Citrate, pH 6.5, 4% w/v Sucrose, 1 mg/mL (50 °C)



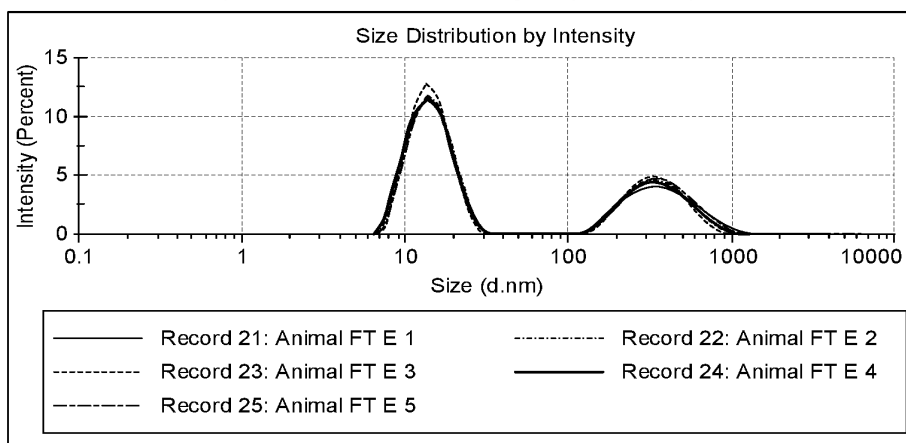
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FIG. 47D

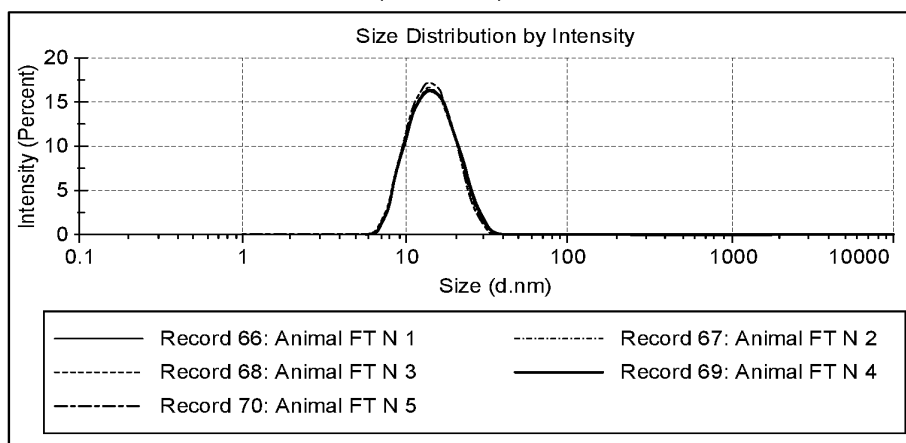
L - 20 mM Citrate, pH 6.5, 4% w/v Sucrose, 2% Mannitol, 1 mg/mL (50 °C)

**FIG. 47E**

E - 20 mM Citrate, pH 6.5, 4% w/v Mannitol, 1 mg/mL (freeze thaw)

**FIG. 47F**

N - 20 mM Citrate, pH 6.5, 4% w/v Sucrose, 2% Mannitol, 0.01% PS80, 10 mg/mL (freeze thaw)



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FIG. 48A

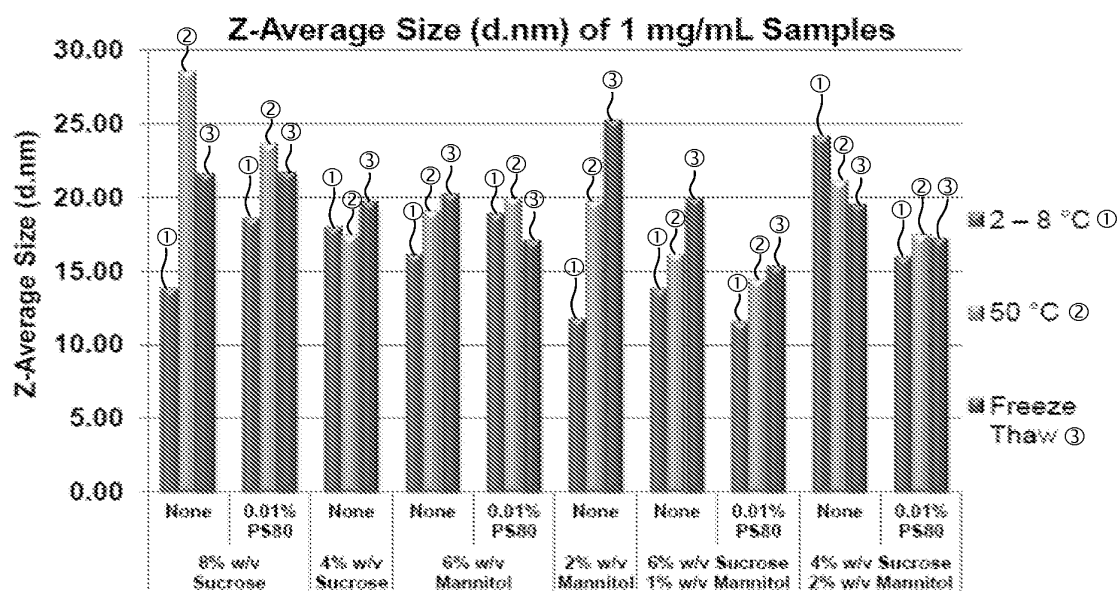
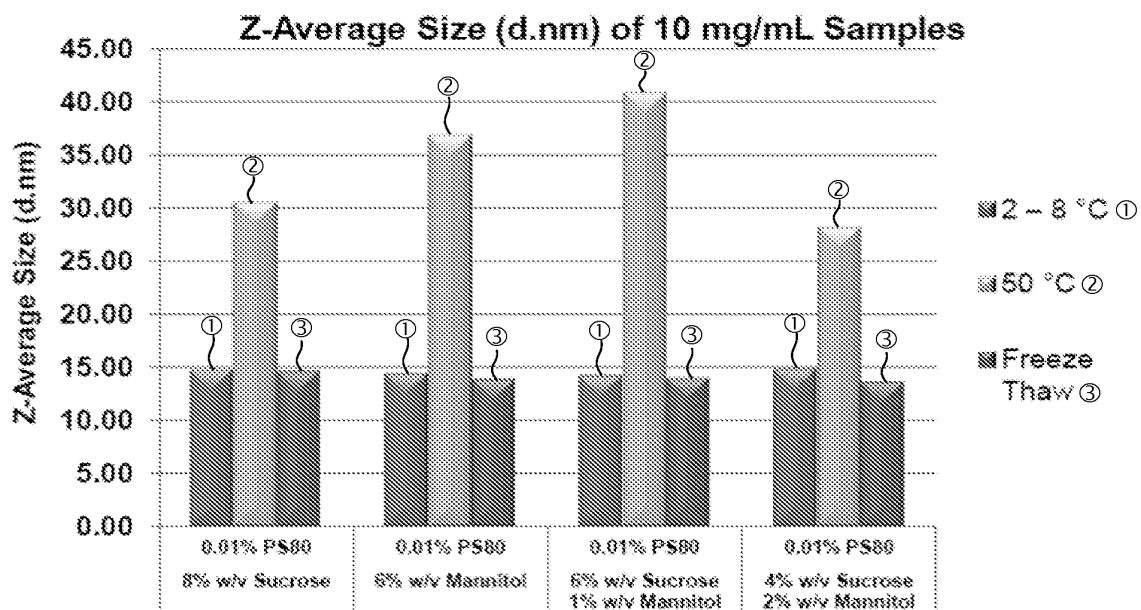


FIG. 48B



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FIG. 48C

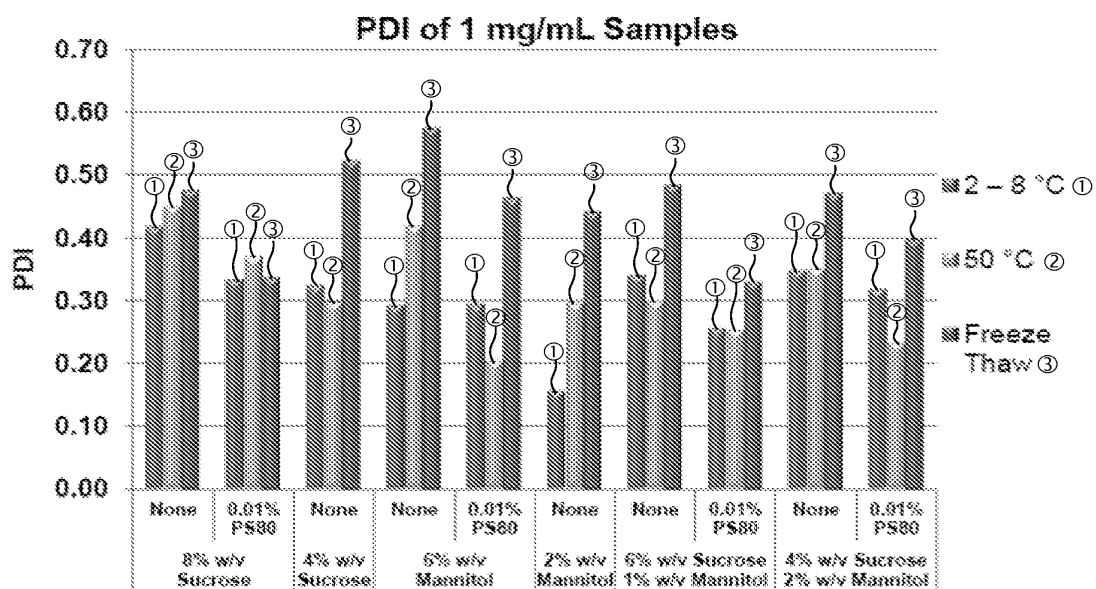
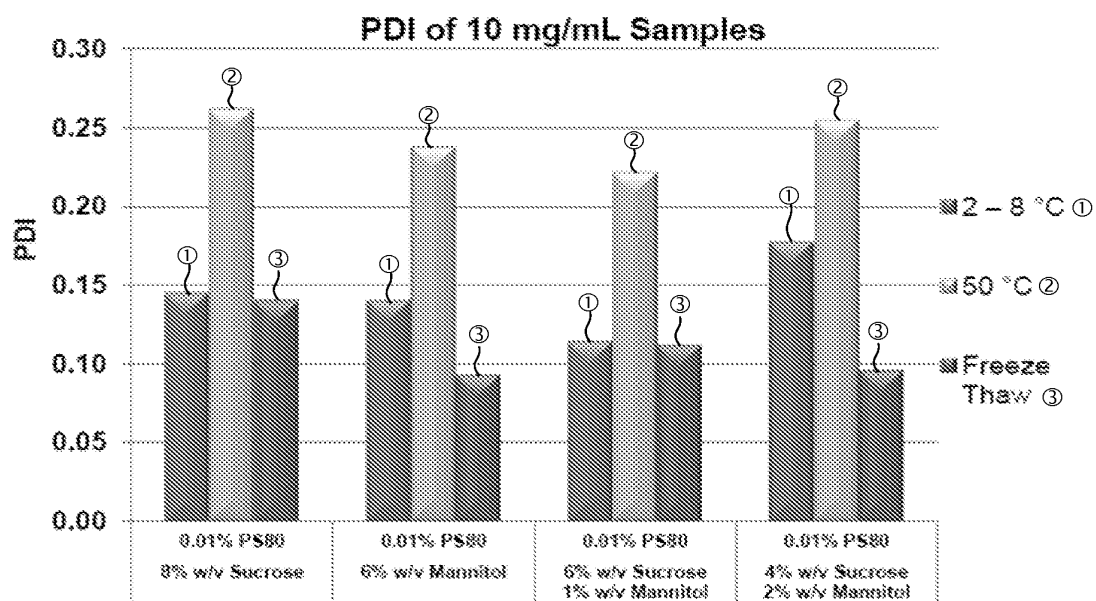


FIG. 48D



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FIG. 48E

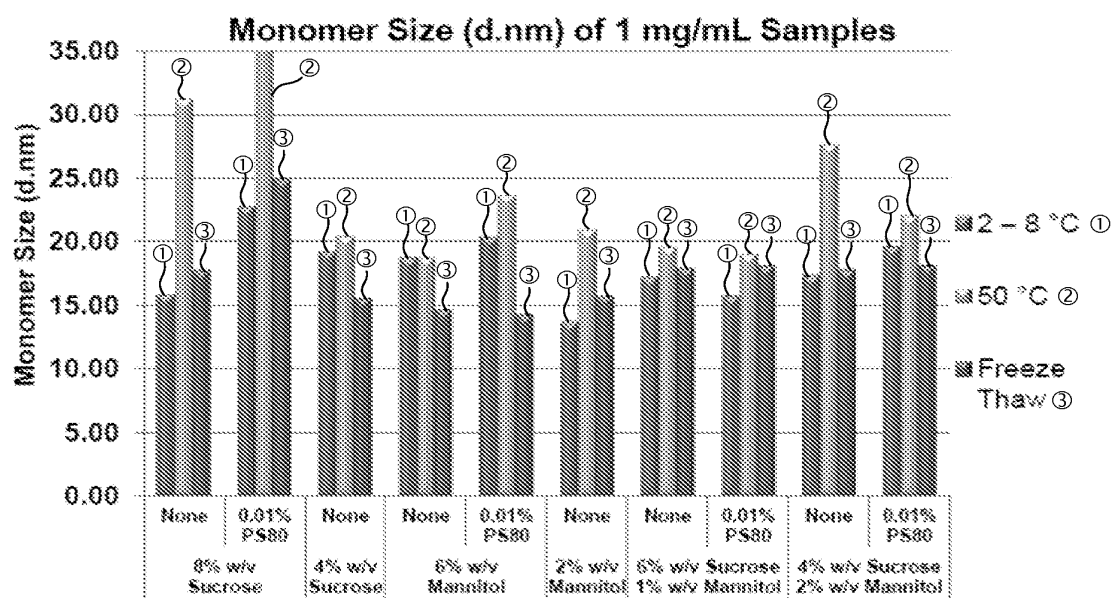
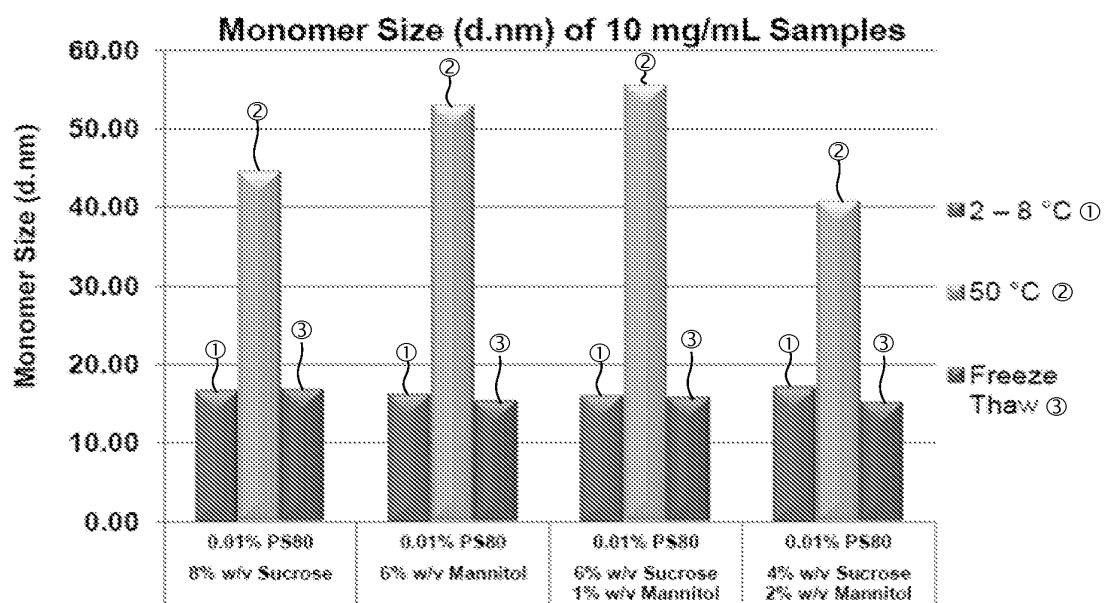


FIG. 48F



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FIG. 48G

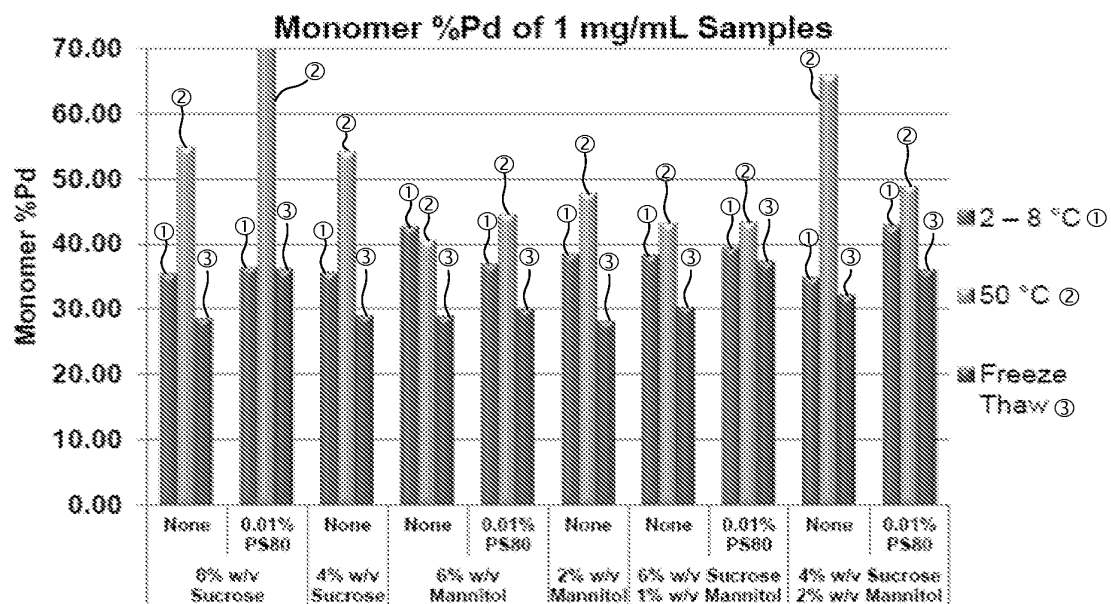
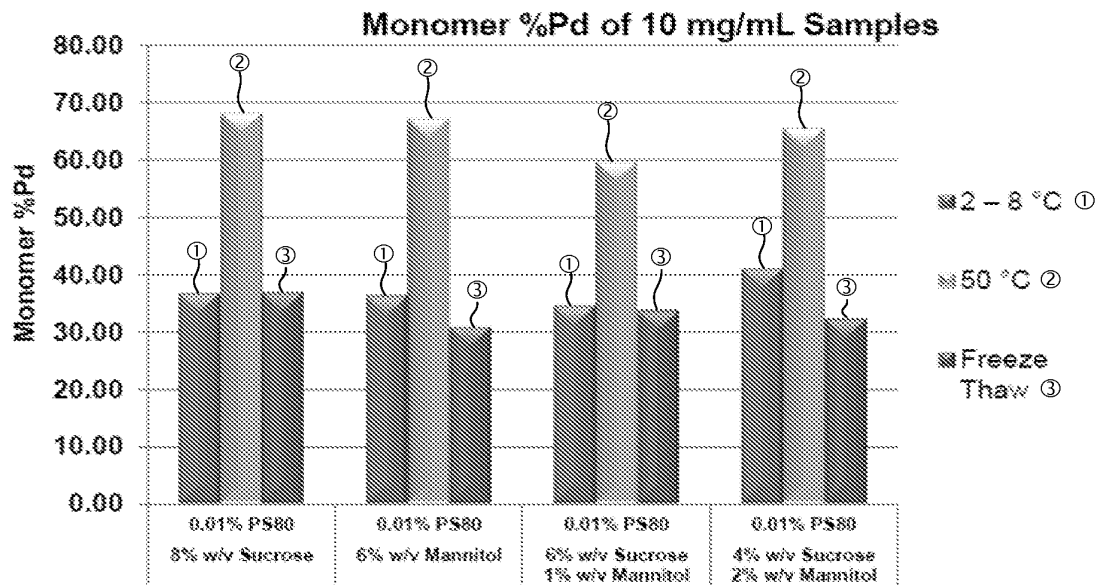


FIG. 48H



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FIG. 49A

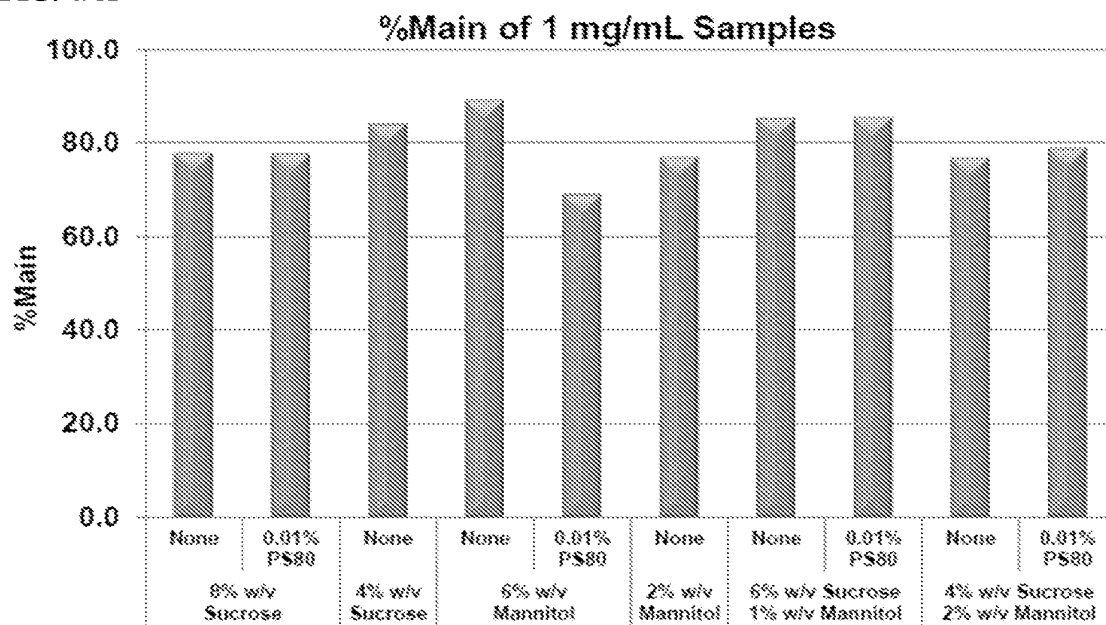
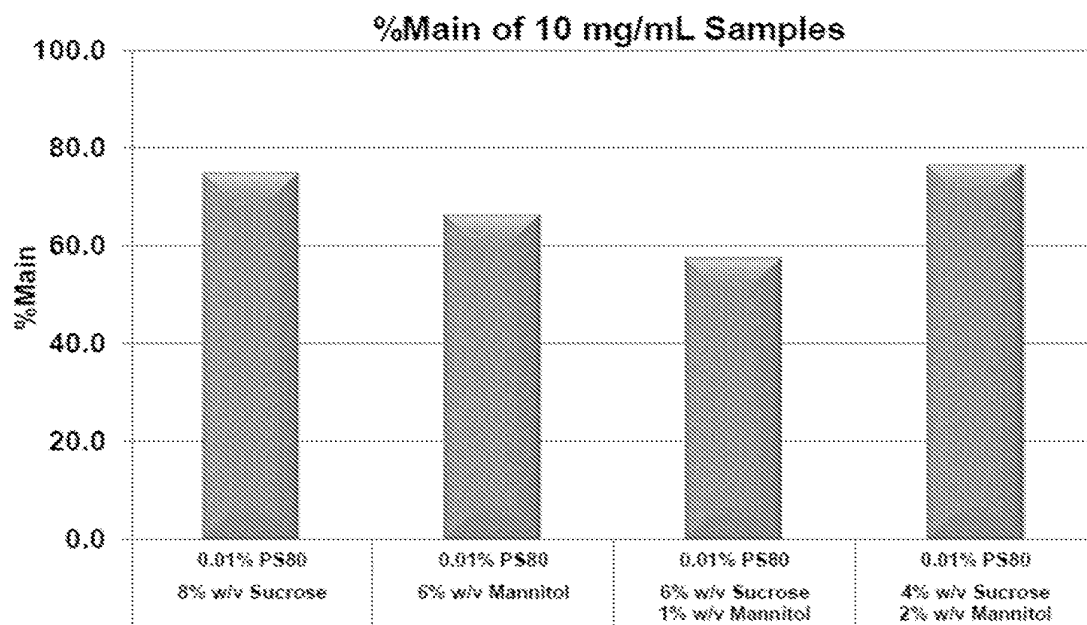


FIG. 49B



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FIG. 49C

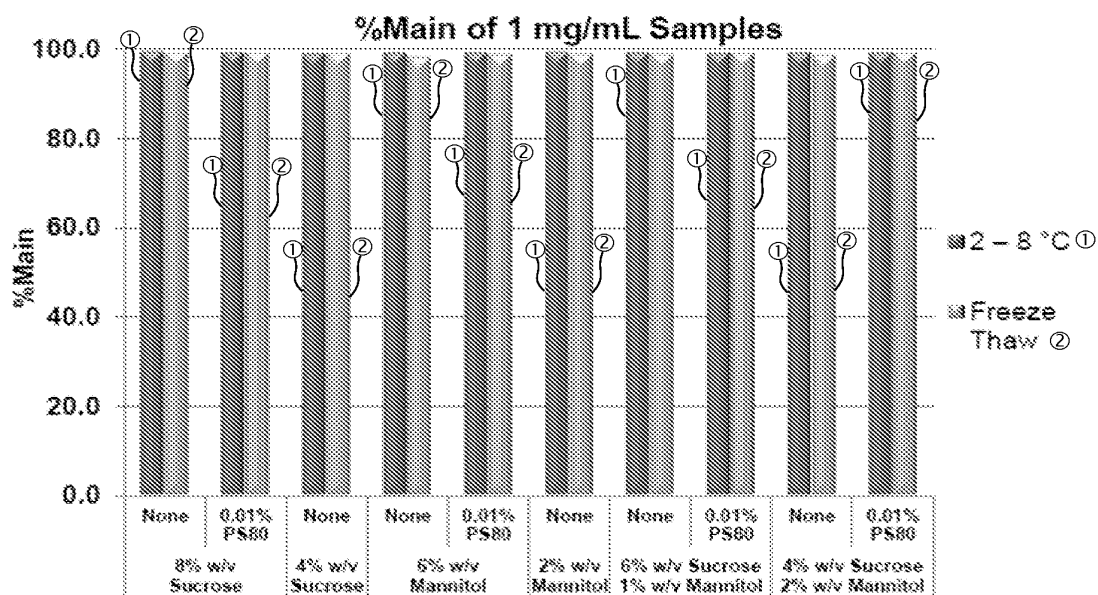
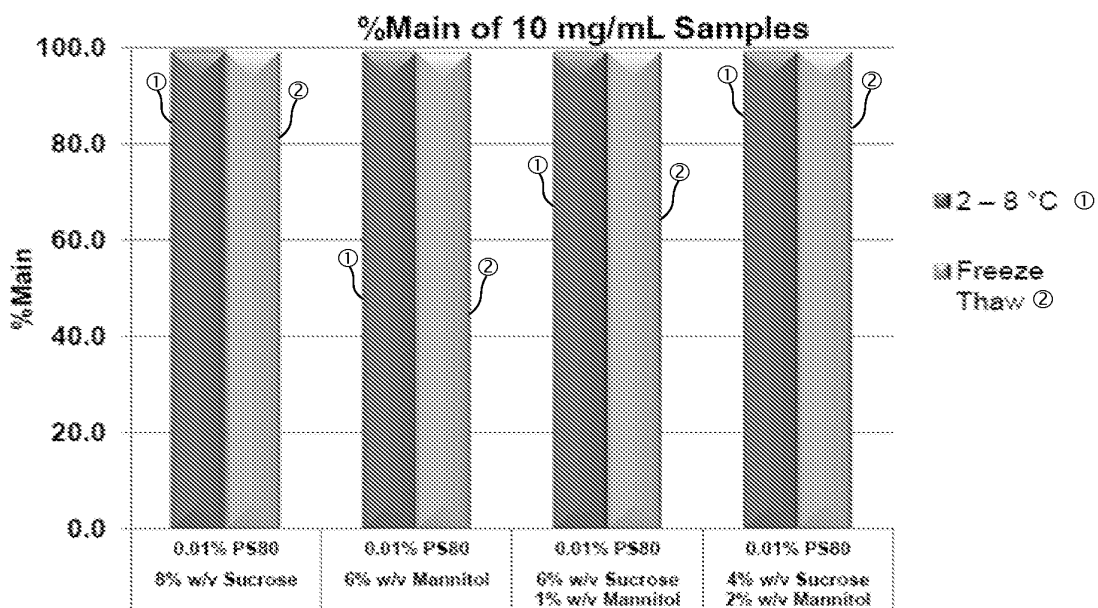


FIG. 49D



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FIG. 50A

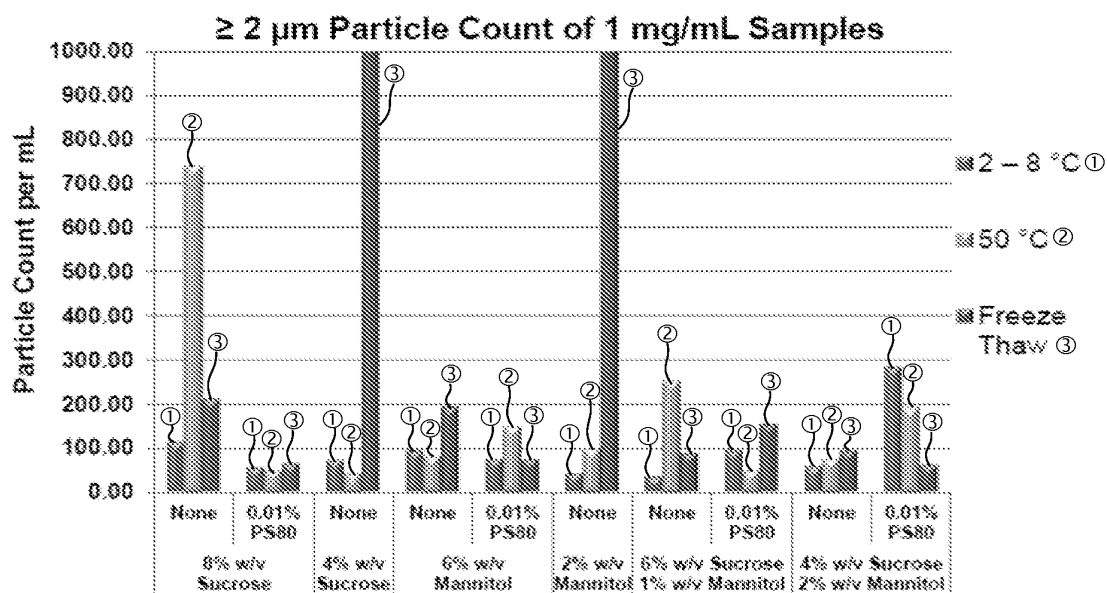
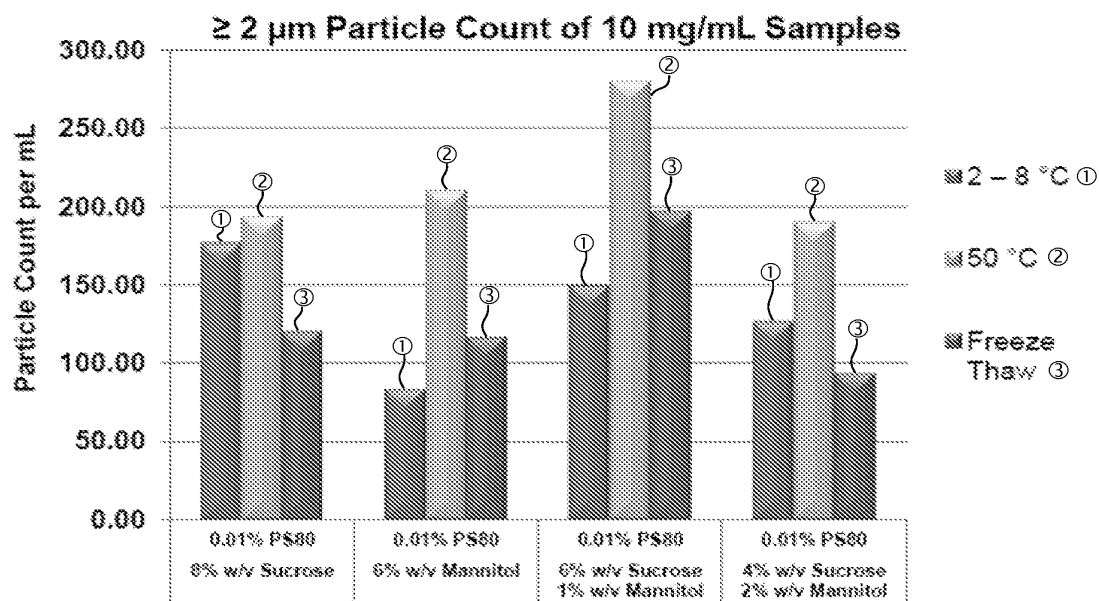


FIG. 50B



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FIG. 50C

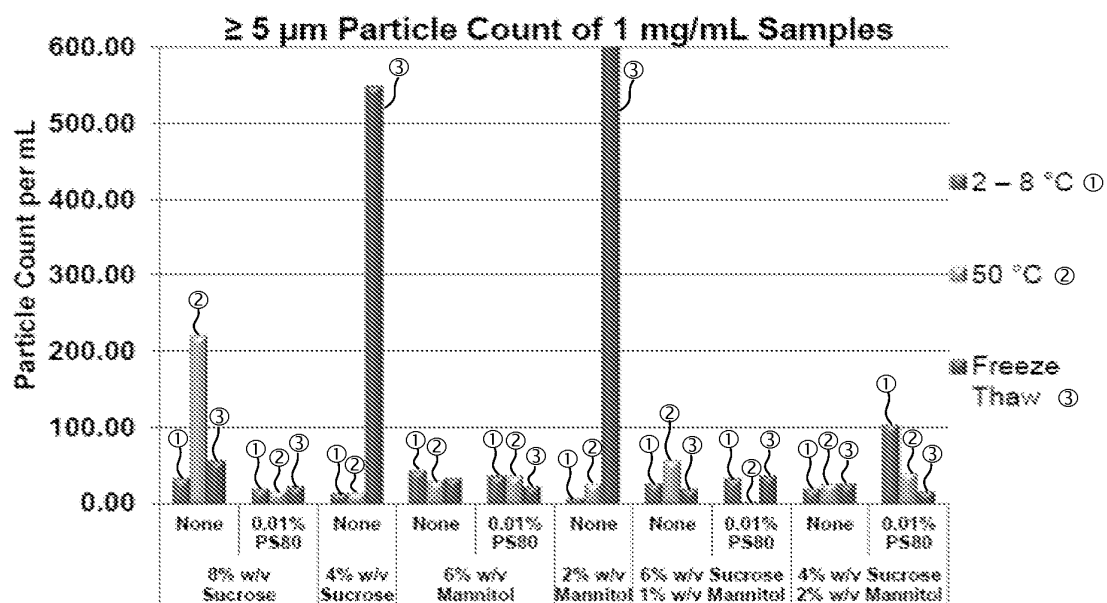
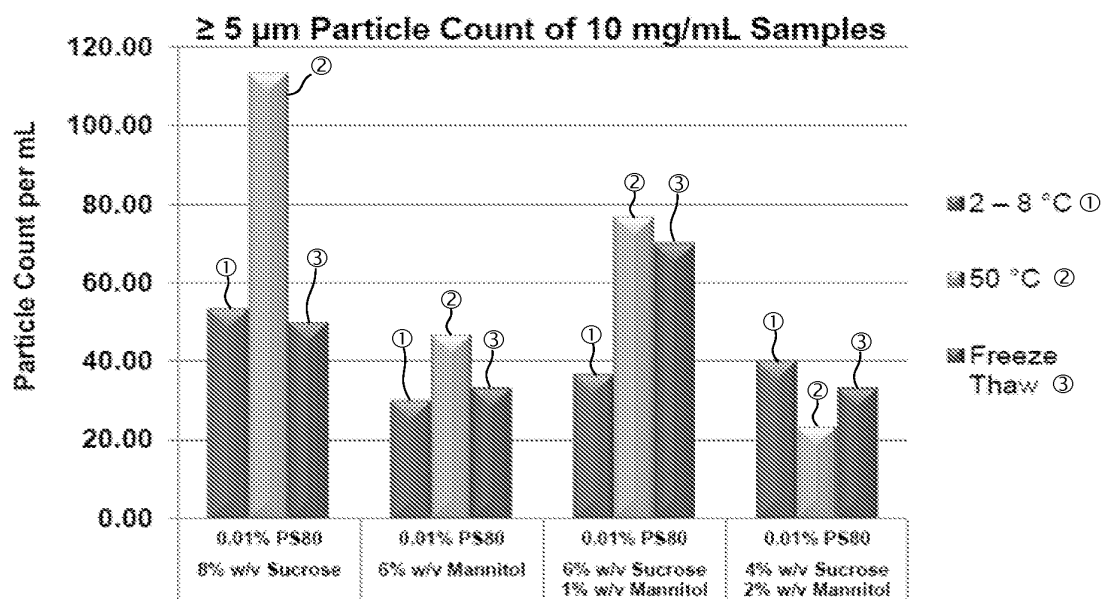


FIG. 50D



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FIG. 50E

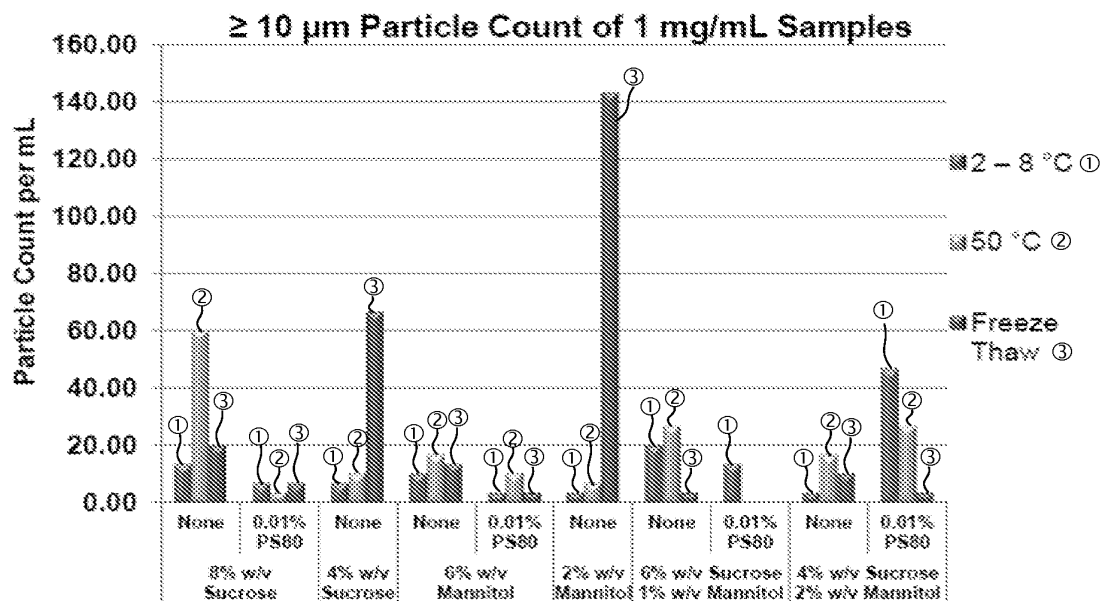
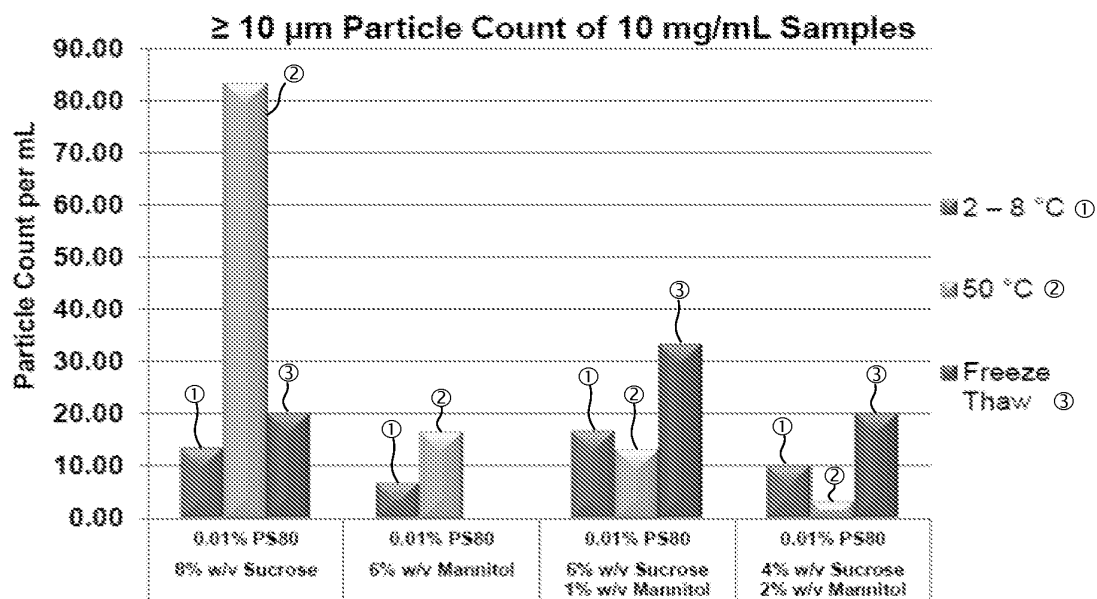


FIG. 50F



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FIG. 50G

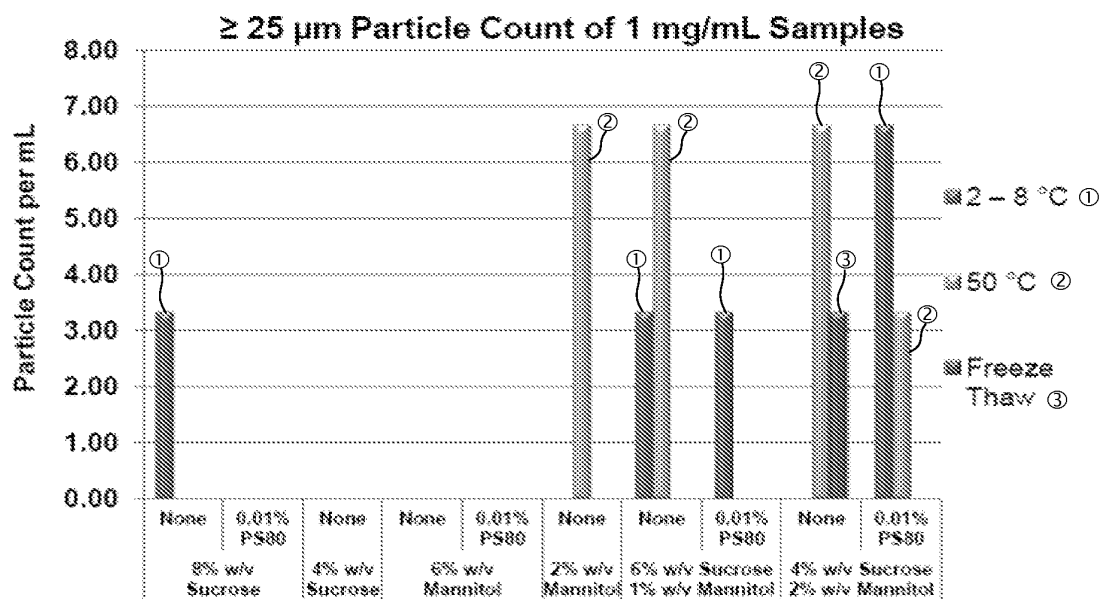


FIG. 50H

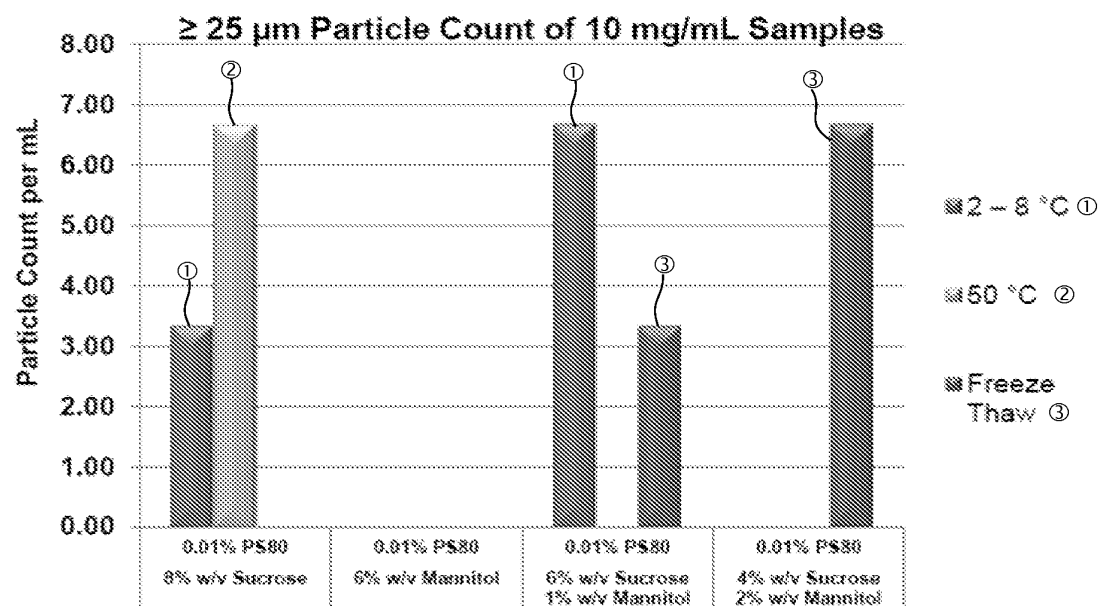


FIG. 51A

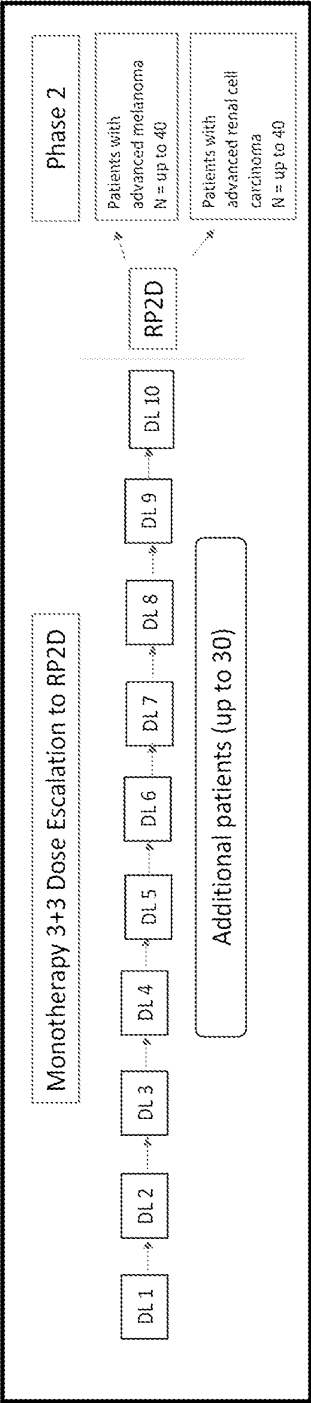
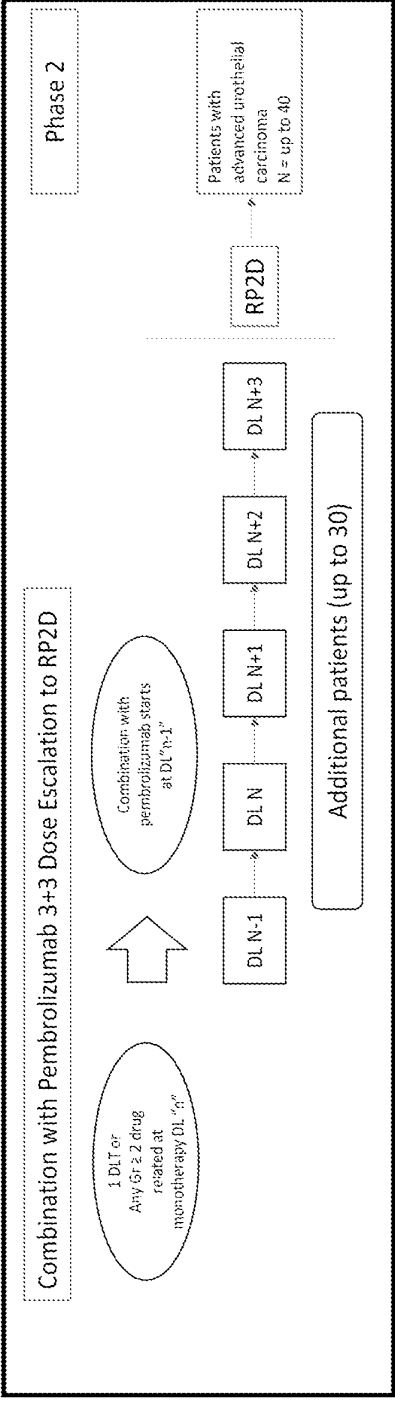


FIG. 51B



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FIG. 52A

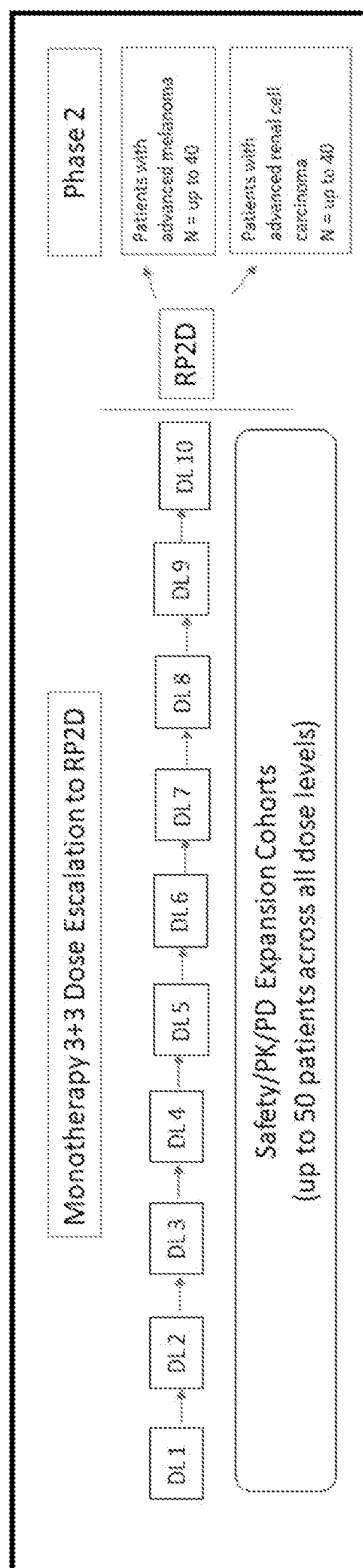
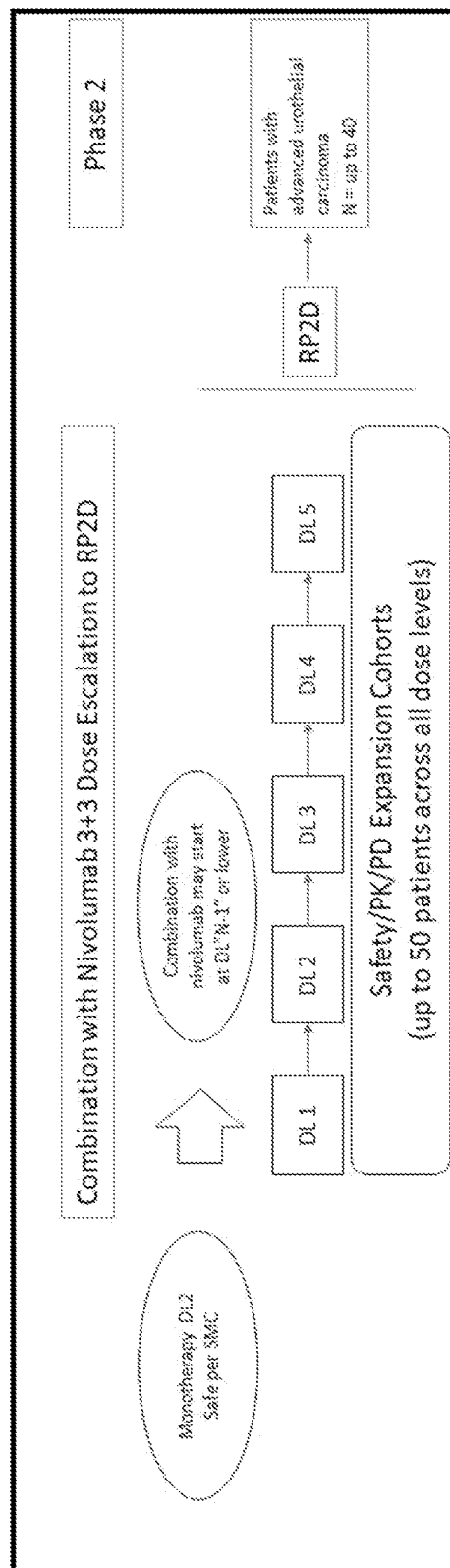


FIG. 52B



INTERNATIONAL SEARCH REPORT

International application No

PCT/US2021/028701

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K9/00 A61K47/12 A61K47/26 C07K14/28
A61K39/395 A61P13/00 A61P35/00

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C12R C07K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EP0-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	WO 2020/086758 A1 (DRAGONFLY THERAPEUTICS INC [US]) 30 April 2020 (2020-04-30) the whole document claims 1-244	1-303
Y	----- KEUNOK JUNG ET AL: "Heterodimeric Fc-fused IL12 shows potent antitumor activity by generating memory CD8 + T cells", ONCOIMMUNOLOGY, vol. 7, no. 7, 6 March 2018 (2018-03-06), page e1438800, XP055653232, DOI: 10.1080/2162402X.2018.1438800 abstract ----- -/--	1-303

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

8 July 2021

Date of mailing of the international search report

16/07/2021

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Felder, Christian

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2021/028701

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>WO 2019/051308 A1 (DRAGONFLY THERAPEUTICS INC [US]) 14 March 2019 (2019-03-14) the whole document paragraph [0233] - paragraph [0260]; claims 1-103</p> <p>-----</p>	1-303

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2021/028701

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2021/028701

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2020086758 A1	30-04-2020	AU 2019366956 A1 CA 3117212 A1 CO 2021006474 A2 KR 20210080460 A SG 11202104136Y A TW 202033547 A WO 2020086758 A1	20-05-2021 30-04-2020 10-06-2021 30-06-2021 28-05-2021 16-09-2020 30-04-2020
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WO 2019051308 A1	14-03-2019	AU 2018329937 A1 AU 2021201494 A1 BR 112020004489 A2 CA 3074840 A1 CA 3112984 A1 CN 111315782 A CN 113004417 A EP 3679071 A1 JP 2020533311 A JP 2021098733 A KR 20200044957 A KR 20210030503 A RU 2021110369 A SG 10202102502V A SG 11202001930Q A US 2020277383 A1 WO 2019051308 A1	19-03-2020 25-03-2021 08-09-2020 14-03-2019 14-03-2019 19-06-2020 22-06-2021 15-07-2020 19-11-2020 01-07-2021 29-04-2020 17-03-2021 01-06-2021 29-04-2021 29-04-2020 03-09-2020 14-03-2019
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(51) International Patent Classification:

A61K 39/395 (2006.01) C07K 16/28 (2006.01)
A61P 35/02 (2006.01) C07K 16/46 (2006.01)

(21) International Application Number:

PCT/US2021/013294

(22) International Filing Date:

13 January 2021 (13.01.2021)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/960,562 13 January 2020 (13.01.2020) US
63/121,637 04 December 2020 (04.12.2020) US

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(72) Inventors; and

(71) Applicants: **CLAPPER, Jonathan** [US/US]; Aptevo Research and Development LLC, 2401 Fourth Avenue, Suite 1050, Seattle, Washington 98121 (US). **BIENVENUE,**

David [US/US]; Aptevo Research and Development LLC, 2401 Fourth Avenue, Suite 1050, Seattle, Washington 98121 (US). **STROMATT, Scott** [US/US]; Aptevo Research and Development LLC, 2401 Fourth Avenue, Suite 1050, Seattle, Washington 98121 (US). **MCMAHAN, Catherine J.** [US/US]; Aptevo Research and Development LLC, 2401 Fourth Avenue, Suite 1050, Seattle, Washington 98121 (US).

(74) Agent: **MCLEOD, Bonnie Weiss et al.**; Cooley LLP, 1299 Pennsylvania Avenue NW, Suite 700, Washington, District of Columbia 20004-2400 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW,

(54) Title: FORMULATIONS FOR PROTEIN THERAPEUTICS

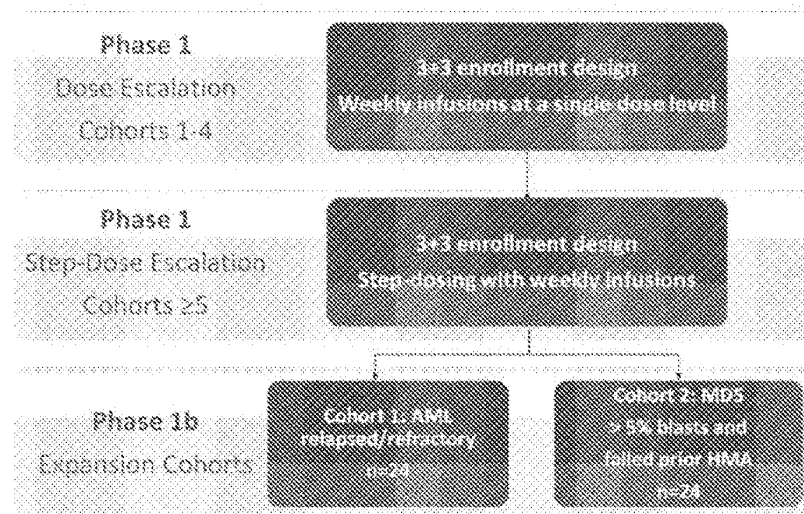


FIG. 2

(57) Abstract: Described herein are compositions comprising protein therapeutics, including multispecific polypeptides and fusion proteins, for intravenous administration. The compositions may comprise, for example, a multispecific protein, a buffer, an excipient, and a surfactant. In some embodiments, the compositions may comprise a multispecific protein, a succinate buffer, sucrose, and polysorbate 80. Also provided herein are clinical methods, including dosing regimens, for administration of the compositions to a subject in need thereof.

[Continued on next page]

SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) **Designated States** (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ,
UG, ZM, ZW); Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM); European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR); OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(ii))

FORMULATIONS FOR PROTEIN THERAPEUTICS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Nos. 63/121,633, filed December 4, 2020, and 62/960,562, filed January 13, 2020, each of which is incorporated by reference herein in its entirety for all purposes.

FIELD OF THE DISCLOSURE

[0002] The disclosure relates to formulations for protein therapeutics. Specifically, the disclosure relates to compositions comprising a bispecific or multispecific protein, a buffer, an excipient, and a surfactant. More specifically, the disclosure relates to formulations for a bispecific or multispecific protein comprising a CD123 binding domain and a CD3 binding domain. The disclosure also relates to clinical methods, including dosing regimens, for administration of the protein therapeutics to a subject in need thereof.

SEQUENCE LISTING

[0003] This application contains a Sequence Listing which has been submitted electronically and is hereby incorporated by reference in its entirety. The Sequence Listing was recorded January 13, 2021, is named APVO_061_02SeqList_ST25.txt, and is about 103 kilobytes in size.

BACKGROUND

[0004] One of the key steps in the development of a successful protein therapeutic is development of a formulation that maintains the physical and chemical integrity of the protein during long-term storage, handling by healthcare professionals, and administration. Protein therapeutics to be administered by the intravenous (i.v.) route are often stored frozen, in a concentrated solution, and diluted in the clinic before use. Moreover, protein therapeutics such as bispecific antibodies and multispecific antibodies, especially those comprising one or more scFv domains, can be prone to formation of aggregates.

[0005] Developing a protein formulation, particularly for administration by the intravenous route, requires careful consideration of many factors, including the properties of the protein, the composition of the formulation, the choice of diluent,

storage temperature, infusion rate, exposure to light, etc. There is a need in the art for stable formulations for protein therapeutics, particularly for administration by the intravenous route.

[0006] There is also a need for dosing strategies to mitigate the risk associated with the effects of cytokine release in patients treated with bispecific and multispecific therapeutics that act by T-cell engagement (i.e., T-cell engagers). This class of therapeutics includes bispecific therapeutics that target CD123 and CD3. mAb14045 (Xencor), a CD123 x CD3 bispecific antibody molecule being evaluated in patients with relapsed or refractory acute myeloid leukemia and other CD123-expressing hematologic malignancies, was placed on a partial clinical hold by the FDA in 2019 due to the deaths of two patients in a Phase I trial, including one death caused by cytokine release syndrome (CRS).

[0007] Dosing strategies designed to reduce the likelihood of severe effects of cytokine release, including cytokine release syndrome, may fail to be therapeutically effective. Accordingly, a need remains for methods to deliver a therapeutically effective dose of a T-cell engager (such as a CD123 x CD3 therapeutic) to a patient in a manner that mitigates risk of toxicity, including cytokine toxicity.

BRIEF SUMMARY

[0008] Described herein are compositions comprising protein therapeutics, including multispecific polypeptides and fusion proteins, for intravenous administration. The compositions may comprise, for example, a multispecific protein, a buffer, an excipient, and a surfactant. In some embodiments, the compositions may comprise a multispecific protein, a succinate buffer, sucrose, and polysorbate 80. In some embodiments, the composition is for intravenous or subcutaneous administration.

[0009] The disclosure provides multispecific polypeptides that are formulated with a succinate buffer and sucrose. In some embodiments, the multispecific polypeptide comprises two or more scFv binding domains. In some embodiments, the multispecific polypeptide forms a homodimer. In other embodiments, the multispecific polypeptide forms a heterodimer. In some embodiments, the multispecific polypeptide is in a format selected from the group consisting of scFv-Fc-scFv (e.g., ADAPTIR®), quadromas, K λ -bodies, dAbs, diabodies, TandAbs, nanobodies, DOCK-AND-LOCKS® (DNLs®), CrossMab Fabs, CrossMab VH-VLs, strand-exchange engineered domain bodies

(SEEDbodies), Affibodies, Fynomers, Kunitz Domains, Albu-dabs, two engineered Fv fragments with exchanged VHs (e.g., a dual-affinity re-targeting molecules (D.A.R.T.s)), scFv x scFv (e.g., BITE), DVD-IG, Conv-bodies, peptibodies, scFv-Igs, SVD-Igs, dAb-Igs, Knobs-in-Holes, IgG1 antibodies comprising matched mutations in the CH3 domain (e.g., DuoBody antibodies) and triomAbs.

[0010] In some embodiments, the disclosure provides a composition comprising a multispecific protein, a buffer, an excipient and a surfactant, wherein the multispecific protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus, or in order from carboxyl-terminus to amino-terminus (i) a first binding domain, (ii) a hinge region, (iii) an immunoglobulin constant region, and (iv) a second binding domain; and the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof. In some embodiments, each polypeptide comprises a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 31.

[0011] In some embodiments, the composition comprises from about 1 mM to about 10 mM succinate, or a pharmaceutically acceptable salt or acid thereof. In some embodiments, the composition comprises about 5 mM succinate, or a pharmaceutically acceptable salt or acid thereof.

[0012] In some embodiments, the excipient comprises or consists of a sugar, such as sucrose. In some embodiments, the composition may comprise about 1% weight/volume (w/v) to about 12% w/v of the sugar. In some embodiments, the composition comprises about 6.5% (w/v) of the sugar.

[0013] In some embodiments, the surfactant comprises or consists of polysorbate 80. In some embodiments, the composition comprises about 0.02% w/v polysorbate 80.

[0014] In some embodiments, the composition comprises from about 0.1 mg/ml to about 10 mg/ml of the multispecific protein. For example, the composition may comprise from about 1 mg/ml to about 5 mg/ml of the multispecific protein. In some embodiments, the composition comprises about 2 mg/ml of the multispecific protein. In some embodiments, the composition comprises about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80. In some embodiments, the composition has a pH from about 4.0 to about 5.5. In some embodiments, the composition has a pH of about 4.8.

[0015] In some embodiments, the immunoglobulin constant region is a human Fc domain. In some embodiments, the immunoglobulin constant region comprises immunoglobulin CH2 and CH3 domains of IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 or IgD.

[0016] In some embodiments, the first binding domain is a CD3 binding domain and the second binding domain is a tumor antigen binding domain. In some embodiments, the polypeptide comprises, from N-terminus to C-terminus, the CD3 binding domain, the hinge region, the immunoglobulin constant region, and the tumor antigen binding domain. In some embodiments, the first domain is a tumor antigen binding domain, and the second binding domain is a CD3 binding domain. In some embodiments, the polypeptide comprises, from N-terminus to C-terminus, the tumor antigen binding domain, the hinge region, the immunoglobulin constant region, and the CD3 binding domain. In some embodiments, the tumor antigen binding domain binds to CD123, PSMA, CD19, CD33, 5T4, or HER2.

[0017] In some embodiments, the first binding domain is a 4-1-BB binding domain and the second binding domain is a tumor antigen binding domain. In some embodiments, the polypeptide comprises, from N-terminus to C-terminus, the 4-1-BB binding domain, the hinge region, the immunoglobulin constant region, and the tumor antigen binding domain. In some embodiments, the first binding domain is a tumor antigen binding domain, and the second binding domain is a 4-1-BB binding domain. In some embodiments, the polypeptide comprises, from N-terminus to C-terminus, tumor antigen binding domain, the hinge region, the immunoglobulin constant region, and the 4-1-BB binding domain. In some embodiments, the tumor antigen binding domain binds to CD123, PSMA, CD19, CD33, 5T4, or HER2.

[0018] In some embodiments, at least one of the first binding domain and the second binding domain comprises (i) an immunoglobulin heavy chain variable region (VH) comprising HCDR1, HCDR2, and HCDR3; and (ii) an immunoglobulin light chain variable region (VL) comprising LCDR1, LCDR2, and LCDR3. In some embodiments, at least one of the first binding domain and the second binding domain is a single chain variable fragment (scFv). In some embodiments, the light chain variable region of the scFv is carboxy-terminal to the heavy chain variable region of the scFv. In some embodiments, the light chain variable region of the scFv is amino-terminal to the heavy chain variable region of the scFv. In some embodiments, the scFv comprises a linker polypeptide. The linker polypeptide may be, for example, between the light chain variable region and the heavy chain variable region of the scFv. In some embodiments,

the linker polypeptide comprises a Gly₄Ser (SEQ ID NO: 128) linker, such as (Gly₄Ser)_n, wherein n = 1-5 (SEQ ID NO: 129).

[0019] In some embodiments, a tumor antigen binding domain is an anti-CD123 scFv comprising a HCDR1 that comprises SEQ ID NO: 10, a HCDR2 that comprises SEQ ID NO: 11, and a HCDR3 that comprises SEQ ID NO: 12; and a LCDR1 that comprises SEQ ID NO: 13, a LCDR2 that comprises SEQ ID NO: 14, and a LCDR3 that comprises SEQ ID NO: 15. In some embodiments, the tumor antigen binding domain is an anti-CD123 scFv comprising a VH comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 136, and a VL comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 134. In some embodiments, the tumor antigen binding domain is an anti-CD123 scFv, and wherein the scFv comprises a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 18.

[0020] In some embodiments, a CD3 binding domain is an anti-CD3 scFv comprising a HCDR1 that comprises SEQ ID NO: 19, a HCDR2 that comprises SEQ ID NO: 20, and a HCDR3 that comprises SEQ ID NO: 21; and a LCDR1 that comprises SEQ ID NO: 22, a LCDR2 that comprises SEQ ID NO: 23, and a LCDR3 that comprises SEQ ID NO: 24. In some embodiments, the CD3 binding domain is an anti-CD3 scFv that comprises a VH comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 383 or 387, and a VL comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 384. In some embodiments, the CD3 binding domain is an anti-CD3 scFv that comprises a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 27.

[0021] In some embodiments, the immunoglobulin constant region comprises one or more mutations to reduce/prevent FcγR binding, ADCC activity, and/or CDC activity. In some embodiments, the immunoglobulin constant region comprises a human IgG1 CH2 domain comprising the substitutions L234A, L235A, G237A, and K322A, according to the EU numbering system. In some embodiments, the immunoglobulin constant region comprises a human IgG1 CH2 domain comprising the substitutions L234A, L235A, G237A, E318A, K320A and K322A, according to the EU numbering system. In some embodiments, the immunoglobulin comprises the sequence of SEQ ID NO: 131, or a sequence at least 90% or at least 95% identical thereto.

[0022] In some embodiments, the hinge region is derived from an immunoglobulin hinge region.

[0023] In some embodiments, each polypeptide comprises an Fc-binding domain linker between the immunoglobulin constant region, and the second binding domain. In some embodiments, the Fc-binding domain linker comprises a Gly₄Ser (SEQ ID NO: 128) sequence. In some embodiments, the Fc-binding domain linker comprises the formula (Gly₄Ser)_n, wherein n = 1-5 (SEQ ID NO: 129).

[0024] In some embodiments, the composition substantially prevents degradation of the multispecific protein. In some embodiments, the composition is substantially stable for at least 1 year at 4°C. In some embodiments, the composition is substantially resistant to formation of aggregates of multispecific protein.

[0025] The disclosure also provides a composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein the fusion protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus (i) a first binding domain that specifically binds to CD123, (ii) a hinge region, (iii) an immunoglobulin constant region, and (iv) a second binding domain that specifically binds to CD3; and (b) the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

[0026] The disclosure also provides a composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein the fusion protein comprises a first binding domain that specifically binds to CD123 and a second binding domain that specifically binds to CD3; and the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

[0027] The disclosure also provides a composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein the fusion protein comprises (i) a first binding domain that specifically binds to CD123, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 10, HCDR2 of SEQ ID NO: 11, and HCDR3 of SEQ ID NO: 12; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 13, LCDR2 of SEQ ID NO: 14, and LCDR3 of SEQ ID NO: 15; and (ii) a second binding domain that specifically binds to CD3, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 19, HCDR2 of SEQ ID NO: 20, and HCDR3 of SEQ ID NO: 21; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 22, LCDR2 of SEQ

ID NO: 23, and LCDR3 of SEQ ID NO: 24; and the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

[0028] The disclosure also provides a composition comprising a fusion protein, about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80, wherein the fusion protein comprises (i) a first binding domain that specifically binds to CD123, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 10, HCDR2 of SEQ ID NO: 11, and HCDR3 of SEQ ID NO: 12; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 13, LCDR2 of SEQ ID NO: 14, and LCDR3 of SEQ ID NO: 15; and (ii) a second binding domain that specifically binds to CD3, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 19, HCDR2 of SEQ ID NO: 20, and HCDR3 of SEQ ID NO: 21; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 22, LCDR2 of SEQ ID NO: 23, and LCDR3 of SEQ ID NO: 24.

[0029] The disclosure also provides a composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein the fusion protein comprises (i) a first binding domain that specifically binds to CD123, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising SEQ ID NO: 136, and an immunoglobulin light chain variable region (VL) comprising SEQ ID NO: 134; and (ii) a second binding domain that specifically binds to CD3, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising SEQ ID NO: 383 or 387; and an immunoglobulin light chain variable region (VL) comprising SEQ ID NO: 384; and the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

[0030] The disclosure also provides a composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein the fusion protein comprises (i) a first binding domain that specifically binds to CD123, wherein the first binding domain comprises SEQ ID NO: 18; and (ii) a second binding domain that specifically binds to CD3, wherein the second binding domain comprises SEQ ID NO: 27; and the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

[0031] The disclosure also provides a composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein the fusion protein is a dimer of two

identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus, or in order from carboxyl-terminus to amino-terminus (i) a first binding domain that specifically binds to CD123, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 10, HCDR2 of SEQ ID NO: 11, and HCDR3 of SEQ ID NO: 12; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 13, LCDR2 of SEQ ID NO: 14, and LCDR3 of SEQ ID NO: 15, (ii) a hinge region of SEQ ID NO: 47, (iii) an immunoglobulin constant region of SEQ ID NO: 33, (iv) a Fc-binding domain linker of SEQ ID NO: 132, and (v) a second binding domain that specifically binds to CD3, wherein the second binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 19, HCDR2 of SEQ ID NO: 20, and HCDR3 of SEQ ID NO: 21; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 22, LCDR2 of SEQ ID NO: 23, and LCDR3 of SEQ ID NO: 24; and the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

[0032] The disclosure also provides a composition comprising a fusion protein, about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80, wherein the fusion protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus (i) a first binding domain that specifically binds to CD123, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 10, HCDR2 of SEQ ID NO: 11, and HCDR3 of SEQ ID NO: 12; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 13, LCDR2 of SEQ ID NO: 14, and LCDR3 of SEQ ID NO: 15, (ii) a hinge region of SEQ ID NO: 47, (iii) an immunoglobulin constant region of SEQ ID NO: 33, (iv) a Fc-binding domain linker of SEQ ID NO: 132, and (v) a second binding domain that specifically binds to CD3, wherein the second binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 19, HCDR2 of SEQ ID NO: 20, and HCDR3 of SEQ ID NO: 21; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 22, LCDR2 of SEQ ID NO: 23, and LCDR3 of SEQ ID NO: 24.

[0033] The disclosure also provides a composition comprising a fusion protein, about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80, wherein the fusion protein comprises or consists of SEQ ID NO: 31;

wherein the composition comprises about 2 mg/ml of the fusion protein; and wherein the composition has a pH of about 4.8.

[0034] The disclosure further provides a method for inhibiting the growth of psoriatic plaques in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a composition of the disclosure.

[0035] The disclosure further provides a method for treating cancer in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of a composition of the disclosure. The cancer may be, for example, a hematological malignancy. For instance, the cancer may be acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), hairy cell leukemia (HCL), blastic plasmacytoid dendritic cell neoplasm, B-cell acute lymphoblastic leukemia (ALL), or chronic myeloid leukemia (CML).

[0036] Also provided are uses of the compositions of the disclosure for treating cancer in a subject. Also provided are uses of the compositions of the disclosure in the manufacture of a medicament for treating cancer. For instance, compositions of the disclosure may be used for treatment of acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). In some embodiments, compositions of the disclosure may be used for treatment of high-risk or high-grade MDS. A composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain may be administered to a subject by IV infusion at a weekly dose of about 0.3, about 1, about 3, about 6, about 9, about 12, about 18, about 20, about 24, about 30, about 36, about 50, about 48, about 60, about 75, or about 100 μ g. To reduce the risk of an adverse event, the first dose may be administered by IV to the patient over several hours, e.g., about 20-24 hours. In some embodiments, the first dose of the composition is administered over a period of about 20-24 hours, the second dose is administered over a period of about 8 hours, the third dose is administered over a period of about 6 hours, and the fourth dose and subsequent doses are administered over a period of about 4 hours. The composition can also be administered to a subject by continuous IV infusion, e.g., continuous IV infusion up to about 72 hours in duration.

[0037] A method for treatment of a patient in need thereof may include administering multispecific protein comprising a CD123 binding domain and a CD3 binding domain to a patient intravenously such that the dosage is increased each week for at least the first two or first three doses. For instance, a composition may be

delivered to the patient by IV infusion according to the following weekly treatment schedule: week 1 dosage: 6 μ g; week 2 dosage: 9 μ g; week 3 dosage: 12 μ g; and week 4 dosage and subsequent week dosages: 12 μ g. In some embodiments, a composition may be administered to a patient intravenously according to the following weekly treatment schedule: week 1 dosage: 6 μ g; week 2 dosage: 9 μ g; week 3 dosage: 12 μ g; and week 4 dosage and subsequent week dosages: 18 μ g. In some embodiments, the highest dose administered to the patient is about 24 μ g, about 36 μ g, about 48 μ g, about 60 μ g, or about 100 μ g. In some embodiments, the highest dose administered to the patient is in the range of about 100 μ g to about 130 μ g.

[0038] In some embodiments, a composition may be administered to a patient in a treatment cycle lasting about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, or more. In some embodiments, the composition may be administered to the patient over more than one treatment cycle, such as 2, 3, 4, 5, 6, 7, 8, or more treatment cycles. In some embodiments the treatment cycle lasts 4 weeks, and may be repeated for up to 6 cycles. In some embodiments, the treatment cycle may be repeated for up to 36 cycles.

[0039] In some embodiments, the composition is administered to a patient intravenously according to the weekly treatment schedule: week 1 dosage: 6 μ g; and week 2 and subsequent week dosages: 9 μ g, and in some embodiments, the composition is administered to a patient intravenously according to the weekly treatment schedule week 1 dosage: 9 μ g; and week 2 and subsequent week dosages: 12 μ g. In some embodiments, the composition is administered to a patient intravenously according to the weekly treatment schedule: week 1 dosage 12 μ g, and week 2 and subsequent week dosages: 18 μ g.

[0040] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient on days 1, 8, 15, and 22. In some embodiments, 6 μ g is administered on day 1, 9 μ g is administered on day 8, 12 μ g is administered on day 15, and 12 μ g is administered on day 22. In some embodiments, 6 μ g is administered on day 1, 9 μ g is administered on day 8, 12 μ g is administered on day 15, and 18 μ g is administered on day 22. In some embodiments, 6 μ g is administered on day 1, 9 μ g is administered on day 8, 9 μ g is administered on day 15, and 9 μ g is administered on day 22. In some embodiments, 9 μ g is administered on day 1, 12 μ g is administered on day 8, 12 μ g is administered on day

15, and 12 μ g is administered on day 22. In some embodiments, 12 μ g is administered on day 1, 18 μ g is administered on day 8, 18 μ g is administered on day 15, and 18 μ g is administered on day 22. In some embodiments, a patient treated according to the methods of the disclosure exhibits a decrease in bone marrow blast percentage, and in some embodiments, a patient exhibits a decrease in absolute blast counts in the blood. In some embodiments, the treatment results in reduction in patient blast levels in the blood by at least 0.5%, at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 45%, at least 50%, or more, compared to the patient's levels immediately before the treatment.

[0041] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient during a 28-day cycle. In some embodiments, the composition is administered to the patient once, twice, three times, or four times each week during the 28-day cycle. In some embodiments, the dosage is increased over the course of the 28-day cycle. In some embodiments, the dosage is decreased over the course of the 28-day cycle. In some embodiments, the dosage is increased each week during the 28-day cycle. In some embodiments, the dosage is decreased each week during the 28-day cycle.

[0042] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient on days 1, 8, 15, and 22 of a first 28-day cycle, and on days 1, 8, 15, and 22 of at least one additional 28-day cycle. In some embodiments, the dose administered on day 22 of the first 28-day cycle is the same as the dose administered on days 1, 8, 15, and 22 of the at least one additional 28-day cycle. In some embodiments, the patient is treated for two, three, four, five, six, seven, eight, or more additional 28-day cycles, wherein administration of the composition occurs on days 1, 8, 15, and 22 of each cycle.

[0043] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient on days 1, 2, 3, 4, 8, 11, 15, and 22 of a first 28-day cycle. In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain

to the patient on days 1, 2, 3, 4, 8, 11, 15, and 22 of a first 28-day cycle, and then subsequently on days 1, 8, 15, and 22 of at least one additional 28-day cycle. In some embodiments, the patient is treated for two, three, four, five, six, seven, eight, or more additional 28-day cycles, wherein administration of the composition occurs on days 1, 8, 15, and 22 of each cycle.

[0044] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient on days 1, 2, 3, 4, 8, 11, 15, 18, 22, and 25 of a first 28-day cycle. In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient on days 1, 2, 3, 4, 8, 11, 15, 18, 22, and 25 of a first 28-day cycle, and then subsequently on days 1, 8, 15, and 22 of at least one additional 28-day cycle. In some embodiments, the patient is treated for two, three, four, five, six, seven, eight, or more additional 28-day cycles, wherein administration of the composition occurs on days 1, 8, 15, and 22 of each cycle.

[0045] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient during a first 28-day cycle, wherein 6 μ g of the multispecific protein is administered on day 1, 9 μ g of the multispecific protein is administered on day 2, 12 μ g of the multispecific protein is administered on day 3, 18 μ g of the multispecific protein is administered on day 4, 18 μ g of the multispecific protein is administered on day 8, 18 μ g of the multispecific protein is administered on day 11, 36 μ g of the multispecific protein is administered on day 15, and 36 μ g of the multispecific protein is administered on day 22 of a first 28-day cycle. In some embodiments, the method further comprises administering the multispecific protein to the patient during at least one additional 28-day cycle, wherein 36 μ g of the multispecific protein is administered on each of days 1, 8, 15, and 22 of the at least one additional 28-day cycle.

[0046] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient during a first 28-day cycle, wherein 6 μ g of the multispecific protein is administered on day 1, 12 μ g of

the multispecific protein is administered on day 2, 18 µg of the multispecific protein is administered on day 3, 24 µg of the multispecific protein is administered on day 4, 24 µg of the multispecific protein is administered on day 8, 24 µg of the multispecific protein is administered on day 11, 48 µg of the multispecific protein is administered on day 15, and 48 µg of the multispecific protein is administered on day 22 of a first 28-day cycle. In some embodiments, the method further comprises administering the multispecific protein to the patient during at least one additional 28-day cycle, wherein 48 µg of the multispecific protein is administered on each of days 1, 8, 15, and 22 of the at least one additional 28-day cycle.

[0047] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient during a first 28-day cycle, wherein 6 µg of the multispecific protein is administered on day 1, 12 µg of the multispecific protein is administered on day 2, 24 µg of the multispecific protein is administered on day 3, 36 µg of the multispecific protein is administered on day 4, 36 µg of the multispecific protein is administered on day 8, 36 µg of the multispecific protein is administered on day 11, 60 µg of the multispecific protein is administered on day 15, and 60 µg of the multispecific protein is administered on day 22 of a first 28-day cycle. In some embodiments, the method further comprises administering the multispecific protein to the patient during at least one additional 28-day cycle, wherein 60 µg of the multispecific protein is administered on each of days 1, 8, 15, and 22 of the at least one additional 28-day cycle.

[0048] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient during a first 28-day cycle, wherein 6 µg of the multispecific protein is administered on day 1, 12 µg of the multispecific protein is administered on day 2, 24 µg of the multispecific protein is administered on day 3, 36 µg of the multispecific protein is administered on day 4, 48 µg of the multispecific protein is administered on day 8, 48 µg of the multispecific protein is administered on day 11, 100 µg of the multispecific protein is administered on day 15, and 100 µg of the multispecific protein is administered on day 22 of a first 28-day cycle. In some embodiments, the method further comprises administering the multispecific protein to the patient during at least one additional 28-day cycle, wherein

100 µg of the multispecific protein is administered on each of days 1, 8, 15, and 22 of the at least one additional 28-day cycle.

[0049] In some embodiments, the highest dose administered to the patient in any one of the aforementioned schemes is increased by about 5% to about 40%, such as about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 26%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%, about 39%, or about 40%. In these embodiments, this increased dose may first be administered to the patient on the first day that the highest dose was previously administered in the aforementioned schemes.

BRIEF DESCRIPTION OF THE DRAWINGS

[0050] FIG. 1A and FIG. 1B are schematics showing the structures of exemplary therapeutic proteins for use with the compositions and methods of the disclosure. FIG. 1A shows a homodimeric protein comprising two identical polypeptides each comprising a CD3 binding domain and an Fc domain. FIG. 1B shows a homodimeric protein comprising two identical polypeptides each comprising a tumor binding domain (e.g., a CD123 binding domain), an Fc domain, and a CD3 binding domain. An exemplary CD123 x CD3 bispecific therapeutic protein is referred to herein as TRI130.

[0051] FIG. 2 is a schematic that shows the design of a Phase 1/1b dose escalation clinical study, wherein TRI130 is administered to patients with relapsed or refractory acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS).

[0052] FIG. 3A-3D show percentage of blasts in bone marrow aspirates, plotted over time for patients in the Phase 1/1b study described in FIG. 2 and Example 3. Data is graphed for patients in cohorts receiving a highest dose of ≥ 12 µg. N = 14 patients evaluable for changes from baseline. Cohorts 6a and 6b tested different step dosing regimens, as shown in Table 10.

[0053] FIG. 4A-4D show serum concentrations of interleukin-6 (IL-6, FIG. 4A), interleukin-10 (IL-10, FIG. 4B), interferon-gamma (IFN-γ, FIG. 4C) and tumor necrosis factor-alpha (TNF-α, FIG. 4D) in patient samples from scheduled blood collections (pre-dose, about 15-30 minutes post-dose, and about 20-26 hours post-dose; N=26) in the Phase 1/1b study described in FIG. 2 and Example 3. The scheduled blood

collections were collected at the first administration of the highest planned dose for each patient. Peak cytokine levels observed at unscheduled collections during IRR/CRS events are shown for comparison (N=6 events in 4 patients).

DETAILED DESCRIPTION

[0054] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited herein, including but not limited to patents, patent applications, articles, books, and treatises, are hereby expressly incorporated by reference in their entirety for any purpose. In the event that one or more of the incorporated documents or portions of documents define a term that contradicts that term's definition in the application, the definition that appears in this application controls. However, mention of any reference, article, publication, patent, patent publication, and patent application cited herein is not, and should not be taken as an acknowledgment, or any form of suggestion, that they constitute valid prior art or form part of the common general knowledge in any country in the world.

[0055] In the present description, any concentration range, percentage range, ratio range, or integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated. It should be understood that the terms "a" and "an" as used herein refer to "one or more" of the enumerated components unless otherwise indicated. The use of the alternative (e.g., "or") should be understood to mean either one, both, or any combination thereof of the alternatives. As used herein, the terms "include" and "comprise" are used synonymously. In addition, it should be understood that the polypeptides comprising the various combinations of the components (e.g., domains or regions) and substituents described herein, are disclosed by the present application to the same extent as if each polypeptide was set forth individually. Thus, selection of particular components of individual polypeptides is within the scope of the present disclosure.

Definitions

[0056] The term "about" when immediately preceding a numerical value means $\pm$ up to 10% of the numerical value. For example, "about 40" means $\pm$ up to 10% of 40 (i.e., from 36 to 44), for example $\pm$ up to 10%, $\pm$ up to 9%, $\pm$ up to 8%, $\pm$ up to 7%, $\pm$

up to 6%, $\pm$ up to 5%, $\pm$ up to 4%, $\pm$ up to 3%, $\pm$ up to 2%, $\pm$ up to 1%, $\pm$ up to less than 1%, or any other value or range of values therein.

[0057] As used herein, substantially has its ordinary meaning as used in the art. For example, "substantially" may mean "significantly," "considerably," "largely," "mostly," or "essentially." In some embodiments, "substantially" may refer to at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%.

[0058] The term "CD123" may refer to any isoform of CD123, also known as Cluster of Differentiation 123, Interleukin-3 receptor alpha chain, and IL3RA. CD123 associates with the beta chain of the interleukin-3 receptor to form the receptor. CD123 is a type I transmembrane glycoprotein, with an extracellular domain comprising a predicted Ig-like domain and two FnIII domains. The CD123-binding domains of the disclosure bind to the extracellular domain of CD123. CD123 is also known as the alpha chain of the human interleukin-3 (IL-3) receptor. CD123 is a type I transmembrane glycoprotein and is a member of the cytokine receptor superfamily. The interleukin-3 receptor is a heterodimer formed by CD123 and the beta chain (CD131). IL-3 binds to CD123, and signal transduction is provided by CD131. IL-3 regulates the function and production of hematopoietic and immune cells and stimulates endothelial cell proliferation (Testa et al., *Biomark Res.* 2:4 (2014)).

[0059] CD123 is overexpressed in many hematologic malignancies, including a subset of acute myeloid leukemia (AML), B-lymphoid leukemia, blastic plasmacytoid dendritic neoplasms (BPDCN) and hairy cell leukemia. While most AML patients respond well to initial therapies, the majority of AML patients are ultimately diagnosed with relapsed or refractory disease (Ramos et al., *J. Clin. Med.* 4:665-695 (2015)). There is a need for molecules targeting CD123 with increased efficiency and potency and reduced adverse effects and that may be used to treat disorders associated with dysregulation of CD123.

[0060] "CD3" is known in the art as a multi-protein complex of six chains (see, e.g., Abbas and Lichtman, 2003; Janeway et al., p. 172 and 178, 1999), which are subunits of the T-cell receptor complex. In mammals, the CD3 subunits of the T-cell receptor complex are a CD3 γ chain, a CD3 δ chain, two CD3 ϵ chains, and a homodimer of CD3 ζ chains. The CD3 γ , CD3 δ , and CD3 ϵ chains are highly related cell surface proteins of the immunoglobulin superfamily containing a single immunoglobulin domain. The

transmembrane regions of the CD3 γ , CD3 δ , and CD3 ϵ chains are negatively charged, which is a characteristic that allows these chains to associate with the positively charged T-cell receptor chains. The intracellular tails of the CD3 γ , CD3 δ , and CD3 ϵ chains each contain a single conserved motif known as an immunoreceptor tyrosine-based activation motif or ITAM, whereas each CD3 ζ chain has three. It is believed the ITAMs are important for the signaling capacity of a TCR complex. CD3 as used in the present disclosure can be from various animal species, including human, monkey, mouse, rat, or other mammals.

[0061] "Cytokine release" or "cytokine storm" or "infusion reaction" refers to the release of cytokines from T-cells. When cytokines are released into the circulation, systemic symptoms such as fever, nausea, chills, hypotension, tachycardia, asthenia, headache, rash, scratchy throat, and dyspnea can result. Some patients may experience severe, life-threatening reactions that result from massive release of cytokines. "Reduced" cytokine release refers to the to the reduction in the release of at least one cytokine (e.g., IFN- γ , TNF- α , IL-6, IL-2, IL-8, IL-10, IL-17, GM-CSF, IL-4, IL-12, IL-13 or IL-1 β) following administration of a bispecific molecule as disclosed herein, as compared to the OKT3 antibody (which binds CD3) or other CD3 binding bispecific molecule. Reduced cytokine release can be measured using *in vitro* assays or *in vivo* assays.

[0062] As used herein, the term "step dosing" or "stepped dosing" or similar terms refers to a dosing regimen wherein a multispecific polypeptide as described herein is administered to a patient on at least a first day and a second day, wherein the dose administered to the patient is either kept constant or increased between the first day and the second day. For example, in some step dosing regimens, a patient may be administered a first, a second, a third, and a fourth dose, wherein each dose is administered on different day, and wherein the second dose is greater than the first dose. The third dose may be greater than the second dose, or may be the same as the second dose. The fourth dose may be greater than the third dose, or may be the same as the third dose. In some embodiments, if the patient has an adverse response to a particular dose, the subsequent dose may be reduced.

[0063] As used herein, the term "binding domain" or "binding region" refers to the domain, region, portion, or site of a protein, polypeptide, oligopeptide, peptide, antibody, or binding domain derived from an antibody, receptor or ligand that possesses the ability to specifically recognize and bind to a target molecule, such as

an antigen, ligand, receptor, substrate, or inhibitor. Exemplary binding domains include, antibodies and antibody-like proteins or domains, antibody heavy and light chain variable regions, and single-chain antibody variable regions (e.g., domain antibodies, sFv, scFv, scFab), receptor ectodomains and ligands (e.g., cytokines, chemokines). In certain embodiments, the binding domain comprises or consists of an antigen binding site (e.g., comprising a variable heavy chain sequence and variable light chain sequence or three light chain complementary determining regions (CDRs) and three heavy chain CDRs from an antibody placed into alternative framework regions (FRs) (e.g., human FRs optionally comprising one or more amino acid substitutions). A variety of assays are known for identifying binding domains of the present disclosure that specifically bind a particular target, including Western blot, ELISA, phage display library screening, and BIAcore® interaction analysis.

[0064] A binding domain or protein comprising a binding domain "specifically binds" a target if it binds the target with an affinity or K_a (i.e., an equilibrium association constant of a particular binding interaction with units of 1/M) equal to or greater than 10^5 M^{-1} , while not significantly binding other components present in a test sample. Binding domains can be classified as "high affinity" binding domains and "low affinity" binding domains. "High affinity" binding domains refer to those binding domains with a K_a of at least 10^7 M^{-1} , at least 10^8 M^{-1} , at least 10^9 M^{-1} , at least 10^{10} M^{-1} , at least 10^{11} M^{-1} , at least 10^{12} M^{-1} , or at least 10^{13} M^{-1} . "Low affinity" binding domains refer to those binding domains with a K_a of up to 10^7 M^{-1} , up to 10^6 M^{-1} , up to 10^5 M^{-1} . Alternatively, affinity can be defined as an equilibrium dissociation constant (K_d) of a particular binding interaction with units of M (e.g., 10^{-5} M to 10^{-13} , or about 500 nM, about 300 nM, about 250 nM, about 200 nM, about 150 nM, about 100 nM, about 50 nM, about 25 nM, about 10 nM, or about 5 nM). Affinities of binding domain polypeptides and single chain polypeptides according to the present disclosure can be readily determined using conventional techniques (see, e.g., Scatchard et al. (1949) Ann. N.Y. Acad. Sci. 51:660; and U.S. Patent Nos. 5,283,173, 5,468,614, or the equivalent).

[0065] As used herein, a "conservative substitution" is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are well-known in the art (see, e.g., PCT Application Publication No. WO 97/09433, page 10, published March 13, 1997; Lehninger, Biochemistry, Second Edition; Worth Publishers, Inc. NY:NY (1975), pp.71-77; Lewin, Genes IV, Oxford University Press, NY and Cell Press, Cambridge,

MA (1990), p. 8). In certain embodiments, a conservative substitution includes a leucine to serine substitution.

[0066] As used herein, the term "derivative" refers to a modification of one or more amino acid residues of a peptide by chemical or biological means, either with or without an enzyme, e.g., by glycosylation, alkylation, acylation, ester formation, or amide formation.

[0067] As used herein, a polypeptide or amino acid sequence "derived from" a designated polypeptide or protein refers to the origin of the polypeptide. In certain embodiments, the polypeptide or amino acid sequence which is derived from a particular sequence (sometimes referred to as the "starting" or "parent" or "parental" sequence) and has an amino acid sequence that is essentially identical to the parent sequence or a portion thereof, wherein the portion consists of at least 10-20 amino acids, at least 20-30 amino acids, or at least 30-50 amino acids, or at least 50-150 amino acids, or which is otherwise identifiable to one of ordinary skill in the art as having its origin in the parent sequence. For example, a binding domain can be derived from an antibody, e.g., a Fab, F(ab')₂, Fab', scFv, single domain antibody (sdAb), etc.

[0068] Polypeptides derived from another polypeptide can have one or more mutations or alterations relative to the parent polypeptide, e.g., one or more amino acid residues which have been substituted with another amino acid residue or which has one or more amino acid insertions or deletions. In such embodiments, polypeptides derived from a parent polypeptide and comprising one or more mutations or alteration are referred to as "variants." As used herein, the term "variant" or "variants" refers to a polynucleotide or polypeptide with a sequence differing from that of a reference polynucleotide or polypeptide but retaining essential properties thereof. Generally, variant polynucleotide or polypeptide sequences are overall closely similar, and, in many regions, identical to the reference polynucleotide or polypeptide. For instance, a variant polynucleotide or polypeptide may exhibit at least about 70%, at least about 80%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity compared to the active portion or full length reference polynucleotide or polypeptide. The polypeptide can comprise an amino acid sequence which is not naturally occurring. Such variations necessarily have less than 100% sequence identity or similarity with the parent polypeptide. In one embodiment, the variant will have an amino acid sequence from

about 60% to less than 100% amino acid sequence identity or similarity with the amino acid sequence of the parent polypeptide. In another embodiment, the variant will have an amino acid sequence from about 75% to less than 100%, from about 80% to less than 100%, from about 85% to less than 100%, from about 90% to less than 100%, from about 95% to less than 100% amino acid sequence identity or similarity with the amino acid sequence of the parent polypeptide.

[0069] As used herein, the term "sequence identity" refers to a relationship between two or more polynucleotide sequences or between two or more polypeptide sequences. When a position in one sequence is occupied by the same nucleic acid base or amino acid residue in the corresponding position of the comparator sequence, the sequences are said to be "identical" at that position. The percentage sequence identity is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of identical positions. The number of identical positions is then divided by the total number of positions in the comparison window and multiplied by 100 to yield the percentage of sequence identity. Percentage of sequence identity is determined by comparing two optimally aligned sequences over a comparison window. The comparison window for polynucleotide sequences can be, for instance, at least about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90, about 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, about 190, about 200, about 300, about 400, about 500, about 600, about 700, about 800, about 900 or about 1000 or more nucleic acids in length. The comparison window for polypeptide sequences can be, for instance, at least about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90, about 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, about 190, about 200, about 300 or more amino acids in length. In order to optimally align sequences for comparison, the portion of a polynucleotide or polypeptide sequence in the comparison window can comprise additions or deletions termed gaps while the reference sequence is kept constant. An optimal alignment is that alignment which, even with gaps, produces the greatest possible number of "identical" positions between the reference and comparator sequences. Percentage "sequence identity" between two sequences can be determined using the version of the program "BLAST 2 Sequences" which was available from the National Center for Biotechnology Information as of September 1, 2004, which program incorporates the programs