the contralateral eye for an ocular disease. In some embodiments, the individual has not received a prior anti-VEGF treatment. In some embodiments, the individual has not received a prior anti-VEGF treatment in the one eye and/or the contralateral eye. In some embodiments, the individual has not received a prior aflibercept treatment. In some embodiments, the individual has not received a prior aflibercept treatment in the one eye and/or the contralateral eye.

[0219] In some embodiments, methods described herein are used to prevent or treat an ocular disease or disorder in a subject who has received prior treatment with bevacizumab, brolucizumab, ranibizumab, faricimab, abicipar pegol, conbercept, OPT-302, KSI-301, injectable sunitinib maleate (GB-102), PAN-90806 (PanOptica), and/or aflibercept. In some embodiments, methods described herein are used to prevent or treat an ocular disease or disorder that is responsive to treatment with bevacizumab, brolucizumab, ranibizumab, and/or aflibercept.

[0220] In some embodiments, the individual was diagnosed with the ocular disease at least 1 day, at least 1 week, at least 1 month, at least 2 months, at least 4 months, at least 6 months, at least 12 months, at least 18 months, at least 24 months, at least 30 months, at least 36 months, at least 42 months, at least 48 months, at least 54 months, at least 60 months, at least 66 months, at least 72 months, at least 78 months, at least 84 months, at least 90 months, 96 months, at least 102 months, at least 108 months, at least 114 months, at least 120 months, at least 126 months, at least 132 months, or more, prior to administration of the unit dose of rAAV particles to the one eye and/or the contralateral eye.

[0221] The following description is presented to enable a person of ordinary skill in the art to make and use the various embodiments. Descriptions of specific devices, techniques, and applications are provided only as examples. Various modifications to the examples described herein will be readily apparent to those of ordinary skill in the art, and the general principles defined herein may be applied to other examples and applications without departing from the spirit and scope of the various embodiments. Thus, the various embodiments are not intended to be limited to the examples described herein and shown, but are to be accorded the scope consistent with the claims.

EXAMPLES

Example 1: A Phase 2, Multi-Center, Randomized, Double-Masked, Active Controlled Study of AAV2.7m8-aflibercept in Subjects with Diabetic Macular Edema.

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[0222] This Example describes a Phase 2, multi-center, randomized, double-masked, active controlled study designed to evaluate the durability of a single intravitreal (IVT) injection of AAV2.7m8-aflibercept in subjects with diabetic macular edema.

I. Study Objectives and Endpoints

A. Primary Objective

[0223] The primary objective of this study was to assess the durability of a single IVT injection of AAV2.7m8-aflibercept.

B. Secondary Objectives

[0224] Secondary objectives of this study included:

- Assessment of the safety and tolerability of AAV2.7m8-aflibercept.
- Evaluation of the effect of AAV2.7m8-aflibercept on macular edema.
- Evaluation of the effect of AAV2.7m8-aflibercept on Best Corrected Visual Acuity (BCVA).
- Evaluation of the effect of AAV2.7m8-aflibercept on Diabetic Retinopathy Severity Scale (DRSS) score.
- Assessment of the need for rescue aflibercept (2 mg IVT).
- Assessment of the effect of a preceding dose of aflibercept (2 mg IVT) prior to AAV2.7m8-aflibercept administration.
- Evaluation of the effect of AAV2.7m8-aflibercept on development of vision threatening complications (anterior segment neovascularization, vitreous hemorrhage, or tractional retinal detachment).

C. Primary Endpoints

[0225] The primary endpoint of this study was the time to worsening of diabetic macular edema (DME) disease activity in the study eye, as defined by the occurrence of either of:

- An increase in central subfield thickness (CST) > 50 μm as assessed by SD-OCT compared to the lower of the two CST measurements recorded at Day 1 or Week 4.
- A loss of > 5 letters in BCVA due to worsening DME disease activity compared to the higher of the two BCVA measurements recorded at Day 1 or Week 4.

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D. Secondary Endpoints

[0226] The secondary endpoints for this study were based on outcome measures for the study eye (unless otherwise specified) and included:

- Incidence and severity of ocular and non-ocular adverse events (AEs).
- Change from Baseline in CST and macular volume over time through Week 48.
- Change from Baseline in BCVA over time through Week 48.
- Frequency of rescue aflibercept (2 mg IVT) in the study eye over time during the study.
- Incidence of 2-step and 3-step improvement in DRSS score over time through Week 48.
- Incidence of 2-step and 3-step worsening in DRSS score over time through Week 48.
- Occurrence of vision threatening complication (anterior segment neovascularization, vitreous hemorrhage, or any other high-risk proliferative diabetic retinopathy (DR), or tractional retinal detachment) over time through Week 48.
- Incidence of CST <300 µm over time through Week 48.
- Incidence of clinically significant findings via physical examinations, ocular examinations, imaging, and laboratory evaluation over time through Week 48.

II. Study Population

A. Inclusion Criteria

[0227] Subjects with newly diagnosed DME (*i.e.*, DME diagnosis within 6 months of screening) that received up to 2 prior injections of anti-VEGF therapy in the study eye were included in this study if they met the following inclusion criteria:

- Age ≥ 18 years of age
- Type 1 or 2 diabetes mellitus.
- Vision impairment due to center involving diabetic macular edema.
- Vision at Screening:
  - Study Eye: BCVA 78 to 50 ETDRS letters, inclusive (approximate Snellen equivalent 20/32 to 20/100).
  - Non-study eye: BCVA 35 ETDRS letters or more (approximate Snellen equivalent of 20/200 or better).
- CST of study eye at Screening visit of ≥ 325µm using Heidelberg Spectralis® with center-involving IRF (center 1 mm).
- A decrease in vision in the study eye determined to be primarily due to DME.

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- Initial DME diagnosis within 6 months from screening.
- Up to 2 prior injections (0, 1, or 2) of anti-VEGF in the study eye.
    - If a prior anti-VEGF has been administered to the study eye, there must have been a meaningful CST response (e.g., ≥ 10% reduction) and no adverse reaction to anti-VEGF (e.g., inflammation).
- A minimum 60-day interval between the last anti-VEGF injection in the study eye and randomization on Day 1.

B. Exclusion Criteria

[0228] Subjects meeting any of the following criteria were excluded from this study:

- Documented anti-AAV2.7m8 neutralizing antibody titer > 1:125 within 6 months prior to randomization.
- Prior ocular gene therapy.
- History of allergy to aflibercept, corticosteroid, or fluorescein dye or sodium fluorescein used in angiography (mild allergy amenable to treatment was allowable).
- History or evidence of any of the following cardiovascular disease within 6 months of dosing:
    - Severe cardiac disease (e.g., New York Heart Association [NYHA] Functional Class III or IV) or clinical evidence of unstable angina.
    - Acute coronary syndrome, myocardial infarction or coronary artery revascularization, cerebrovascular accident (CVA), transient ischemic attack (TIA).
    - Ventricular tachyarrhythmias requiring ongoing treatment, or uncontrolled arrhythmia.
    - Uncontrolled hypertension defined as systolic blood pressure (SBP) >160 mmHg or a diastolic blood pressure (DBP) >100 mmHg, despite using BP–lowering medication within the screening period. If BP-lowering medications were required, subject should have been on a stable dose of the same medication continuously for 30 days prior to randomization.
- Any history of ongoing bleeding disorders. The use of aspirin or other anticoagulants (e.g., Factor Xa inhibitors) was not an exclusion criterion.
- Uncontrolled diabetes defined as HbA1C >10%, or history of diabetic ketoacidosis within 3 months prior to randomization, or subjects who, within the last 3 months, initiated

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intensive insulin treatment (a pump or multiple daily injection) or planned to do so in the next 3 months.

- History of systemic autoimmune disease requiring treatment with systemic steroids or immunosuppressive treatments (e.g. methotrexate, adalimumab).
- Systemic drugs known to cause macular edema (e.g., fingolomod, tamoxifen, chloroquine/hydroxychloroquine) or any prior systemic anti-VEGF therapy.
- Known to be positive for HIV or active viral hepatitis (unless documented cure after treatment for Hepatitis C); known history of syphilis
- Known severe renal impairment, as indicated by estimated CrCl <30 mL/min (by Cockcroft-Gault calculation); need or anticipated need for hemodialysis during the study period.
- Any febrile illness within 1 week prior to randomization.

[0229] In addition, subjects meeting any of the following ocular exclusion criteria in the study eye were excluded from this study:

- High-risk proliferative diabetic retinopathy (PDR) at time of screening, defined as: any vitreous or preretinal hemorrhage, neovascularization elsewhere >1/2-disc area within an area equivalent to standard ETDRS 7-field on clinical examination, or neovascularization of disc > 1/3-disc area on clinical examination.
- Any prior focal or grid laser photocoagulation or any prior pan retinal photocoagulation (PRP) in the study eye.
- Any anti-VEGF therapy in the preceding 60 days prior to randomization (up to 2 prior anti-VEGF injections were allowed but they cannot have occurred in the prior 60 days).
- History of anterior segment neovascularization (e.g., neovascularization of the iris [NVI] or neovascular glaucoma [NVG]), significant vitreous hemorrhage, fibrovascular proliferation or tractional retinal detachment.
- Examination evidence of structural abnormalities at the fovea (e.g., dense hard exudates, pigment abnormalities, foveal atrophy, vitreomacular traction or epiretinal membrane), either on clinical examination or OCT, thought to be contributing to macular edema or visual impairment.
- History of retinal disease in the study eye other than diabetic retinopathy including age-related macular degeneration (in either eye), retinal vein occlusion, retinal arterial occlusion, pathologic myopia, etc.

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- Any current or history of ocular disease other than DME that could reduce the potential for visual improvement, confound assessment of the macula or require medical or surgical intervention during the study (e.g. significant cataract, macular traction) or any evidence of posterior subcapsular cataract.
- History of cataract extraction or Yttrium Aluminum Garnet (YAG) capsulotomy within 3 months before Day 1.
- History of retinal detachment (with or without repair) in the study eye.
- History of trabeculectomy or glaucoma shunt or minimally invasive glaucoma surgery (MIGS).
- History of vitrectomy or other filtration surgery.
- Aphakia or presence of an anterior chamber intraocular lens.
- Uncontrolled ocular hypertension or glaucoma in the study eye at time of randomization (defined as IOP >22 mmHg despite treatment with anti-glaucoma medication) or current use of >2 IOP lowering medications.
- Any history of intraocular or periocular steroid treatment for any ocular condition (e.g., IVT Triesence, Iluvien or Ozurdex).
- Refractive surgery within the 90 day period prior to Screening.
- Previous penetrating keratoplasty, endothelial keratoplasty, or ocular radiation.
- Any prior vitreoretinal surgery.

[0230] In addition, subjects meeting any of the following ocular exclusion criteria in the study eye or in the non-study eye (*i.e.*, the "fellow eye") were excluded from this study:

- Any history of uveitis or intraocular inflammation (grade trace or above) except mild anticipated post-operative inflammation that resolved.
- History of IOP elevation related to topical steroid administration.
- Known history of ocular Herpes Simplex Virus (HSV), Varicella-zoster virus (VZV), or Cytomegalovirus (CMV) including viral uveitis, retinitis or keratitis.
- Evidence of external ocular infection, including conjunctivitis, chalazion or significant blepharitis.
- History of ocular toxoplasmosis.

III. Study Design

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A. Study Treatment

[0231] This was a multi-center, randomized, double-masked, controlled, parallel-group study designed to evaluate the efficacy, safety and tolerability of a single 0.10 mL IVT injection of AAV2.7m8-aflibercept. Two doses of AAV2.7m8-aflibercept were investigated.

[0232] Subjects with initial diagnosis of DME within 6 months of screening and that have received up to 2 prior injections of anti-VEGF therapy were eligible for enrollment.

[0233] Approximately 33 eligible subjects were randomized to receive one of two doses of AAV2.7m8-aflibercept (6×10^{11} vg/eye or 2×10^{11} vg/eye), or to a control arm to receive a sham ocular injection with a preceding aflibercept injection. Subjects who were assigned to receive AAV2.7m8-aflibercept were further randomized to receive a preceding aflibercept or sham ocular injection. The study arms are summarized below:

- Arm 1 (n=6): Subjects receive AAV2.7m8-aflibercept at a dose of 6×10^{11} vg/eye with a preceding aflibercept dose.
- Arm 2 (n=6): Subjects receive AAV2.7m8-aflibercept at a dose of 6×10^{11} vg/eye without a preceding aflibercept dose.
- Arm 3 (n=6): Subjects receive AAV2.7m8-aflibercept at a dose of 2×10^{11} vg/eye with a preceding aflibercept dose.
- Arm 4 (n=6): Subjects receive AAV2.7m8-aflibercept at a dose of 2×10^{11} vg/eye without a preceding aflibercept dose.
- Arm 5 (n=9): Subjects receive aflibercept only (active control).

[0234] To maintain masking of the treatment assignment, subjects assigned to the arms with no preceding aflibercept on Day 1 or to the arm with no AAV2.7m8-aflibercept on Day 8 received a sham ocular injection on the corresponding visit. Only one eye per subject was selected as the study eye. If both eyes were eligible, the eye with the worse BCVA was selected as the study eye.

[0235] Both AAV2.7m8-aflibercept and aflibercept were administered via IVT injection. IVT injections were not performed if active inflammation was present. Aseptic technique with povidone-iodine was used with topical or subconjunctival anesthesia. The sham ocular injection procedure was done under the same conditions but with an empty syringe without a needle (using the blunt end) pressed against the eye to mimic an injection.

[0236] A summary of the arms in this study is provided in **Table 17**.

Table 17. Study Arms.

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Arm	N	Day 1 IVT Aflibercept or Sham	Day 8 AAV2.7m8-aflibercept Dose (vg/eye)
1	6	Aflibercept	6×10^{11}
2	6	Sham	6×10^{11}
3	6	Aflibercept	2×10^{11}
4	6	Sham	2×10^{11}
5	9	Aflibercept	Sham

[0237] After the assigned IVT injections on Day 1 and Day 8, all subjects were followed up on Week 2, Week 4, and then every 4 weeks up to Week 48 after Day 1 (e.g., Week 8, Week 12, Week 16, etc.). To maintain masking, both subjects and personnel conducting assessments were masked to the treatment assignments throughout the study.

[0238] All subjects were followed for 48 weeks after randomization.

B. Prophylactic Topical Steroid Regimen

[0239] All subjects were administered a prophylactic 7-week topical corticosteroid regimen of difluprednate (0.05%; e.g., Durezol) starting on Day 1. Subjects were instructed to self-administer difluprednate four times per day (QID) for 4 weeks (*i.e.*, from Day 1 to Day 28), followed by three times per day (TID) for 1 week (*i.e.*, for 7 days), followed by two times per day (BID) for 1 week (*i.e.*, 7 days), and finally once per day (QD) for 1 week (*i.e.*, 7 days). Tapering was not commenced in the presence of active inflammation. This regimen was prolonged should signs of inflammation have occurred. A summary of the difluprednate regimen is provided in **Table 2**.

Table 2. Difluprednate Regimen.

Study Days	Total Number of Days	Difluprednate Administrations per Day
Days 1 to 28	28	4 times
Days 29 to 42	14	3 times
Days 43 to 56	14	2 times
Days 57 to 70	14	1 time
Day 71	0	STOP – if no signs of inflammation

C. Rescue Treatment

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[0240] Starting at Week 8, subjects received rescue aflibercept (2 mg IVT) if they met any of the following:

- Increase in CST > 50 μm as assessed by SD-OCT compared to the lower of the two CST measurements recorded at Day 1 or Week 4.
- Loss of > 5 letters in BCVA due to worsening DME disease activity compared to the higher of the two BCVA measurements recorded at Day 1 or Week 4.

[0241] Aflibercept was not injected in eyes with active inflammation. A minimum of 21 days was required between rescue aflibercept injections.

D. Medications and Treatments

[0242] The following medications were prohibited during the study:

- Any systemic anti-VEGF agent, including bevacizumab.
- Systemic drugs known to cause macular edema (e.g., fingolomod, tamoxifen, chloroquine/hydroxychloroquine).
- Any anti-VEGF agent in the study eye other than the study drug or aflibercept IVT 2 mg.
- IVT steroids in the study eye (e.g., Ozurdex or Illuvien Triesence).
- Systemic immunosuppressive drugs (e.g., intravenous steroids, methotrexate, azathioprine, ciclosporin, adalimumab, infliximab, etanercept). Inhaled or topical steroids and NSAIDs were allowed.

[0243] Subjects who developed high-risk PDR in the study eye received panretinal photocoagulation (PRP) after receiving rescue aflibercept.

[0244] Subjects with visually significant cataract were not enrolled in the study, but if a cataract developed during the study, cataract surgery in the study eye was permitted if clinically indicated and was scheduled > 90 days after AAV2.7m8-aflibercept administration and/or > 7 days after the last injection of aflibercept.

[0245] Subjects who developed DME in the fellow (non-study) eye were permitted to receive standard of care therapy.

IV. Study Assessments

A. General Physical Examination and Vital Signs

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[0246] A general physical examination (PE) was conducted at the screening and End of Study (EOS) or Early Termination visit. The PE consisted of body system examination for general appearance, neurologic, HEENT (head, eyes, ears, nose, and throat), neck, cardiovascular, respiratory, abdomen, extremities, skin, weight, and height. At the EOS or Early Termination visit, the physical examination assessed if any changes in the subject's physical condition had occurred since the Screening examination. A targeted physical examination was conducted as needed for the evaluation of AEs.

[0247] Vital signs consisted of blood pressure, pulse rate, body temperature, and respiratory rate. A 12-lead Electrocardiogram (ECG) was taken for each subject at Screening and EOS or Early Termination Visit.

B. Laboratory Tests, Vector Expression and Immune Response

[0248] The following Clinical Laboratory Tests were conducted for the study: chemistry, complete blood count, HbA1C, urinalysis, and HLA-B27 genotyping.

[0249] Subjects' samples (both blood and/or aqueous humor) were collected to measure the following:

- Total antibodies to AAV2.7m8: serum for total anti-AAV2.7m8 antibodies were measured in an ELISA assay.
- Neutralizing antibodies to AAV2.7m8: serum for neutralizing anti-AAV2.7m8 antibodies were measured in a cell-based assay.
- Anti-aflibercept antibodies: serum for the humoral immune response against aflibercept was measured in an ELISA assay.
- Aflibercept protein expression: Serum and aqueous humor samples were collected for the presence of aflibercept protein and measured in a MesoScale Discovery assay.
- Cell-mediated immune response: cellular immunity against AAV2.7m8 capsid and aflibercept protein were measured in an ELISPOT assay.

C. Full Ophthalmic Examination

[0250] Study assessments included an ophthalmologic exam, Intraocular Pressure (IOP), and indirect ophthalmoscopy.

[0251] The ophthalmic examination consisted of an external examination of the eye and adnexa, routine screening for eyelid/pupil responsiveness (including but not limited to blepharoptosis, abnormal pupil shape, unequal pupils, abnormal reaction to light, and afferent pupillary defect), and

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slit-lamp examination (eyelids, conjunctiva, cornea, lens, iris, anterior chamber). The slit-lamp examination examined the anterior ocular structures and was used for grading any findings. If any finding was noted during the slit-lamp examination, at any visit, the severity was graded and the finding was described as clinically significant or not clinically significant.

[0252] IOP measurements were performed using a Goldmann applanation tonometer or Tono-pen™. The same method of IOP measurement was used throughout the study for each individual subject. IOP measurements were performed prior to any IVT injection and prior to dilating eyes, using the same method throughout the study. Day 1 and Day 8 visits required pre-injection and post-injection (30 minutes after injection) IOP measurements.

[0253] The dilated indirect ophthalmoscopy examination included an evaluation of posterior segment abnormalities of the vitreous, optic nerve, peripheral retina, and retinal vasculature. If any finding was noted during the ophthalmoscopy, at any visit, the severity was graded and the finding was described as clinically significant or not clinically significant. Day 1 and Day 8 visits required pre-injection and post-injection indirect ophthalmoscopy assessments.

D. Refraction and Visual Acuity

[0254] Refraction and BCVA were measured. Visual acuity measurements were measured at a starting distance of 4 meters, prior to dilating eyes.

E. Imaging

[0255] Spectral Domain Optical Coherence Tomography (SD-OCT) is an interferometric technique that provides depth-resolved tissue structure information encoded in the magnitude and delay of the back-scattered light by spectral analysis of the interference fringe pattern. If a subject received anti-VEGF injections at visits prior to study randomization, OCTs from those visits were collected and delivered to the central reading center.

[0256] Optical Coherence Tomography Angiography (OCT-A) is an imaging technology that provides volumetric, three-dimensional maps of the retina and choroid as well as information on blood flow. There are two types of OCT-A, swept-source and spectral-domain. Swept-source imaging was used where available. If a swept-source instrument was not available and a spectral-domain instrument was available, then the spectral domain instrument was used.

[0257] Standardized procedures for the collection of ultra-wide field fundus digital photographic images of the retina, optic disc, and macula were followed. In addition, photographs of the iris were taken prior to dilation.

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[0258] A standardized procedure for examining the retinal circulation and vessel permeability using a dye-tracing method was followed. This involved injection of sodium fluorescein into the systemic circulation, after which an angiogram was obtained by digitally photographing the fluorescence emitted after illumination of the retina with blue light at a wavelength of 490 nm.

F. Laboratory, Biomarker and Other Biological Specimens

[0259] Aqueous humor samples were collected and analyzed for levels of aflibercept, VEGF-A, neutralizing antibodies (NAbs), and additional biomarkers. Vitreous humor samples were obtained and analyzed for aflibercept concentrations and other biomarkers.

G. Safety

[0260] After study treatment administration, all clinically significant adverse events (AEs) were reported. Each subject was followed until a) the end of the AE reporting period at 30 days after the last study visit or b) for any ongoing study treatment related AEs and/or serious AEs (SAEs) until resolved or stable. Any clinically significant safety assessment that was associated with DME was not reported as an AE or SAE, unless judged to be more severe than expected for the subject's condition. Progression of the disease under study was captured as an efficacy outcome.

[0261] Adverse events of special interest for this study included:

- Sight-threatening adverse events: an adverse event was considered to be sight-threatening if it met one or more of the following criteria:
  - Caused a decrease of $\geq$ 30 letters in BCVA compared with the prior visit.
  - Required surgical or medical intervention (i.e., conventional surgery, vitrectomy) to prevent permanent loss of sight.
  - Caused severe intraocular inflammation (i.e., endophthalmitis, 4+ anterior chamber cell/flare, or 4+ vitreous cells).

[0262] All of the above-listed sight-threatening adverse events were reported as serious adverse events, listing the underlying cause (if known) of the event as the primary event term.

H. Efficacy

[0263] The efficacy of AAV2.7m8-aflibercept in the treatment of DME was assessed by the following measures. Baseline values for BCVA and SD-OCT endpoints referred to pre-treatment measurements taken on the Day 1 visit when aflibercept IVT or sham ocular injection were administered.

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- BCVA: Vision was assessed primarily through BCVA expressed as an ETDRS score (number of letters correctly read). Maintenance of vision was classified if the subject has lost fewer than 15 letters in the ETDRS score compared to Baseline. Calculated endpoints included the mean change from Baseline, the percent gaining at least 15 letters compared to Baseline, and the percent losing 15 or more letters compared to Baseline.
- Central subfield thickness: SD-OCT was performed using approved equipment and standard techniques to evaluate thickness and fluid compared to Baseline values. Endpoints included CST and macular volume.
- Aflibercept re-treatments: The incidence and timing of aflibercept injections given post-AAV2.7m8-aflibercept treatment over time.
- For each timepoint, Diabetic Retinopathy Severity Scale (DRSS) was determined using ultra-wide field color fundus photography and compared to Day 1.
- Vision threatening complications (anterior segment neovascularization, diabetic macular edema, high-risk PDR development, vitreous hemorrhage, or tractional retinal detachment), as determined by ultra-wide field imaging and clinical examination.

I. Statistical Analyses

[0264] The main analysis population included all randomized subjects who received the study treatment on Day 8 (AAV2.7m8-aflibercept IVT or Sham ocular injection). All safety and efficacy variables were summarized descriptively by treatment arm. Mean, standard deviation (SD), median and range were provided for continuous variables; and frequency counts and percentages were provided for categorical variables. Confidence intervals of the means and percentages were provided at both 90% and 95% levels. Kaplan-Meier survival analysis was utilized to derive median time to the first occurrence of DME disease worsening. All rescue aflibercept (2 mg IVT) received by each subject during the study were summarized using statistical models for recurrent events. Mean cumulative function (MCF) curve over time was plotted for the mean cumulative number of injections. Mixed-effect models for repeated measures (MMRM) were employed to explore the treatment effect on the change over time in BCVA and CST. The treatment effect on DRSS changes over time was explored using generalized mixed models for categorical outcomes. An interim analysis (IA) occured after all subjects have been followed for 24 weeks.

V. Results

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[0265] One subject, a 56-year-old American Indian or Alaskan Native female with Diabetic Macular Edema (OU) in Arm 1 (e.g., who received treatment with a dose of 6E11 vector genomes of AAV2.7m8-aflibercept) experienced a serious adverse event of hypotony approximately 30 weeks after being randomized to study drug vs. sham in this study. Per the current Investigator Brochure (edition 4.0), hypotony is unexpected for this study drug. The patient remained on study and continued to be treated and followed closely. Initiation of steroid therapy was recommended if IOP ≤ 10 mmHg was observed, even in the absence of signs of clinical inflammation.

[0266] Although the present disclosure has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the present disclosure. The disclosures of all patent and scientific literature cited herein are expressly incorporated in the entirety by reference.

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CLAIMS

1. A method for treating glaucoma in an individual, the method comprising administering a unit dose of recombinant adeno-associated virus (rAAV) particles to one eye of the individual, wherein the individual is a human, and wherein the rAAV particles comprise

a) a nucleic acid encoding a polypeptide comprising an amino acid sequence with at least about 95% identity to the amino acid sequence of SEQ ID NO: 35 and flanked by AAV2 inverted terminal repeats (ITRs), and

b) an AAV2 capsid protein comprising an amino acid sequence LGETTRP (SEQ ID NO: 14) inserted between positions 587 and 588 of the capsid protein, wherein the amino acid residue numbering corresponds to an AAV2 VP1 capsid protein.

2. The method of claim 1, wherein the glaucoma is neovascular glaucoma.

3. A method for reducing intraocular pressure in an individual, the method comprising administering a unit dose of recombinant adeno-associated virus (rAAV) particles to one eye of the individual, wherein the individual is a human, and wherein the rAAV particles comprise

a) a nucleic acid encoding a polypeptide comprising an amino acid sequence with at least about 95% identity to the amino acid sequence of SEQ ID NO: 35 and flanked by AAV2 inverted terminal repeats (ITRs), and

b) an AAV2 capsid protein comprising an amino acid sequence LGETTRP (SEQ ID NO: 14) inserted between positions 587 and 588 of the capsid protein, wherein the amino acid residue numbering corresponds to an AAV2 VP1 capsid protein.

3.a A method of reducing intraocular pressure in an individual, the method comprising administering a unit dose of rAAV particles to one eye of the individual, wherein the individual is a human, and wherein the rAAV particles comprise

a) a nucleic acid encoding a polypeptide under the control of a strong promoter that is active in the ciliary body and flanked by AAV2 inverted terminal repeats (ITRs), and

b) an AAV2 capsid protein comprising an amino acid sequence LGETTRP (SEQ ID NO: 14) inserted between positions 587 and 588 of the capsid protein, wherein the amino acid residue numbering corresponds to an AAV2 VP1 capsid protein.

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4. The method of claim 3, wherein the individual has glaucoma.

5. The method of claim 4, wherein the glaucoma is neovascular glaucoma.

6. The method of any one of claims 1-5, wherein the unit dose of rAAV particles is about 6×10^{11} vector genomes per eye (vg/eye) or less.

7. The method of any one of claims 1-6, wherein the unit dose of rAAV particles is between about 6×10^{10} to about 6×10^{11} vector genomes per eye (vg/eye).

8. The method of any one of claims 1-7, wherein the unit dose of rAAV particles is between about 6×10^{10} to about 2×10^{11} vector genomes per eye (vg/eye).

9. The method of any one of claims 1-8, wherein the unit dose of rAAV particles is between about 2×10^{11} to about 6×10^{11} vector genomes per eye (vg/eye).

10. The method of claim 9, wherein the unit dose of rAAV particles is about 2×10^{11} or about 6×10^{11} vector genomes per eye (vg/eye).

11. The method claim 10, wherein the unit dose of rAAV particles is about 2×10^{11} vector genomes per eye (vg/eye).

12. The method claim 11, wherein the unit dose of rAAV particles is about 6×10^{11} vector genomes per eye (vg/eye).

13. The method of any one of claims 1-12, further comprising administering a unit dose of rAAV particles to the contralateral eye of the individual.

14. The method of claim 13, wherein the administering the unit dose of rAAV particles to the contralateral eye is up to about 2 weeks after administering the unit dose of rAAV particles to the one eye.

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15. The method of claim 14, wherein:

(a) the administering the unit dose of rAAV particles to the contralateral eye is on the same day as the administering the unit dose of rAAV particles to the one eye; or

(b) the administering the unit dose of rAAV particles to the contralateral eye is between about 1 day to about 14 days after administering the unit dose of rAAV particles to the one eye.

16. The method of any one of claims 13-15, wherein the unit dose of rAAV particles administered to the contralateral eye of the individual comprises the same or less vector genomes per eye (vg/eye) than the unit dose of rAAV particles administered to the one eye of the individual.

17. The method of claim 16, wherein the administering the unit dose of rAAV particles to the contralateral eye is at least about 2 weeks after administering the unit dose of rAAV particles to the one eye.

18. The method of claim 15, wherein the unit dose of rAAV particles administered to the contralateral eye of the individual comprises more vector genomes per eye (vg/eye) than the unit dose of rAAV particles administered to the one eye of the individual.

19. The method of any one of claims 1-18, wherein the nucleic acid comprises the nucleic acid sequence of SEQ ID NO: 40 or a sequence having at least 85% identity thereto.

20. The method of any one of claims 1-19, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO: 35.

21. The method of any one of claims 1-20, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO: 41.

22. The method of any one of claims 1-21, wherein the polypeptide is aflibercept.

23. The method of any one of claims 1-22, wherein the nucleic acid further comprises a first enhancer region, a promoter region, a 5'UTR region, a second enhancer region, and a polyadenylation site.

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24. The method of any one of claims 1-23, wherein the nucleic acid comprises, in the 5' to 3' order:

(a) a first enhancer region;

(b) a promoter region;

(c) a 5'UTR region;

(d) a nucleic acid encoding a polypeptide comprising an amino acid sequence with at least about 95% identity to the amino acid sequence of SEQ ID NO: 35;

(e) a second enhancer region; and

(f) a polyadenylation site;

and flanked by AAV2 inverted terminal repeats (ITRs).

25. The method of claim 23 or claim 24, wherein the first enhancer region comprises a CMV sequence comprising the sequence of SEQ ID NO: 22 or a sequence having at least 85% identity thereto.

26. The method of any one of claims 23-25, wherein the promoter region comprises a CMV sequence comprising the sequence of SEQ ID NO: 23 or a sequence having at least 85% identity thereto.

27. The method of any one of claims 24-26, wherein the nucleic acid encoding a polypeptide comprises the nucleic acid sequence of SEQ ID NO: 40 or a sequence having at least 85% identity thereto.

28. The method of any one of claims 24-27, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO: 35 or a sequence having at least 95% identity thereto.

29. The method of any one of claims 24-28, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO: 41 or a sequence having at least 95% identity thereto.

30. The method of any one of claims 24-29, wherein the polypeptide is aflibercept.

31. The method of any one of claims 23-30, wherein the 5'UTR region comprises, in 5' to 3' order, a TPL sequence comprising the sequence of SEQ ID NO: 24 or a sequence having at least

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85% identity thereto, and an eMLP sequence comprising the sequence of SEQ ID NO: 25 or a sequence having at least 85% identity thereto.

32. The method of any one of claims 23-31, wherein the second enhancer region comprises a full EES sequence comprising the sequence of SEQ ID NO: 26 or a sequence having at least 85% identity thereto.

33. The method of any one of claims 23-32, wherein the polyadenylation site comprises a HGH polyadenylation site comprising the sequence of SEQ ID NO: 27 or a sequence having at least 85% identity thereto.

34. The method of any one of claims 1-22, wherein the nucleic acid further comprises (a) a first enhancer region comprising a CMV sequence comprising the sequence of SEQ ID NO: 22 or a sequence having at least 85% identity thereto; (b) a promoter region, comprising a CMV sequence comprising the sequence of SEQ ID NO: 23 or a sequence having at least 85% identity thereto; (c) a 5'UTR region comprising, in 5' to 3' order, a TPL sequence comprising the sequence of SEQ ID NO: 24 or a sequence having at least 85% identity thereto, and an eMLP sequence comprising the sequence of SEQ ID NO: 25 or a sequence having at least 85% identity thereto; (d) a second enhancer region comprising a full EES sequence comprising the sequence of SEQ ID NO: 26 or a sequence having at least 85% identity thereto; and (e) a HGH polyadenylation site comprising the sequence of SEQ ID NO: 27 or a sequence having at least 85% identity thereto.

35. The method of any one of claims 1-34, wherein the nucleic acid comprises the sequence of SEQ ID NO: 39 or a sequence having at least 85% identity thereto.

36. The method of any one of claims 1-35, wherein the AAV2 capsid protein comprises the amino acid sequence LALGETTRPA (SEQ ID NO: 1) inserted between positions 587 and 588 of the capsid protein, wherein the amino acid residue numbering corresponds to an AAV2 VP1 capsid protein.

37. The method of any one of claims 1-36, wherein the AAV2 capsid protein comprises the amino acid sequence LGETTRP (SEQ ID NO: 14) inserted between positions 587 and 588 of the AAV2 VP1 comprising the sequence of SEQ ID NO: 13.

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38. The method of any one of claims 1-37, wherein the AAV2 capsid protein comprises the amino acid sequence LALGETTRPA (SEQ ID NO: 1) inserted between positions 587 and 588 of the AAV2 VP1 comprising the sequence of SEQ ID NO: 13.

39. The method of any one of claims 1-36, wherein the rAAV particles comprise an AAV2 VP1 capsid protein comprising a GH loop that comprises the amino acid sequence of SEQ ID NO: 38 or an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 38.

40. The method of any one of claims 1-39, wherein the administration of the unit dose of rAAV particles to the one eye and/or the contralateral eye is by intravitreal administration.

41. The method of any one of claims 1-40, wherein the unit dose of rAAV particles is in a pharmaceutical formulation.

42. The method of claim 41, wherein the pharmaceutical formulation comprises the rAAV particles, sodium chloride, sodium phosphate and a surfactant.

43. The method of claim 42, wherein the pharmaceutical formulation comprises about 150 to about 200 mM sodium chloride, about 1 to about 10 mM monobasic sodium phosphate, about 1 to about 10 mM dibasic sodium phosphate, about 0.0005% (w/v) to about 0.005% (w/v) poloxamer 188, and about 6×10^{13} to about 6×10^{10} vector genomes (vg) per mL (vg/mL) of the rAAV particles, wherein the pharmaceutical formulation has a pH of about 7.0 to about 7.5.

44. The method of claim 43, wherein the pharmaceutical formulation comprises about 180 mM sodium chloride, about 5 mM monobasic sodium phosphate, about 5 mM dibasic sodium phosphate, about 6×10^{12} vg/mL of the rAAV particles, and about 0.001% (w/v) poloxamer 188, wherein the pharmaceutical formulation has a pH of about 7.3.

45. The method of claim 43, wherein the pharmaceutical formulation comprises about 180 mM sodium chloride, about 5 mM monobasic sodium phosphate, about 5 mM dibasic sodium phosphate, about 2×10^{12} vg/mL of the rAAV particles, and about 0.001% (w/v) poloxamer 188, wherein the pharmaceutical formulation has a pH of about 7.3.

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46. The method of claim 43, wherein the pharmaceutical formulation comprises about 180 mM sodium chloride, about 5 mM monobasic sodium phosphate, about 5 mM dibasic sodium phosphate, about 6×10^{13} vg/mL of the rAAV particles, and about 0.001% (w/v) poloxamer 188, wherein the pharmaceutical formulation has a pH of about 7.3.

47. The method of any one of claims 1-46, wherein the unit dose of rAAV particles administered to the one eye and/or to the contralateral eye in a volume of about 25 µL to about 250 µL.

48. The method of claim 47, wherein the unit dose of rAAV particles administered to the one eye and/or to the contralateral eye comprises a volume of about 100µL.

49. The method of claim 48, wherein the unit dose of rAAV particles administered to the one eye and/or to the contralateral eye comprises a volume of about 30µL.

50. The method of any one of claims 1-49, wherein the individual received prior treatment for the ocular neovascular disease with an anti-VEGF agent.

51. The method of claim 50, wherein the individual has received 1 or 2 injections of an anti-VEGF agent in the one eye and/or in the contralateral eye prior to administration of the rAAV particles in the one eye and/or in the contralateral eye.

52. The method of claim 50 or 51, wherein the anti-VEGF agent is aflibercept.

53. The method of any one of claims 1-49, wherein the individual has not received prior treatment for the ocular neovascular disease with an anti-VEGF agent.

54. The method of any one of claims 1-53, wherein the unit dose of rAAV particles is administered in combinations with administration of an anti-VEGF agent.

55. The method of claim 54, comprising administering the unit dose of rAAV particles to the one eye of the individual about 1 week or about 7 days after administration of the anti-VEGF agent.

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56. The method of claim 54 or claim 55, comprising administering the anti-VEGF agent to the one eye of the individual on Day 1, and administering the unit dose of rAAV particles to the one eye of the individual on Day 8.

57. The method of any one of claims 54-56, wherein the anti-VEGF agent comprises aflibercept.

58. The method of claim 57, where the aflibercept is administered at a dose of about 2 mg by intravitreal injection.

59. The method of any one of claims 1-58, wherein the unit dose of rAAV particles is administered in combination with steroid treatment.

60. The method of claim 59, wherein the steroid treatment is a corticosteroid treatment.

61. The method of claim 59 or claim 60, wherein the steroid treatment is a systemic steroid treatment.

62. The method of any one of claims 59-61, wherein the steroid treatment is an oral steroid treatment.

63. The method of any one of claims 59-62, wherein the steroid treatment is a prednisone treatment.

64. The method of claim 59 or claim 60, wherein the steroid treatment is a topical steroid treatment.

65. The method of claim 64, wherein the steroid treatment is a difluprednate treatment.

66. The method of any one of claims 59-65, wherein the steroid is administered before, during and/or after administration of the unit dose of rAAV particles to the one eye and/or to the contralateral eye.

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67. The method of any one of claims 64-66, wherein the topical steroid comprises difluprednate 0.05% at a dose of about 1μg to about 3 μg.

68. The method of any one of claims 64-67, wherein the topical steroid comprises difluprednate 0.05% at a dose of about 2.5μg.

69. A unit dose of about 6×10^{11} vector genomes (vg) or less of recombinant adeno- associated virus (rAAV) particles for use in a method for treating glaucoma in an individual, the method comprising administering said unit dose to one eye of the individual, wherein the individual is a human, and wherein the rAAV particles comprise:

a) a nucleic acid encoding a polypeptide comprising an amino acid sequence with at least about 95% identity to the amino acid sequence of SEQ ID NO: 35 and flanked by AAV2 inverted terminal repeats (ITRs), and

b) an AAV2 capsid protein comprising an amino acid sequence LGETTRP (SEQ ID NO: 14) inserted between positions 587 and 588 of the capsid protein, wherein the amino acid residue numbering corresponds to an AAV2 VP1 capsid protein.

70. A unit dose of rAAV particles for use in a method for reducing intraocular pressure in an eye of an individual in need thereof, the method comprising administering said unit dose to one eye of the individual, wherein the individual is a human, and wherein the rAAV particles comprise:

a) a nucleic acid encoding a polypeptide comprising an amino acid sequence with at least about 95% identity to the amino acid sequence of SEQ ID NO: 35 and flanked by AAV2 inverted terminal repeats (ITRs), and

b) an AAV2 capsid protein comprising an amino acid sequence LGETTRP (SEQ ID NO: 14) inserted between positions 587 and 588 of the capsid protein, wherein the amino acid residue numbering corresponds to an AAV2 VP1 capsid protein.

71. The unit does of claim 70, wherein the individual has glaucoma.

72. The unit dose of claim 69 or 71, wherein the glaucoma is neovascular glaucoma.

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ATGGTCAGCTACTGGGACACCGGGGTCCTGCTGTGCGGCGCTGCTCAGCCTGTCTGC
TTCTCACAGGATCTAGTTCCGGAAGTGATACCGGTAGACCTTTCGTAGAGATGTA
CAGTGAAATCCCCGAAATTATACACATGACTGAAGGAAGGGAGCTCGTCATTCC
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ACTTTGATCCCTGATGGAAAACGCATAATCTGGGACAGTAGAAAGGGCTTCATC
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TGTAACCCGGAGTGACCAAGGATTGTACACCTGTGCAGCATCCAGTGGGCTGAT
GACCAAGAAGAACAGCACATTTGTCAGGGTCCATGAAAAGGACAAAACTCACA
CATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTT
CCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATG
CGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGT
GGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACA
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AGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACC
CTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTG
GTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAG
CCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTC
TTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTC
TTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCC
CTCTCCCTGTCTCCGGGTAAATGA

FIG. 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/071937

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/00 A61K31/573 A61K31/713 A61K35/761 A61K38/17
A61K45/06 A61P27/02 C12N7/00 C12N15/86
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 A61P C12N

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, BIOSIS, Sequence Search, EMBASE, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[x] Further documents are listed in the continuation of Box C. [x] See patent family annex.

* Special categories of cited documents :
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Date of the actual completion of the international search	Date of mailing of the international search report
22 August 2022	30/08/2022

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International application No
PCT/US2022/071937

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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Patents WO2022025660A1

Stable pharmaceutical preparation

Abstract

A stable pharmaceutical preparation according to the present invention comprises: a recombinant fusion protein; a surfactant; a saccharide or a derivative thereof; and a buffer agent. The stable pharmaceutical preparation according to the present invention has a low viscosity while comprising a recombinant fusion protein, maintains excellent stability in long-term storage, accelerated, and severe conditions, and can be intraocularly administered.

Classifications

- A61K9/08 Solutions

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WO2022025660A1
WIPO (PCT)

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Other languages: French, Korean

Inventor: 강수정, 강봉우, 노지원, 신연경, 오준석, 이지원, 최창훈

Worldwide applications

2021 · EP AU WO CN KR CA TW

Application PCT/KR2021/009870 events

- Priority claimed from KR10-2020-0096434
- 2021-07-29 · Application filed by (주)셀트리온
- 2021-07-29 · Priority to CN202180058213.8A
- 2021-07-29 · Priority to EP21851412.3A
- 2021-07-29 · Priority to AU2021315381A
- 2021-07-29 · Priority to CA3190325A
- 2022-02-03 · Publication of WO2022025660A1

Info: Patent citations (8), Legal events, Similar documents, Priority and Related Applications

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Claims (44)

Hide Dependent

1. (A) a recombinant fusion protein;

   (B) a surfactant;

   (C) sugars or derivatives thereof; and

   (D) A stable pharmaceutical formulation comprising a buffer.

2. The method of claim 1,

   A stable pharmaceutical formulation, characterized in that the pharmaceutical formulation is liquid.

3. According to claim 1,

   (A) A stable pharmaceutical formulation, characterized in that the recombinant fusion protein is a VEGF (Vascular Endothelial Growth Factor) antagonist.

4. According to claim 1,

   (A) A stable pharmaceutical formulation, characterized in that the recombinant fusion protein comprises a human VEGF receptor extracellular domain.

5. According to claim 1,

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(A) A stable pharmaceutical formulation, characterized in that the recombinant fusion protein comprises human VEGF receptor extracellular domain 1, 2 or a mixture thereof.

6. 5. The method according to claim 4 or 5,

(A) A stable pharmaceutical formulation, characterized in that the recombinant fusion protein comprises the Fc region of human immunoglobulin G (IgG).

7. According to claim 1,

(A) The recombinant fusion protein is

(1) a VEGF receptor extracellular region 1 element comprising amino acids 27 to 129 of SEQ ID NO: 2;

(2) a VEGF receptor extracellular region 2 element comprising amino acids 130 to 231 of SEQ ID NO: 2; and

(3) A stable pharmaceutical formulation comprising an Fc region element comprising amino acids 232 to 457 of SEQ ID NO: 2.

8. According to claim 1,

(A) A stable pharmaceutical formulation, characterized in that the recombinant fusion protein comprises Aflibercept.

9. According to claim 1,

(A) A stable pharmaceutical formulation, characterized in that the concentration of the recombinant fusion protein is 5 to 100 mg/ml.

10. According to claim 1,

(B) A stable pharmaceutical formulation, characterized in that the surfactant comprises a polysorbate, a poloxamer or a mixture thereof.

11. 11. The method of claim 10,

(B) A stable pharmaceutical formulation, characterized in that the surfactant comprises polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80 or a mixture of two or more thereof.

12. 12. The method of claim 11,

(B) A stable pharmaceutical formulation, characterized in that the surfactant comprises polysorbate 20.

13. According to claim 1,

(B) A stable pharmaceutical formulation, characterized in that the concentration of the surfactant is 0.01 to 0.1% (w/v).

14. According to claim 1,

(C) the sugar comprises a monosaccharide, a disaccharide, an oligosaccharide, a polysaccharide, or a mixture of two or more thereof, and (C) the derivative of the sugar comprises a sugar alcohol, a sugar acid or a mixture thereof.

15. According to claim 1,

(C) A stable pharmaceutical formulation, characterized in that the sugar or derivative thereof comprises sorbitol, mannitol, trehalose, sucrose or a mixture of two or more thereof.

16. 16. The method of claim 15,

(C) A stable pharmaceutical formulation, characterized in that the sugar or derivative thereof comprises trehalose.

17. According to claim 1,

(C) A stable pharmaceutical preparation, characterized in that the concentration of sugar or its derivative is 1 to 20% (w/v).

18. According to claim 1,

(D) A stable pharmaceutical formulation, characterized in that the buffer comprises an amino acid.

19. According to claim 1,

(D) A stable pharmaceutical formulation, characterized in that the buffer comprises a free amino acid, an amino acid salt or a mixture thereof.

20. 20. The method of claim 19,

(D) A stable pharmaceutical formulation, characterized in that the buffer comprises histidine, histidine salt or a mixture thereof.

21. According to claim 1,

(D) A stable pharmaceutical formulation, characterized in that the content of the buffer is 1 to 20 mM.

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22. According to claim 1,

A stable pharmaceutical formulation, characterized in that it does not contain acetic acid, citric acid, phosphoric acid or a mixture thereof.

23. According to claim 1,

(B) A stable pharmaceutical formulation, characterized in that it further comprises an isotonic agent.

24. 24. The method of claim 23,

(B) A stable pharmaceutical formulation, characterized in that the isotonic agent comprises sodium chloride, potassium chloride, calcium chloride or a mixture of two or more thereof.

25. 24. The method of claim 23,

(B) A stable pharmaceutical formulation, characterized in that the content of the isotonic agent is 30 mM or less.

26. The method of claim 1,

A stable pharmaceutical formulation, characterized in that the pH is 5.0 to 7.0.

27. According to claim 1,

A stable pharmaceutical formulation, characterized in that it does not contain NaF, KBr, NaBr, Na_2SO_4, NaSCN, K_2SO_4 or mixtures thereof.

28. According to claim 1,

A stable pharmaceutical preparation, characterized in that it does not contain a chelating agent.

29. (A) (1) a VEGF receptor extracellular region 1 element comprising amino acids 27 to 129 of SEQ ID NO: 2, (2) a VEGF receptor extracellular region 2 element comprising amino acids 130 to 231 of SEQ ID NO: 2, and (3)) 5 to 100 mg/ml of a recombinant fusion protein comprising an Fc region element comprising amino acids 232 to 457 of SEQ ID NO: 2;

(B) 0.01 to 0.1% (w/v) surfactant;

(C) 1 to 20% (w/v) of a sugar or derivative thereof; and

(D) Buffer 1-20 mM

A stable pharmaceutical formulation comprising a.

30. 30. The method of claim 29,

(B) A stable pharmaceutical formulation, characterized in that it further comprises 30 mM or less of an isotonic agent.

31. (A) Vascular Endothelial Growth Factor (VEGF) antagonists;

(B) polysorbates;

(C) sugars or derivatives thereof; and

(D) histidine

A stable pharmaceutical formulation comprising a.

32. 32. The method of claim 31,

(B) A stable pharmaceutical formulation, characterized in that it further comprises sodium chloride.

33. (A) (1) a VEGF receptor extracellular region 1 element comprising amino acids 27 to 129 of SEQ ID NO: 2, (2) a VEGF receptor extracellular region 2 element comprising amino acids 130 to 231 of SEQ ID NO: 2, and (3)) 5 to 100 mg/ml of a recombinant fusion protein comprising an Fc region element comprising amino acids 232 to 457 of SEQ ID NO: 2;

(B) 0.01 to 0.1% (w/v) polysorbate;

(C) 1 to 20% (w/v) of a sugar or derivative thereof; and

(D) histidine 1-20 mM

A stable pharmaceutical formulation comprising a.

34. 34. The method of claim 33,

(B) A stable pharmaceutical formulation, characterized in that it further comprises 30 mM or less sodium chloride.

35. 35. The method of any one of claims 1 to 34,

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A stable pharmaceutical formulation with 50 or less insoluble foreign particles of 10.00 μm or more and less than 400.00 μm, measured by HIAC when stored at 5±3° C. for 9 months.

36. 35. The method of any one of claims 1 to 34,

A stable pharmaceutical preparation showing an active ingredient content of 98% or more after 10 days of storage at 8±3°C.

37. 35. The method according to any one of claims 1 to 34,

A stable pharmaceutical formulation showing a high molecular weight component content of 1% or less after 10 days of storage at 5±3°C.

38. 35. The method of any one of claims 1 to 34,

A stable pharmaceutical formulation showing a low molecular weight component content of 0.65% or less after 10 days of storage at 5±3°C.

39. 35. The method of any one of claims 1 to 34,

A stable pharmaceutical formulation exhibiting a charge variant content of 87% or more when stored at 5±3° C. for 9 months.

40. 35. The method of any one of claims 1 to 34,

A stable pharmaceutical formulation that exhibits 90% or more of VEGF binding affinity when stored at 5±3° C. for 9 months.

41. 35. The method of any one of claims 1 to 34,

A stable pharmaceutical preparation, characterized in that it is for intraocular administration.

42. 35. The method of any one of claims 1 to 34,

A stable pharmaceutical preparation, characterized in that it does not undergo a reconstitution step, a dilution step, or both before use.

43. A glass vial filled with the stable pharmaceutical formulation according to any one of claims 1 to 34.

44. 35. A pre-filled syringe filled with the stable pharmaceutical formulation according to any one of claims 1 to 34.

Description

stable pharmaceutical formulations

The present invention relates to a pharmaceutical preparation capable of stably preserving a drug capable of inhibiting Vascular Endothelial Growth Factor (VEGF) and capable of intraocular administration.

As release of vascular endothelial growth factor (VEGF) contributes to increased vascular permeability of the eye and inadequate new blood vessel growth, neovascular (wet) age-related macular degeneration, retinal vein occlusion (central retinal vein occlusion or branch retinal vein occlusion) macular area it can cause visual impairment due to tumors, visual impairment due to diabetic macular edema, and visual impairment due to choroidal neovascularization due to pathological myopia. Therefore, inhibiting VEGF is an effective method for treating ophthalmic diseases associated with angiogenesis.

The component name of the VEGF inhibitory drug is Aflibercept, and it is a kind of recombinant fusion protein capable of inhibiting Type A and Type B VEGF and PlGF (Placental Growth Factor). Pharmaceutical preparations including this drug should be administered by intraocular injection and should be performed directly by a qualified physician with experience in intraocular administration.

For long-term storage of the VEGF inhibitory agent, a formulation containing histidine, trehalose, and polysorbate 20 was developed as a stabilizer, and sodium chloride was added as an isotonic agent to have an osmotic pressure similar to the inside of the eyeball for the purpose of reducing pain during administration. However, it was confirmed that the stability of the drug was reduced when sodium chloride, an isotonic agent, was present, and to improve this, the concentration of sodium chloride was applied at a concentration capable of maintaining an appropriate osmotic pressure.

The formulation of the present invention derived improved results in terms of drug stabilization than the aflibercept liquid formulation of Korean Patent Application Laid-Open No. 10-2009-0018807, and the improved level was directly compared and evaluated through Experimental Examples (Experimental Example) One).

Therefore, the superiority of the stable pharmaceutical formulation containing the VEGF inhibitory agent aflibercept of the present invention was finally confirmed.

Accordingly, an object of the present invention is to provide a pharmaceutical formulation that is stable for long-term storage while including aflibercept, a VEGF inhibitory agent.

In addition, another problem to be solved by the present invention is to provide a pharmaceutical formulation having excellent long-term storage stability based on excellent stability under accelerated conditions and harsh conditions.

In addition, another problem to be solved by the present invention is to provide a stable pharmaceutical preparation capable of intraocular administration.

A stable pharmaceutical preparation according to an embodiment of the present invention comprises (A) a recombinant fusion protein, (B) a surfactant, (C) a sugar or derivative thereof, and (D) a buffer.

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In one embodiment of the present invention, the pharmaceutical preparation may be in a liquid form.

In one embodiment of the present invention, (A) the recombinant fusion protein may include a vascular endothelial growth factor (VEGF) antagonist.

In one embodiment of the present invention, (A) the recombinant fusion protein may comprise a human VEGF receptor extracellular region.

In one embodiment of the present invention, (A) the recombinant fusion protein may comprise human VEGF receptor extracellular region 1, 2, or a mixture thereof.

In one embodiment of the present invention, (A) the recombinant fusion protein may include an Fc region of immunoglobulin G (IgG), preferably an Fc region of human immunoglobulin G (IgG).

In one embodiment of the present invention, (A) the recombinant fusion protein comprises (1) a VEGF receptor extracellular region 1 element comprising amino acids 27 to 129 of SEQ ID NO: 2, (2) amino acids 130 to 231 of SEQ ID NO: 2 It may include a VEGF receptor extracellular region 2 element and (3) an Fc region element comprising amino acids 232 to 457 of SEQ ID NO: 2. The amino acid of SEQ ID NO: 2 may be encoded by the polynucleotide of SEQ ID NO: 1.

In one embodiment of the present invention, (A) the recombinant fusion protein may include aflibercept.

In one embodiment of the present invention, (A) the concentration of the recombinant fusion protein may be 5 to 100 mg/ml.

In one embodiment of the present invention, (B) the surfactant may include polysorbate, poloxamer, or a mixture thereof.

In one embodiment of the present invention, (B) the surfactant may include polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80, or a mixture of two or more thereof.

In one embodiment of the present invention, (B) the surfactant may include polysorbate 20.

In one embodiment of the present invention, (B) the concentration of the surfactant may be 0.01 to 0.1% (w / v).

In one embodiment of the present invention, (C) sugar comprises a monosaccharide, disaccharide, oligosaccharide, polysaccharide, or a mixture of two or more thereof, and (C) a derivative of sugar may include a sugar alcohol, sugar acid, or a mixture thereof. .

In one embodiment of the present invention, (C) sugar or a derivative thereof may include sorbitol, mannitol, trehalose, sucrose, or a mixture of two or more thereof.

In one embodiment of the present invention, (C) sugar or a derivative thereof may include trehalose.

In one embodiment of the present invention, the concentration of (C) sugar or a derivative thereof may be 1 to 20% (w/v).

In one embodiment of the present invention, (D) the buffer may include an amino acid.

In one embodiment of the present invention, (D) the buffer may include a free amino acid, an amino acid salt, or a mixture thereof.

In one embodiment of the present invention, (D) the buffer may include histidine, histidine salt, or a mixture thereof.

In one embodiment of the present invention, (D) the content of the buffer may be 1 to 20 mM.

In one embodiment of the present invention, the stable pharmaceutical formulation of the present invention may not contain acetic acid, citric acid, phosphoric acid, or mixtures thereof.

In one embodiment of the present invention, the stable pharmaceutical formulation of the present invention may further comprise (E) an isotonic agent.

In one embodiment of the present invention, (E) the tonicity agent may include sodium chloride (NaCl), potassium chloride (KCl), calcium chloride (CaCl), or a mixture of two or more thereof.

In one embodiment of the present invention, (E) the content of the tonicity agent may be 30 mM or less.

In one embodiment of the present invention, the formulation of the present invention may have a pH of 5.0 to 7.0.

In one embodiment of the present invention, the formulation of the present invention may not include NaF, KBr, NaBr, Na_2SO_4, NaSCN, K_2SO_4 or a mixture thereof.

In one embodiment of the present invention, the preparation of the present invention may not contain a chelating agent.

A stable pharmaceutical formulation according to an embodiment of the present invention is (A) (1) a VEGF receptor extracellular region 1 element comprising amino acids 27 to 129 of SEQ ID NO: 2, (2) amino acids 130 to 231 of SEQ ID NO: 2 5 to 100 mg/ml of a recombinant fusion protein comprising a VEGF receptor extracellular region 2 element comprising and (3) an Fc region element comprising amino acids 232 to 457 of SEQ ID NO: 2, (B) 0.01 to 0.1% of a surfactant (w/v), (C) 1 to 20% (w/v) of a sugar or derivative thereof, (D) 1 to 20 mM of a buffer and (E) 30 mM or less of an isotonic agent. The amino acid of SEQ ID NO: 2 may be encoded by the polynucleotide of SEQ ID NO: 1.

A stable pharmaceutical formulation according to an embodiment of the present invention includes (A) a vascular endothelial growth factor (VEGF) antagonist; (B) polysorbate; (C) sugars or derivatives thereof, and (D) histidine.

The stable pharmaceutical formulation according to an embodiment of the present invention may further comprise (E) sodium chloride.

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A stable pharmaceutical formulation according to an embodiment of the present invention is (A) (1) a VEGF receptor extracellular region 1 element comprising amino acids 27 to 129 of SEQ ID NO: 2, (2) amino acids 130 to 231 of SEQ ID NO: 2 5-100 mg/ml of a recombinant fusion protein comprising a VEGF receptor extracellular region 2 element comprising, and (3) an Fc region element comprising amino acids 232 to 457 of SEQ ID NO: 2; (B) 0.01 to 0.1% (w/v) polysorbate; (C) 1 to 20% (w/v) of a sugar or derivative thereof; and (D) 1 to 20 mM histidine.

The stable pharmaceutical formulation according to an embodiment of the present invention may further comprise (E) sodium chloride 30 mM or less.

In one embodiment of the present invention, the stable pharmaceutical formulation may have 50 or less insoluble foreign particles having a size of 10.00 μm or more and less than 400.00 μm, measured by HIAC when stored at 5±3° C. for 9 months.

In one embodiment of the present invention, the stable pharmaceutical formulation may exhibit an active ingredient content of 98% or more after 10 days of storage at 5±3°C.

In one embodiment of the present invention, the stable pharmaceutical formulation may exhibit a high molecular weight component content of 1% or less after 10 days of storage at 5±3°C.

In one embodiment of the present invention, the stable pharmaceutical formulation may exhibit a low molecular weight component content of 0.05% or less after 10 days of storage at 5±3°C.

In one embodiment of the present invention, the stable pharmaceutical formulation may exhibit a charge variant content of 87% or more when stored at 5±3° C. for 9 months.

In one embodiment of the present invention, the stable pharmaceutical formulation may exhibit VEGF binding affinity of 90% or more when stored at 5±3° C. for 9 months.

In one embodiment of the present invention, the stable pharmaceutical formulation may be for intraocular administration, preferably for intravitreal administration.

In one embodiment of the present invention, the stable pharmaceutical preparation may be one that does not undergo a reconstitution step, a dilution step, or both before use.

A glass vial according to an embodiment of the present invention is filled with the stable pharmaceutical formulation.

A pre-filled syringe according to an embodiment of the present invention is filled with the stable pharmaceutical agent.

The stable pharmaceutical preparation according to the present invention contains the recombinant fusion protein, has low viscosity, maintains excellent stability in long-term storage conditions, accelerated conditions and harsh conditions, and can be administered intraocularly.

[Stable pharmaceutical formulations]

The stable pharmaceutical preparation according to the present invention comprises (A) a recombinant fusion protein; (B) a surfactant; (C) sugars or derivatives thereof; and (D) a buffer.

In the specification of the present application, the term "not including" means not including the corresponding component at all. In addition, the term refers to those that do not substantially include the component, that is, include in a range that does not affect the activity of the fusion protein, stability and viscosity of the pharmaceutical formulation, for example, based on the total weight of the pharmaceutical formulation. as 0 to 1% (w/v), 0 to 1 ppm (w/v), or 0 to 1 ppb (w/v). The pharmaceutical formulation may be in a liquid form, but is not limited thereto.

(A) Recombinant fusion protein

The recombinant fusion protein of the present invention may be a vascular endothelial growth factor (VEGF) antagonist. In addition, the recombinant fusion protein of the present invention is (1) a VEGF receptor extracellular region 1 element comprising amino acids 27 to 129 of SEQ ID NO: 2, (2) a VEGF receptor extracellular region comprising amino acids 130 to 231 of SEQ ID NO: 2 2 and (3) an Fc region element comprising amino acids 232 to 457 of SEQ ID NO:2. The amino acid of SEQ ID NO: 2 may be encoded by the polynucleotide of SEQ ID NO: 1.

The recombinant fusion protein of the present invention may include a human VEGF receptor extracellular region, more specifically, the human VEGF receptor extracellular region 1, 2, or a mixture thereof, but is not limited thereto.

The recombinant fusion protein may include an Fc region of human immunoglobulin G (IgG), but is not limited thereto.

The recombinant fusion protein may include, but is not limited to, aflibercept.

The concentration of the recombinant fusion protein of the present invention can be freely adjusted within a range that does not substantially adversely affect the stability and viscosity of the stable pharmaceutical preparation according to the present invention. In one embodiment of the present invention, the concentration of the recombinant fusion protein may be 5 to 100 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 5 to 95 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 5 to 90 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 5 to 85 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 5 to 80 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 5 to 75 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 5 to 70 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 5 to 65 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 5 to 60 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 5 to 55 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 5-50 mg/ml. In another embodiment of the

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present invention, the concentration of the recombinant fusion protein may be 5 to 45 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 5 to 40 mg/ml.

Also, in another embodiment of the present invention, the concentration of the recombinant fusion protein may be 10 to 100 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 10 to 90 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 10-80 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 10 to 70 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 10 to 60 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 10-50 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 10 to 40 mg/ml.

Also, in another embodiment of the present invention, the concentration of the recombinant fusion protein may be 20 to 100 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 20 to 90 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 20 to 80 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 20 to 70 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 20 to 60 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 20-50 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 20 to 40 mg/ml.

In addition, in another embodiment of the present invention, the concentration of the recombinant fusion protein may be 30 to 100 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 30 to 90 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 30 to 80 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 30 to 70 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 30 to 60 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 30-50 mg/ml. In another embodiment of the present invention, the concentration of the recombinant fusion protein may be 30-40 mg/ml.

(B) surfactant

Examples of surfactants include polyoxyethylene sorbitan fatty acid esters (eg polysorbate), polyoxyethylene alkyl ethers (eg Brij), alkylphenylpolyoxyethylene ethers (eg Triton-X) , polyoxyethylene-polyoxypropylene copolymers (eg, Poloxamer, Pluronic), sodium dodecyl sulfate (SDS), poloxamers and mixtures thereof, and the like.

In one embodiment of the present invention, the surfactant may include polysorbate, poloxamer, or a mixture thereof, specifically, polyoxyethylene sorbitan fatty acid ester (polysorbate), poloxamer, or a mixture thereof. can do. More specifically, it may include, but is not limited to, polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80, or a mixture of two or more thereof. In another embodiment of the present invention, the polysorbate may include, but is not limited to, polysorbate 20.

In one embodiment of the present invention, the concentration of the surfactant can be freely adjusted within a range that does not adversely affect the stability and viscosity of the stable pharmaceutical formulation according to the present invention. For example, the concentration of the surfactant is preferably 0.001 to 5% (w/v), 0.005 to 2% (w/v), more preferably 0.01 to 1% (w/v), 0.01 to 0.5% (w/v), 0.01 to 0.1% (w/v), or 0.01 to 0.05% (w/v), but is not limited thereto.

(C) sugars or derivatives of sugars

The sugars may include monosaccharides, disaccharides, oligosaccharides, polysaccharides, or a mixture of two or more thereof. Examples of monosaccharides include, but are not limited to, glucose, fructose, galactose, and the like. Examples of disaccharides include, but are not limited to, sucrose, lactose, maltose, trehalose, and the like. Examples of oligosaccharides include, but are not limited to, fructooligosaccharides, galactooligosaccharides, mannan oligosaccharides, and the like. Examples of polysaccharides include, but are not limited to, starch, glycogen, cellulose, chitin, pectin, and the like.

The sugar derivative may include a sugar alcohol, a sugar acid, or a mixture thereof. Examples of sugar alcohols include glycerol, erythritol, threitol, arabitol, xylitol, ribitol, mannitol, sorbitol, galactitol, fusitol, iditol, inositol, boiemitol, isomalt, maltitol, lactitol, maltotriitol , maltotetraitol, polyglycitol, and the like, but are not limited thereto. Examples of sugar acids include, but are not limited to, aldonic acid (such as glyceric acid), ulosonic acid (such as neuramic acid), uronic acid (such as glucuronic acid), and aldaric acid (such as tartaric acid).

In one embodiment of the present invention, the sugar or its derivative may include sorbitol, mannitol, trehalose, sucrose, or a mixture of two or more thereof. In another embodiment of the present invention, the sugar or derivative thereof may include trehalose, but is not limited thereto.

In one embodiment of the present invention, the concentration of sugar or a derivative thereof can be freely adjusted within a range that does not substantially adversely affect the stability and viscosity of the pharmaceutical formulation according to the present invention. For example, the concentration of the sugar or its derivative may be 0.1 to 30% (w/v), 0.5 to 25% (w/v), and more preferably 1 to 20% (w/v). . According to one embodiment of the present invention, it may be 0.5 to 20% (w/v) or 1 to 25% (w/v). According to an embodiment of the present invention, it may be 1 to 20% (w/v), 2 to 15% (w/v), 4 to 13% (w/v), or 8 to 12% (w/v). .

(D) buffer

Buffers are neutralizing substances that minimize changes in pH caused by acids or alkalis. Examples of buffers include phosphate, acetate, succinate, gluconate, and citrate. However, there are amino acids such as glutamate and histidine.

In one embodiment of the present invention, an amino acid may be included as a buffer, and more specifically, a free amino acid, an amino acid salt, or a mixture thereof may be included, but is not limited thereto. In one embodiment of the present invention, the buffer may include histidine, histidine salt, or a mixture thereof. However, the present invention is not limited thereto.

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When a histidine salt is used as the buffer, for example, histidine chloride, histidine acetate, histidine phosphate, histidine sulfate and the like may be included. It may be preferable to include histidine as a buffer in terms of pH control and stability, but is not limited thereto. In addition, other types of acids may be included for pH adjustment, but according to one embodiment of the present invention, acetic acid, citric acid, phosphoric acid, or mixtures thereof may not be included, but is not limited thereto.

In one embodiment of the present invention, the content of the buffering agent can be freely adjusted within a range that does not substantially adversely affect the stability and viscosity of the pharmaceutical formulation according to the present invention. In one embodiment of the present invention, the content of the buffer may be 1 to 20 mM or 1 to 10 mM. In one embodiment of the present invention, the content of the buffer may be 2 to 20 mM or 2 to 10 mM. In one embodiment of the present invention, the content of the buffer may be 3 to 20 mM or 3 to 10 mM. In one embodiment of the present invention, the content of the buffer may be 4 to 20 mM or 4 to 10 mM. In one embodiment of the present invention, the content of the buffer may be 5 to 20 mM or 5 to 10 mM. In one embodiment of the present invention, the content of the buffer may be 6 to 20 mM or 6 to 10 mM. In one embodiment of the present invention, the content of the buffer may be 1 to 20 mM. In one embodiment of the present invention, the content of the buffer may be 6.4 to 9.6 mM, but is not limited thereto.

(E) isotonic agents

In one embodiment of the present invention, (E) may additionally include an isotonic agent.

The isotonic agent may include sodium chloride, potassium chloride, calcium chloride or a mixture of two or more thereof.

In one embodiment of the present invention, the content of the isotonic agent can be freely adjusted within a range that does not substantially adversely affect the stability and viscosity of the pharmaceutical preparation according to the present invention. In one embodiment of the present invention, the content of the isotonic agent may be 30 mM or less. In one embodiment of the present invention, the content of the isotonic agent may be 0.00001 to 30 mM, 0.0001 to 30 mM, 0.001 to 30 mM, 0.01 to 30 mM, 0.1 to 30 mM, or 1 to 30 mM. In one embodiment of the present invention, the content of the isotonic agent may be 0.00001 to 30 mM, 0.00001 to 25 mM, or 0.00001 to 20 mM. In one embodiment of the present invention, the content of the isotonic agent may be 0.0001 to 30 mM, 0.0001 to 25 mM, or 0.0001 to 20 mM. In one embodiment of the present invention, the content of the isotonic agent may be 0.001 to 30 mM, 0.001 to 25 mM, or 0.001 to 20 mM. In one embodiment of the present invention, the content of the isotonic agent may be 0.01 to 30 mM, 0.01 to 25 mM, or 0.01 to 20 mM. In one embodiment of the present invention, the content of the isotonic agent may be 0.1 to 30 mM, 0.1 to 25 mM, or 0.1 to 20 mM. In one embodiment of the present invention, the content of the isotonic agent may be 1 to 30 mM, 1 to 25 mM, or 1 to 20 mM. In one embodiment of the present invention, the content of the isotonic agent may be 2 to 30 mM, 2 to 25 mM, or 2 to 20 mM. In one embodiment of the present invention, the content of the isotonic agent may be 3 to 30 mM, 3 to 25 mM, or 3 to 20 mM. In one embodiment of the present invention, the content of the isotonic agent may be 5 to 18 mM, 6 to 17 mM, or 7.2 to 15.6 mM, but is not limited thereto.

(F) pH

In one embodiment of the present invention, the stable pharmaceutical composition may have a pH of 5.0 to 7.0, 5.0 to 6.5, 5.5 to 7.0, or 5.5 to 6.5. When the pH is within this range, it can exhibit excellent long-term stability and low viscosity. The pH can be adjusted using a buffer. In other words, when the buffer is included in a predetermined amount, the pH in the above range can be exhibited without a separate pH adjusting agent. In one embodiment of the present invention, the composition may not include acetic acid, citric acid, phosphoric acid, or a mixture thereof. When acetic acid (acetate) citric acid (citrate), phosphoric acid, or mixtures thereof are used as buffers, it may be difficult to achieve a pH in the above range. When an acid (eg, hydrochloric acid) or a base (eg, sodium hydroxide) is additionally included as a separate pH adjusting agent, the stability of the target recombinant fusion protein may be reduced.

(G) other ingredients

In one embodiment of the present invention, the stable pharmaceutical preparation may not contain a preservative. Examples of preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride, benzethonium chloride, phenol, butyl alcohol, benzyl alcohol, alkyl parabens, catechol, resorcinol, cyclohexanol, 3-pentane Ol, m-cresol, and the like. The inclusion of preservatives may not help improve stability.

In addition, in one embodiment of the present invention, the stable pharmaceutical preparation may not contain NaF, KBr, NaBr, Na_2SO_4, NaSCN, K_2SO_4 or a mixture thereof, and does not contain a chelating agent. However, the present invention is not limited thereto.

(H) "stable" pharmaceutical formulations

The term "stable" in the "stable" pharmaceutical formulation of the present invention means that the recombinant fusion protein according to the present invention substantially improves its physical stability and/or chemical stability and/or biological activity during the manufacturing process and/or upon storage/storage. means to hold Various analytical techniques for determining the stability of an antibody are readily available in the art. Stability can be measured at a selected temperature and for a selected period of time.

Physical stability can be assessed by methods known in the art, which include measuring the sample apparent attenuation of light (absorption or optical density). This light attenuation measurement is related to the turbidity of the formulation. In addition, the content of high molecular weight components, the content of low molecular weight components, the amount of intact protein, the number of insoluble foreign matter particles, etc. can be measured for physical stability.

Chemical stability can be assessed, for example, by detecting and quantifying a chemically altered form of the recombinant fusion protein. Chemical stability includes charge changes (eg, occurring as a result of deamidation or oxidation) that can be assessed, for example, by ion exchange chromatography. Charge variants (acidic or basic peaks) and the like can be measured for chemical stability.

Biological activity can be assessed by methods known in the art, for example, antigen binding affinity can be measured through ELISA.

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In one embodiment of the present invention, the term "stable" pharmaceutical agent refers to a pharmaceutical agent satisfying one or more of the following.

(H)-1 turbidity

- A pharmaceutical formulation having an absorbance A_{600} of 0 to 0.0700 or 0 to 0.0400 measured with a spectrophotometer after storage at a temperature of 5±3° C. for 4 weeks;

- A pharmaceutical formulation having an absorbance A_{600} of 0 to 0.0700 or 0 to 0.0400 measured with a spectrophotometer after storage for 4 weeks at a temperature of 10 ° C, 15 ° C, 20 ° C, 25 ° C, 30 ° C, or 35 ° C ;

- a pharmaceutical formulation having an absorbance A_{600} of 0 to 0.0700 or 0 to 0.0400 measured with a spectrophotometer after storage for 4 weeks at a temperature of 40±2° C., a relative humidity of 75±5%, and a closed condition;

(H)-2 main component content (main peak)

- A pharmaceutical formulation containing 98 to 99% of the main component as measured by SE-HPLC after storage for 9 months at a temperature of 5±3° C.;

- A pharmaceutical formulation containing 89 to 92% of the main component as measured by SE-HPLC after storage for 4 weeks at a temperature of 40±2° C., a relative humidity of 75±5% and a closed condition;

(H)-3 high molecular weight component (peak with retention time ahead of the main peak (intact recombinant fusion protein))

- A pharmaceutical formulation having 98 to 100% of high molecular weight components measured by SE-HPLC after storage for 9 months at a temperature of 5±3° C.;

- A pharmaceutical formulation containing 7 to 10% of high molecular weight components as measured by SE-HPLC after storage for 4 weeks at a temperature of 40±2° C., a relative humidity of 75±5% and a closed condition;

(H)-4 low molecular weight component (peak with retention time posterior to the main peak (intact recombinant fusion protein))

- A pharmaceutical formulation having a low molecular weight component of 0.0 to 0.1% as measured by SE-HPLC after storage for 9 months at a temperature of 5±3°C;

- A pharmaceutical formulation containing 0.0 to 1.2% of low molecular weight components measured by SE-HPLC after storage for 4 weeks at a temperature of 40±2° C., a relative humidity of 75±5% and a closed condition;

(H)-5 intact one recombinant human VEGF receptor extracellular region 1, one recombined human VEGF receptor extracellular region 2, and the content of the Fc portion of the recombinant human IgG (1VEGF1 + 1VEGF2 + Fc)

- One intact recombined human VEGF receptor extracellular domain 1, 1 recombined human VEGF receptor extracellular domain 2 and recombined human IgG measured by reduced CE-SDS after storage at a temperature of 5±3° C. for 9 months of a pharmaceutical formulation in which the content of some of the Fc (1VEGF1 + 1VEGF2 + Fc) is 96 to 98%;

- Intact 1 recombined human VEGF receptor extracellular domain 1, 1 recombined human VEGF receptor measured by reduced CE-SDS after storage for 4 weeks at a temperature of 40±2°C, relative humidity of 75±5%, and airtight conditions a pharmaceutical preparation in which the content of the extracellular region 2 and the Fc portion of recombinant human IgG (1VEGF1 + 1VEGF2 + Fc) is 90 to 96%;

(H)-6 insoluble foreign matter particles

- A pharmaceutical formulation in which the number of insoluble foreign particles (2.00 μm≤, <400.00 μm) measured by HIAC after storage for 9 months at a temperature of 5±3° C. is 0 to 100;

- A pharmaceutical formulation in which the number of insoluble foreign particles (10.00 μm≤, <400.00 μm) measured by HIAC after storage for 9 months at a temperature of 5±3° C. is 0 to 50;

- A pharmaceutical formulation in which the number of insoluble foreign particles (25.00 μm≤, <400.00 μm) measured by HIAC after storage for 9 months at a temperature of 5±3° C. is 0 to 5;

- The number of insoluble foreign particles (2.00μm≤, <400.00μm) measured by HIAC after storage for 4 weeks at a temperature of 40±2°C, a relative humidity of 75±5%, and a closed condition is 0 to 100 pharmaceutical formulations ;

- The number of insoluble foreign particles (10.00μm≤, <400.00μm) measured by HIAC after storage for 4 weeks at a temperature of 40±2°C, a relative humidity of 75±5%, and a closed condition is 0 to 50 pharmaceutical formulations ;

- The number of insoluble foreign particles (25.00μm≤, <400.00μm) measured by HIAC after storage for 4 weeks at a temperature of 40±2°C, a relative humidity of 75±5%, and a closed condition is 0 to 5 pharmaceutical formulations ;

- A pharmaceutical formulation in which the number of insoluble foreign particles (1.00 μm≤, <400.00 μm) measured by MFI after storage for 9 months at a temperature of 5±3° C. is 0 to 5,000;

- A pharmaceutical formulation in which the number of insoluble foreign particles (10.00 μm≤, <400.00 μm) measured by MFI after storage for 9 months at a temperature of 5±3° C. is 0 to 100;

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- A pharmaceutical formulation in which the number of insoluble foreign particles (25.00 μm≤, <400.00 μm) measured by MFI after storage for 9 months at a temperature of 5±3° C. is 0 to 15;

- A pharmaceutical formulation in which the number of insoluble foreign particles (10.00 μm≤, <100.00 μm) measured by MFI after storage for 4 weeks at a temperature of 5±3° C. is 0 to 1,000;

- A pharmaceutical formulation in which the number of insoluble foreign particles (10.00 μm≤, <100.00 μm) measured by MFI after storage for 4 weeks at a temperature of 5±3° C. is 0 to 100;

- A pharmaceutical formulation in which the number of insoluble foreign particles (25.00 μm≤, <100.00 μm) measured by MFI after storage for 4 weeks at a temperature of 5±3° C. is 0 to 100;

- A pharmaceutical formulation in which the number of insoluble foreign particles (25.00 μm≤, <100.00 μm) measured by MFI after storage for 4 weeks at a temperature of 5±3° C. is 0 to 10;

- A pharmaceutical formulation in which the number of insoluble foreign particles (1.00 μm≤, <100.00 μm) measured by MFI after storage for 4 weeks at a temperature of 40±2° C. is 0 to 5,000;

- A pharmaceutical formulation in which the number of insoluble foreign particles (1.00 μm≤, <100.00 μm) measured by MFI after storage for 4 weeks at a temperature of 40±2° C. is 0 to 2,500;

- A pharmaceutical formulation in which the number of insoluble foreign particles (10.00 μm≤, <100.00 μm) measured by MFI after storage for 4 weeks at a temperature of 40±2° C. is 0 to 1,000;

- A pharmaceutical formulation in which the number of insoluble foreign particles (10.00 μm≤, <100.00 μm) measured by MFI after storage at a temperature of 40±2° C. for 4 weeks is 0 to 100;

- A pharmaceutical formulation in which the number of insoluble foreign particles (25.00 μm≤, <100.00 μm) measured by MFI after storage for 4 weeks at a temperature of 40±2° C. is 0 to 100;

- A pharmaceutical formulation in which the number of insoluble foreign particles (25.00 μm≤, <100.00 μm) measured by MFI after storage for 4 weeks at a temperature of 40±2° C. is 0 to 10;

- The number of insoluble foreign particles (1.00㎛≤, <400.00㎛) measured by MFI after storage for 4 weeks at a temperature of 40±2℃, a relative humidity of 75±5%, and a closed condition is 0 to 20,000 pharmaceutical formulations ;

- The number of insoluble foreign particles (10.00㎛≤, <400.00㎛) measured by MFI after storage for 4 weeks at a temperature of 40±2℃, a relative humidity of 75±5%, and a closed condition is 0 to 70 pharmaceutical formulations ;

- The number of insoluble foreign particles (25.00㎛≤, <400.00㎛) measured by MFI after storage for 4 weeks at a temperature of 40±2℃, a relative humidity of 75±5%, and a closed condition is 0 to 15 pharmaceutical formulations ;

(H)-7 oxidation rate

- a pharmaceutical formulation having an oxidation rate of 0 to 7% of heavy chain Met 192 measured by LC-MS after storage at a temperature of 5±3° C. for 4 weeks;

- a pharmaceutical formulation having an oxidation rate of 0 to 6% of heavy chain Met 192 measured by LC-MS after storage for 4 weeks at a temperature of 40±2° C., a relative humidity of 75±5%, and a closed condition;

(H)-8 charge variant

- A pharmaceutical formulation having a relative content of peak 11 of 89 to 91% in theoretical peak 4 measured by cIEF after storage at a temperature of 5±3° C. for 4 weeks

- a pharmaceutical formulation in which the sum of peak 11 in theoretical peak 4 measured by cIEF after storage for 9 months at a temperature of 5±3° C. is 86 to 88%;

- A pharmaceutical formulation in which the sum of the theoretical peaks 4 to 11 measured by cIEF is 86 to 88% after storage for 4 weeks at a temperature of 40±2° C., a relative humidity of 75±5%, and a closed condition;

- A pharmaceutical formulation having a relative content of peak 11 of 86 to 88% in theoretical peak 4 measured by cIEF after storage for 4 weeks at a temperature of 40±2° C., relative humidity of 75±5%, and sealed conditions

(H)-9 VEGF binding affinity

- A pharmaceutical formulation having a VEGF binding affinity of 75 to 95% measured by ELISA after storage at a temperature of 5±3° C. for 9 months;

- A pharmaceutical formulation having a VEGF binding affinity of 60 to 75% measured by ELISA after storage for 4 weeks at a temperature of 40±2° C., a relative humidity of 75±5%, and a closed condition;

[Method for preparing stable pharmaceutical preparations]

The stable pharmaceutical formulation of the present invention can be prepared using a known method, and is not limited to a specific method. For example, after adjusting the pH while adding a buffer to a solution containing a surfactant, sugar or derivative thereof, and an isotonic agent, the

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recombinant fusion protein may be added to the mixed solution to prepare a pharmaceutical formulation. In addition, after preparing a solution containing some excipients in the final step of the purification process, the remaining ingredients may be added to prepare a pharmaceutical formulation. For example, after preparing a solution containing the recombinant fusion protein, buffer, sugar or derivative thereof, and isotonicity agent in the final step of the purification process, a pharmaceutical formulation may be prepared by adding a surfactant to the solution.

In addition, the preparation may not include a freeze-drying process during manufacture.

If the freeze-drying process is not included, for example, the pharmaceutical preparation of the present invention may be prepared and placed in an airtight container such as a glass vial or pre-filled syringe, which is a primary packaging material, immediately after treatment such as sterilization.

[How to use stable pharmaceutical preparations]

The stable pharmaceutical formulation according to the present invention can be used to treat diseases caused by leakage of VEGF. Examples of diseases caused by VEGF leakage include neovascular (wet) age-related macular degeneration, retinal vein occlusion (central retinal vein occlusion or branch retinal vein occlusion), visual impairment due to macular edema, visual impairment due to diabetic macular edema, and pathologic myopia. visual impairment due to choroidal neovascularization due to

The stable pharmaceutical formulation according to the present invention may be used once or several times, and when administered, it should be administered by a qualified physician with experience in intraocular administration (injection).

Concentrations of other components including the recombinant fusion protein in the pharmaceutical formulation are as described above, and the total volume of the pharmaceutical formulation may be 0.05 to 0.3 ml.

The dosage and administration period of the pharmaceutical agent may vary depending on the type of disease, the severity and course of the disease, the patient's health and response to treatment, and the judgment of the treating physician, and the recommended dosage is 2 mg (equal to about 50 microliters), but the dosing interval and timing of administration are not limited. For example, after intraocular administration of 2 mg based on the concentration of the recombinant fusion protein as one product containing the pharmaceutical formulation, the same dosage may be administered every 2 weeks or 2 months at the shortest. Dosing intervals longer than 16 weeks have not been studied.

In one embodiment of the present invention, the stable pharmaceutical preparation may not undergo a reconstitution step, a dilution step, or both before use.

[Treatment method and stabilization method]

The present invention also provides a patient with a disease due to VEGF leakage (A) a recombinant fusion protein; (B) a surfactant; (C) sugars or derivatives thereof; and (D) a method of treatment using a stable pharmaceutical formulation comprising a buffer.

The present invention also provides conditions for a pharmaceutical agent that stabilizes the recombinant fusion protein, comprising: (A) a recombinant fusion protein; (B) a surfactant; (C) sugars or derivatives thereof; and (D) a method for stabilizing a stable pharmaceutical formulation comprising a buffer.

In one embodiment of the treatment method or stabilization method, the pharmaceutical formulation may be in a liquid form.

In one embodiment of the treatment method or stabilization method, (A) the recombinant fusion protein may include a vascular endothelial growth factor (VEGF) antagonist.

In one embodiment of the treatment method or stabilization method, (A) the recombinant fusion protein may include a human VEGF receptor extracellular region.

In one embodiment of the treatment method or stabilization method, (A) the recombinant fusion protein may include human VEGF receptor extracellular region 1, 2, or a mixture thereof.

In one embodiment of the above treatment method or stabilization method, (A) the recombinant fusion protein may include an Fc region of immunoglobulin G (IgG), preferably an Fc region of human immunoglobulin G (IgG).

In one embodiment of the treatment or stabilization method, (A) the recombinant fusion protein comprises (1) a VEGF receptor extracellular region 1 element comprising amino acids 27 to 129 of SEQ ID NO: 2, (2) amino acid 130 of SEQ ID NO: 2 to 231 VEGF receptor extracellular region 2 element and (3) an Fc region element comprising amino acids 232 to 457 of SEQ ID NO:2.

In one embodiment of the treatment method or stabilization method, (A) the recombinant fusion protein may include aflibercept.

In one embodiment of the treatment method or stabilization method, (A) the concentration of the recombinant fusion protein may be 5 to 100 mg/ml.

In one embodiment of the treatment method or stabilization method, (B) the surfactant may include polysorbate, poloxamer, or a mixture thereof.

In one embodiment of the treatment method or stabilization method, (B) the surfactant may include polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80, or a mixture of two or more thereof.

In one embodiment of the treatment method or stabilization method, (B) the surfactant may include polysorbate 20.

In one embodiment of the treatment method or stabilization method, (B) the concentration of the surfactant may be 0.01 to 0.1% (w/v).

In one embodiment of the treatment or stabilization method, (C) the sugar comprises a monosaccharide, a disaccharide, an oligosaccharide, a polysaccharide, or a mixture of two or more thereof, and (C) the derivative of the sugar is a sugar alcohol, a sugar acid, or a mixture thereof. may include

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In one embodiment of the treatment or stabilization method, (C) sugar or a derivative thereof may include sorbitol, mannitol, trehalose, sucrose, or a mixture of two or more thereof.

In one embodiment of the treatment method or stabilization method, (C) sugar or a derivative thereof may include trehalose.

In one embodiment of the treatment or stabilization method, the concentration of (C) sugar or a derivative thereof may be 1 to 20% (w/v).

In one embodiment of the treatment or stabilization method, (D) the buffer may include an amino acid.

In one embodiment of the treatment method or the stabilization method, (D) the buffer may include a free amino acid, an amino acid salt, or a mixture thereof.

In one embodiment of the treatment or stabilization method, (D) the buffer may include histidine, histidine salt, or a mixture thereof.

In one embodiment of the treatment or stabilization method, the content of (D) buffer may be 1 to 20 mM.

In one embodiment of the treatment or stabilization method, the stable pharmaceutical preparation of the present invention may not contain acetic acid, citric acid, phosphoric acid, or a mixture thereof.

In one embodiment of the treatment or stabilization method, (E) may additionally include an isotonic agent.

(E) the isotonic agent may comprise sodium chloride, potassium chloride, calcium chloride or a mixture of two or more thereof.

In one embodiment of the treatment or stabilization method, (E) the content of the isotonic agent may be 30 mM or less.

In one embodiment of the treatment or stabilization method, the formulation of the present invention may have a pH of 5.0 to 7.0.

In one embodiment of the treatment or stabilization method, the formulation of the present invention may not contain NaF, KBr, NaBr, Na_2SO_4, NaSCN, K_2SO_4 or a mixture thereof.

In one embodiment of the present invention, the preparation of the present invention may not contain a chelating agent.

A stable pharmaceutical formulation according to an embodiment of the treatment method or stabilization method is (A) (1) a VEGF receptor extracellular region 1 element comprising amino acids 27 to 129 of SEQ ID NO: 2, (2) amino acid of SEQ ID NO: 2 5 to 100 mg/ml of a recombinant fusion protein comprising a VEGF receptor extracellular region 2 element comprising 130 to 231 and (3) an Fc region element comprising amino acids 232 to 457 of SEQ ID NO: 2, (B) surfactant 0.01 to 0.1% (w/v), (C) 1 to 20% (w/v) of sugar or a derivative thereof, and (D) 1 to 20 mM of a buffer. The pharmaceutical formulation according to an embodiment of the treatment method or stabilization method may further include (E) 30 mM or less of an isotonic agent.

The pharmaceutical formulation according to an embodiment of the treatment method or stabilization method includes (A) a vascular endothelial growth factor (VEGF) antagonist; (B) polysorbate; (C) sugars or derivatives thereof; and (D) histidine. The pharmaceutical formulation according to an embodiment of the treatment or stabilization method may further include (E) sodium chloride.

The pharmaceutical formulation according to an embodiment of the treatment or stabilization method is (A) (1) VEGF receptor extracellular region 1 element comprising amino acids 27 to 129 of SEQ ID NO: 2, (2) amino acid 130 of SEQ ID NO: 2 5-100 mg/ml of a recombinant fusion protein comprising a VEGF receptor extracellular region 2 element comprising 231 to 231, and (3) an Fc region element comprising amino acids 232 to 457 of SEQ ID NO:2; (B) 0.01 to 0.1% (w/v) polysorbate; (C) 1 to 20% (w/v) of a sugar or derivative thereof; and (D) 1 to 20 mM histidine. The pharmaceutical formulation according to one embodiment of the treatment method or stabilization method may further include (E) sodium chloride 30 mM or less.

In the stable pharmaceutical formulation according to an embodiment of the treatment or stabilization method, the number of insoluble foreign particles of 10.00 μm or more and less than 400.00 μm, measured by HIAC when stored at 5±3° C. for 9 months, may be 50 or less.

The stable pharmaceutical formulation according to an embodiment of the treatment method or stabilization method may exhibit an active ingredient content of 98% or more after 10 days of storage at 5±3°C.

The stable pharmaceutical formulation according to an embodiment of the treatment method or stabilization method may exhibit a high molecular weight component content of 1% or less after 10 days of storage at 5±3°C.

The stable pharmaceutical formulation according to an embodiment of the treatment method or stabilization method may exhibit a low molecular weight component content of 0.05% or less after 10 days of storage at 5±3°C.

The stable pharmaceutical formulation according to an embodiment of the treatment method or stabilization method may exhibit a charge variant content of 87.5% or more when stored at 5±3° C. for 9 months.

The stable pharmaceutical formulation according to an embodiment of the treatment method or stabilization method may exhibit a VEGF binding affinity of 90% or more when stored at 5±3° C. for 9 months.

In one embodiment of the treatment method or stabilization method, the stable pharmaceutical formulation may be for intraocular administration, preferably intravitreal administration.

In one embodiment of the treatment method or stabilization method, the stable pharmaceutical preparation may not be subjected to a reconstitution step, a dilution step, or both before use.

A glass vial according to an embodiment of the treatment method or stabilization method may be filled with the stable pharmaceutical agent.

A pre-filled syringe according to an embodiment of the treatment method or stabilization method may be filled with the stable pharmaceutical agent.

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[product]

The present invention also relates to the above stable pharmaceutical formulation; And it provides a product comprising a container containing the stable pharmaceutical formulation in a closed state.

The stable pharmaceutical formulation is as described above.

In one embodiment of the present invention, the container may be formed from a material such as glass, polymer (plastic), or metal, but is not limited thereto. In one embodiment of the present invention, the container is, but is not limited to, a bottle, a vial, a cartridge, a syringe (pre-filled syringe), or a tube. In one embodiment of the present invention, the container may be a glass or polymer vial, or a glass or polymer pre-filled syringe.

The specific product form of the vial, cartridge, pre-filled syringe, etc. and the method of filling the stable pharmaceutical formulation into the vial, cartridge, pre-filled syringe, etc. are easy for those of ordinary skill in the art to which the present invention pertains can be obtained or practiced. For example, US Pat. Nos. 4,861,335, 6,331,174, etc. disclose specific product forms and filling methods of pre-filled syringes. Commercially available products as the vial, cartridge, pre-filled syringe, etc. may be used as it is, or a separately customized product may be used in consideration of the physical properties, administration site, dosage, etc. of the stable pharmaceutical formulation.

In one embodiment of the present invention, the product may further include instructions for providing a method of use, a method of storage, or both of the stable pharmaceutical formulation. The method of use includes a treatment for a disease caused by VEGF leakage, and may include an administration route, dosage, and timing of administration.

In one embodiment of the present invention, the product may include other tools necessary from a commercial and user point of view, for example, needles, syringes, and the like.

Hereinafter, the present invention will be described in detail by way of Examples. The following examples are only for illustrating the present invention, and the scope of the present invention is not limited by the following examples.

Example

With respect to the recombinant fusion protein used in the Experimental Examples below, aflibercept cultured and purified at Celltrion Lab was used.

The following method was used as a method for measuring the physical stability, chemical stability and biological activity of the pharmaceutical formulation used in the Experimental Example below.

- Turbidity

The absorbance at 600 nm was measured using a UV-Vis spectrophotometer.

- Main ingredient content

The main component content (%) was measured using size exclusion high performance liquid chromatography (Size Exclusion HPLC).

- High molecular weight component content

The content (pre-peak; %) of the high molecular weight component was measured using size exclusion high performance liquid chromatography (Size Exclusion HPLC).

- Low molecular weight ingredient content

The content (post-peak; %) of low molecular weight components was measured using size exclusion high performance liquid chromatography (Size Exclusion HPLC).

- Content of one recombinant human VEGF receptor extracellular region 1, one recombined human VEGF receptor extracellular region 2, and Fc part (1VEGF1 + 1VEGF2 + Fc) of recombinant human IgG

Using Reduced Capillary Electrophoresis-Sodium Dodecyl Sulfate (R CE-SDS), one intact recombined human VEGF receptor extracellular domain 1, one recombined human VEGF receptor extracellular domain 2 and The content (%) of the recombinant human IgG Fc part (1VEGF1 + 1VEGF2 + Fc) was measured.

- Number of insoluble foreign particles (Sub-visible particles)

The number of insoluble foreign particles was measured using Micro Flow Imaging (MFI) and a light-shielding type particle counter (model name: HIAC 9703).

- Oxidation

The oxidation rate (%) of heavy chain Met 192 was measured through peptide mapping by liquid chromatography (LC-MS) through mass spectrometry.

- Charge variant (relative content of peak 4 to peak 11 out of 12 peaks)

Acidic and basic peaks (%) were measured by capillary Iso-Electric Focusing (cIEF).

- VEGF binding affinity

VEGF binding affinity (%) was measured by enzyme-linked immunosorbent assay (ELISA).

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Experimental Example 1: Comparison according to the concentration of histidine buffer; comparison of pH 5.5 - pH 6.5; Comparison according to concentration of sodium chloride isotonic agent; Comparison according to trehalose concentration; and confirming the superiority of the formulation of the present invention

With respect to the pharmaceutical formulation used in Experimental Example 1, each buffer was prepared to suit each pH, trehalose and sodium chloride were added thereto, a recombinant fusion protein was added thereto, and a surfactant was added to prepare the samples in Table 1. . The specific content of each component is as described in Table 1. In Examples 1-9 and Comparative Example 1, aflibercept purified by Celltrion Labs was added, and Comparative Example 2 was an Aylia product of Regeneron Co., Ltd., in the same pharmaceutical formulation as Comparative Example 1, and afliber manufactured by another company. Samples with sept added. The target capacity for filling a glass vial for each sample was 0.278 ml.

The pharmaceutical formulations prepared according to Examples 1-9 and Comparative Examples 1-2 were stored at a temperature of 5±3° C., a temperature of 40±2° C., and a relative humidity of 75±5%, and at a temperature of 5±3° C. for 0 weeks. Then, stability after 2 weeks and 4 weeks, and stability after 2 weeks and 4 weeks at 40±2°C temperature and 75±5% relative humidity were measured. The results are shown in Table 2-15.

Configuration of Experimental Example 1

division	buffer	pH	sugar	isotonic agent	Surfactants	protein concentration
Example 1	Histidine 8.0 mM	5.5	Trehalose 10% (w/v)	sodium chloride 13.0 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL
Example 2	Histidine 8.0 mM	6.0	Trehalose 10% (w/v)	sodium chloride 13.0 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL
Example 3	Histidine 8.0 mM	6.5	Trehalose 10% (w/v)	sodium chloride 13.0 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL
Example 4	Histidine 6.4 mM	6.0	Trehalose 10% (w/v)	sodium chloride 13.0 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL
Example 5	Histidine 9.6 mM	6.0	Trehalose 10% (w/v)	sodium chloride 13.0 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL
Example 6	Histidine 8.0 mM	6.0	Trehalose 10% (w/v)	sodium chloride 10.4 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL
Example 7	Histidine 8.0 mM	6.0	Trehalose 10% (w/v)	sodium chloride 15.6 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL
Example 8	Histidine 8.0 mM	6.0	Trehalose 8 % (w/v)	sodium chloride 13.0 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL
Example 9	Histidine 8.0 mM	6.0	Trehalose 12% (w/v)	sodium chloride 13.0 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL
Comparative Example 1	Sodium Phosphate 10 mM	6.2	Sucrose 5% (w/v)	sodium chloride 40.0 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL
Comparative Example 2	Sodium Phosphate 10 mM	6.2	Sucrose 5% (w/v)	sodium chloride 40.0 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL

turbidity

division	5±3°C after 0 weeks	5±3°C 2 weeks later	5±3°C after 4 weeks	40±2°C, 75±5% 2 weeks later	40±2°C, 75±5% after 4 weeks
Example 1	0.0154	0.0163	0.0275	0.0181	0.0143
Example 2	0.0230	0.0196	0.0252	0.0188	0.0258
Example 3	0.0214	0.0214	0.0207	0.0212	0.0203
Example 4	0.0200	0.0192	0.0164	0.0283	0.0350
Example 5	0.0156	0.0172	0.0206	0.0365	0.0245
Example 6	0.0150	0.0163	0.0240	0.0153	0.0190
Example 7	0.0124	0.0165	0.0162	0.0229	0.0191
Example 8	0.0157	0.0259	0.0176	0.0194	0.0210
Example 9	0.0126	0.0212	0.0394	0.0153	0.0379
Comparative Example 1	0.0203	0.0197	0.0207	0.0240	0.0191
Comparative Example 2	0.0105	0.0178	0.0170	0.0197	0.0181

Looking at Table 2, it can be seen that the turbidity of all Examples 1-9, which sets the range of the formulation of the present invention even after 4 weeks at 5±3° C. temperature condition, is very low, so that it is a transparent pharmaceutical formulation. In particular, it can be seen that the absorbance is 0.0400 or less even after 4 weeks at a temperature of 40±2°C and a relative humidity of 75±5%. It can be seen that the results are similar to those of the existing formulation (Comparative Example 1-2).

Intact active ingredient content (main peak %)

division	5±3°C after 0 weeks	5±3°C 2 weeks later	5±3°C after 4 weeks	40±2°C, 75±5% 2 weeks later	40±2°C, 75±5% after 4 weeks
Example 1	99.33	99.32	99.18	94.52	89.41

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Example 2	99.04	98.96	98.84	94.85	91.13
Example 3	98.89	98.76	98.61	94.96	91.47
Example 4	98.98	98.89	98.88	95.11	91.31
Example 5	99.08	98.94	98.95	95.11	91.27
Example 6	99.14	99.04	98.86	95.17	91.89
Example 7	98.91	98.92	98.95	95.20	91.27
Example 8	99.06	99.03	98.88	94.49	89.88
Example 9	99.01	99.04	98.94	95.63	92.22
Comparative Example 1	98.90	98.61	98.79	92.00	86.07
Comparative Example 2	98.09	97.95	97.98	92.97	89.34

Referring to Table 3, it can be seen that the main component content of all Examples 1-9 that sets the range of the formulation of the present invention after 4 weeks at 5±3° C. temperature condition is a pharmaceutical formulation similar to the results of Comparative Example 1-2. On the other hand, after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the content of the main component of Examples 1-9 is higher than that of Comparative Examples 1-2, so the formulation of the present invention has the target recombinant fusion protein more It can be seen that it is an improved pharmaceutical formulation that can be stored stably.

High molecular weight component content (%)

division	5±3℃ after 0 weeks	5±3℃ 2 weeks later	5±3℃ after 4 weeks	40±2℃, 75±5% 2 weeks later	40±2℃, 75±5% after 4 weeks
Example 1	0.66	0.68	0.82	4.70	9.44
Example 2	0.96	1.03	1.15	4.69	8.24
Example 3	1.10	1.23	1.35	4.97	8.43
Example 4	1.02	1.08	1.10	4.47	8.10
Example 5	0.90	1.06	1.01	4.41	8.12
Example 6	0.86	0.94	1.13	4.35	7.56
Example 7	1.07	1.06	1.04	4.36	8.12
Example 8	0.88	0.96	1.10	5.07	9.47
Example 9	0.98	0.94	0.93	3.94	7.15
Comparative Example 1	1.10	1.38	1.19	7.45	13.29
Comparative Example 2	1.89	2.01	1.99	6.94	10.53

Looking at Table 4, it can be seen that after 4 weeks at 5±3° C. temperature condition, the high molecular weight component content of all Examples 1-9 setting the range of the formulation of the present invention is a pharmaceutical formulation similar to the result of Comparative Example 1-2. have. On the other hand, after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the content of the high molecular weight component of Examples 1-9 is lower than that of Comparative Examples 1-2, so the formulation of the present invention is the target recombinant fusion protein It can be seen that this is an improved pharmaceutical formulation that can be stored more stably.

Low molecular weight component content (%)

division	5±3℃ after 0 weeks	5±3℃ 2 weeks later	5±3℃ after 4 weeks	40±2℃, 75±5% 2 weeks later	40±2℃, 75±5% after 4 weeks
Example 1	0.02	0.00	0.01	0.77	1.15
Example 2	0.00	0.01	0.01	0.47	0.64
Example 3	0.01	0.01	0.04	0.08	0.10
Example 4	0.00	0.03	0.02	0.42	0.59
Example 5	0.02	0.00	0.03	0.48	0.62
Example 6	0.00	0.01	0.00	0.48	0.55
Example 7	0.01	0.02	0.01	0.43	0.61
Example 8	0.05	0.02	0.02	0.44	0.65
Example 9	0.01	0.01	0.13	0.43	0.63
Comparative Example 1	0.00	0.01	0.02	0.54	0.64
Comparative Example 2	0.02	0.04	0.03	0.09	0.13

Referring to Table 5, it can be seen that after 4 weeks at 5±3° C. temperature condition, the low molecular weight component content of all Examples 1-9 setting the range of the formulation of the present invention is a pharmaceutical formulation similar to the results of Comparative Example 1-2. have. In addition, it can be seen that after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the low molecular weight component content of Examples 2-9 is a pharmaceutical formulation having a level similar to that of Comparative Example 1. On the other hand, through Example 1, it was confirmed that the pH 5.5 condition was somewhat disadvantageous compared to Comparative Example 1-2 in terms of the content of low molecular weight components, so it was confirmed that it was necessary to adjust the target pH and pH range by a little. Experimental results of adjusting the pH range are included in Experimental Example 2.

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Content (%) of one recombinant human VEGF receptor extracellular region 1, one recombined human VEGF receptor extracellular region 2 and a portion of recombinant human IgG Fc (1VEGF1 + 1VEGF2 + Fc)

division	5±3℃ after 0 weeks	5±3℃ 2 weeks later	5±3℃ after 4 weeks	40±2℃, 75±5% 2 weeks later	40±2℃, 75±5% after 4 weeks
Example 1	95.43	96.97	98.30	95.18	90.60
Example 2	97.33	97.65	98.30	95.23	93.80
Example 3	97.57	98.04	98.30	97.32	96.20
Example 4	97.53	97.45	98.30	95.98	94.00
Example 5	97.45	97.83	98.30	95.69	93.80
Example 6	97.41	97.27	98.30	95.67	94.10
Example 7	96.65	98.35	97.70	95.74	93.80
Example 8	97.58	97.43	98.20	96.42	93.80
Example 9	97.52	96.33	97.90	95.71	93.90
Comparative Example 1	97.57	97.27	98.40	96.35	93.60
Comparative Example 2	97.89	97.15	98.30	96.74	96.40

Referring to Table 6, after 4 weeks at 5±3° C. temperature condition, one recombined human VEGF receptor extracellular region 1, one recombined human VEGF receptor of all Examples 1-9 to set the scope of the formulation of the present invention it can be seen that the content of the extracellular region 2 and the recombinant human IgG Fc part (1VEGF1 + 1VEGF2 + Fc) is a pharmaceutical preparation similar to the results of Comparative Example 1-2. In addition, after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, one recombined human VEGF receptor extracellular region 1 and one recombined human VEGF receptor extracellular region 2 of Examples 2-9 And it can be seen that the content of the recombinant human IgG Fc part (1VEGF1 + 1VEGF2 + Fc) is a pharmaceutical preparation at a level similar to that of Comparative Example 1-2. However, through Example 1, the pH 5.5 condition is one recombinant human VEGF receptor extracellular region 1, one recombined human VEGF receptor extracellular region 2, and a portion of the recombinant human IgG Fc (1VEGF1 + 1VEGF2 + Fc) Since it is somewhat disadvantageous compared to Comparative Example 1-2 in terms of content, it was confirmed that it was necessary to slightly increase the target pH and pH range. The results are included in Experimental Example 2.

Number of insoluble foreign matter particles measured by HIAC (2.00㎛≤, <400.00㎛, particles/mL)

division	5±3℃ after 0 weeks	5±3℃ 2 weeks later	5±3℃ after 4 weeks	40±2℃, 75±5% 2 weeks later	40±2℃, 75±5% after 4 weeks
Example 1	18	170	115	112	77
Example 2	68	150	53	62	70
Example 3	30	20	13	15	78
Example 4	15	25	12	43	20
Example 5	105	28	12	60	87
Example 6	53	42	7	38	52
Example 7	33	28	7	53	55
Example 85	8	40	7	28	68
Example 9	33	68	13	142	73
Comparative Example 1	53	72	12	57	48
Comparative Example 2	-	-	-	-	-

Referring to Table 7, the number of insoluble foreign particles (2.00㎛≤, <400.00㎛) measured by HIAC of all Examples 1-9 that sets the range of the formulation of the present invention after 4 weeks at 5±3°C temperature condition Similar to the results of Comparative Example 1, it can be seen that it is a very clean pharmaceutical preparation. In addition, even after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the number of insoluble foreign particles measured by HIAC of Examples 1-9 (2.00 μm≤, <400.00 μm) was similar to that of Comparative Example 1 it can be seen that the pharmaceutical preparation is at a similar level.

Number of insoluble foreign matter particles measured by HIAC (10.00㎛≤, <400.00㎛, particles/mL)

division	5±3℃ after 0 weeks	5±3℃ 2 weeks later	5±3℃ after 4 weeks	40±2℃, 75±5% 2 weeks later	40±2℃, 75±5% after 4 weeks
Example 1	5	10	3	23	8
Example 2	5	23	13	7	2
Example 3	7	2	0	3	15
Example 4	2	2	0	17	3
Example 5	7	7	0	10	10
Example 6	10	7	0	10	20
Example 7	25	2	0	13	3
Example 8	0	8	0	5	3
Example 9	5	7	0	13	2
Comparative Example 1	3	12	0	8	3

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Comparative Example 2	-	-	-	-	-

Referring to Table 8, after 4 weeks at 5±3° C. temperature condition, the number of insoluble foreign particles (10.00 μm≤, <400.00 μm) measured by HIAC of all Examples 1-9 that set the range of the formulation of the present invention Similar to the results of Comparative Example 1, it can be seen that it is a very clean pharmaceutical preparation. In addition, even after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the number of insoluble foreign particles measured by HIAC of Examples 1-9 (10.00 μm≤, <400.00 μm) was similar to that of Comparative Example 1 It can be seen that the pharmaceutical preparation is at a similar level.

Number of insoluble foreign matter particles measured by HIAC particles/mL (25.00㎛≤, <400.00㎛, particles/mL)

division	5±3℃ after 0 weeks	5±3℃ 2 weeks later	5±3℃ after 4 weeks	40±2℃, 75±5% 2 weeks later	40±2℃, 75±5% after 4 weeks
Example 1	2	0	0	2	0
Example 2	0	5	0	0	0
Example 3	0	0	0	2	0
Example 4	0	0	0	2	0
Example 5	0	3	0	0	0
Example 6	3	0	0	0	2
Example 7	5	0	0	2	0
Example 8	0	2	0	0	0
Example 9	0	3	0	0	0
Comparative Example 1	0	0	0	0	0
Comparative Example 2	-	-	-	-	-

Looking at Table 9, the number of insoluble foreign particles (25.00㎛≤, <400.00㎛) measured by HIAC of all Examples 1-9 that sets the range of the formulation of the present invention after 4 weeks at 5±3°C temperature condition Similar to the results of Comparative Example 1, it can be seen that it is a very clean pharmaceutical preparation. In addition, even after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the number of insoluble foreign particles measured by HIAC of Examples 1-9 (25.00 μm≤, <400.00 μm) was similar to that of Comparative Example 1 It can be seen that the pharmaceutical preparation is at a similar level.

Number of insoluble foreign matter particles measured by MFI (1.00㎛≤, <100.00㎛, particles/mL)

division	5±3℃ after 0 weeks	5±3℃ 2 weeks later	5±3℃ after 4 weeks	40±2℃, 75±5% 2 weeks later	40±2℃, 75±5% after 4 weeks
Example 1	-	2596	-	2885	6418
Example 2	8406	1116	1906	1208	6484
Example 3	5255	7519	2436	1678	3768
Example 4	14701	-	3744	514	8556
Example 5	16433	1367	3819	1438	3190
Example 6	5135	990	4996	1979	2812
Example 7	6262	566	3459	1666	18324
Example 8	7976	5378	4145	1559	4109
Example 9	8554	672	4055	1609	11022
Comparative Example 1	6996	371	3197	496	3999
Comparative Example 2	-	-	-	-	-

Referring to Table 10, the number of insoluble foreign particles (1.00㎛≤, <100.00㎛) measured by MFI of all Examples 1-9 that sets the range of the formulation of the present invention after 4 weeks at 5±3°C temperature condition Similar to the results of Comparative Example 1, it can be seen that it is a very clean pharmaceutical preparation. In addition, even after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the number of insoluble foreign particles measured by MFI of Examples 1-9 (1.00 μm≤, <100.00 μm) was similar to that of Comparative Example 1 It can be seen that the pharmaceutical preparation is at a similar level.

Number of insoluble foreign matter particles measured by MFI (10.00 μm≤, <100.00 μm, particles/mL)

division	5±3℃ after 0 weeks	5±3℃ 2 weeks later	5±3℃ after 4 weeks	40±2℃, 75±5% 2 weeks later	40±2℃, 75±5% after 4 weeks
Example 1	-	126	-	31	35
Example 2	22	5	22	10	51
Example 3	51	27	14	20	33
Example 4	13	-	26	8	48
Example 5	17	9	19	15	18
Example 6	19	26	40	31	51
Example 7	19	7	18	22	40
Example 8	26	17	34	20	21

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Example 9	22	18	30	96	68
Comparative Example 1	9	13	31	4	19
Comparative Example 2	-	-	-	-	-

Referring to Table 11, the number of insoluble foreign particles (10.00 μm≤, <100.00 μm) measured by MFI of all Examples 1-9 that set the range of the formulation of the present invention after 4 weeks at 5±3° C. temperature condition Similar to the results of Comparative Example 1, it can be seen that it is a very clean pharmaceutical preparation. In addition, even after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the number of insoluble foreign particles measured by the MFI of Examples 1-9 (10.00 μm≤, <100.00 μm) was similar to that of Comparative Example 1 It can be seen that the pharmaceutical preparation is at a similar level.

Number of insoluble foreign matter particles measured by MFI (25.00㎛≤, <100.00㎛, particles/mL)

division	5±3℃ after 0 weeks	5±3℃ 2 weeks later	5±3℃ after 4 weeks	40±2℃, 75±5% 2 weeks later	40±2℃, 75±5% after 4 weeks
Example 1	-	5	-	11	0
Example 2	4	0	0	0	9
Example 3	26	3	4	3	9
Example 4	0	-	11	4	8
Example 5	3	0	2	2	2
Example 6	3	6	2	4	15
Example 7	0	0	4	7	7
Example 8	5	2	4	0	0
Example 9	7	4	6	7	6
Comparative Example 1	0	3	2	0	0
Comparative Example 2	-	-	-	-	-

Referring to Table 12, the number of insoluble foreign particles (25.00㎛≤, <100.00㎛) measured by MFI of all Examples 1-9 that sets the range of the formulation of the present invention after 4 weeks at 5±3°C temperature condition Similar to the results of Comparative Example 1, it can be seen that it is a very clean pharmaceutical preparation. In addition, even after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the number of insoluble foreign particles measured by MFI of Examples 1-9 (25.00 μm≤, <100.00 μm) was similar to that of Comparative Example 1 It can be seen that the pharmaceutical preparation is at a similar level.

Oxidation rate (Met192, %)

division	5±3℃ after 0 weeks	5±3℃ 2 weeks later	5±3℃ after 4 weeks	40±2℃, 75±5% 2 weeks later	40±2℃, 75±5% after 4 weeks
Example 1	4.9	3.5	5.1	4.0	6.1
Example 2	5.6	4.0	4.9	4.0	4.9
Example 3	3.8	4.6	4.1	4.1	4.9
Example 4	4.7	4.3	6.5	4.4	4.5
Example 5	4.4	3.8	4.4	4.6	4.6
Example 6	4.9	3.7	3.8	5.5	5.1
Example 7	5.2	4.7	3.7	6.6	4.7
Example 8	4.2	5.5	5.3	4.2	4.6
Example 9	4.1	4.7	5.3	4.5	3.9
Comparative Example 1	4.2	3.8	4.3	4.3	5.8
Comparative Example 2	4.6	3.2	3.7	4.0	4.4

Looking at Table 13, it can be seen that the oxidation rate (Met192) of all Examples 1-9, which sets the range of the formulation of the present invention, after 4 weeks at 5±3° C. temperature condition, is a pharmaceutical formulation similar to the result of Comparative Example 1-2. have. In addition, it can be seen that even after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the oxidation rate (Met192) of Examples 1-9 is a pharmaceutical preparation having a level similar to that of Comparative Example 1-2.

Charge variant (relative content of peak 4 to peak 11 out of 12 peaks in total, %)

division	5±3℃ after 0 weeks	5±3℃ 2 weeks later	5±3℃ after 4 weeks	40±2℃, 75±5% 2 weeks later	40±2℃, 75±5% after 4 weeks
Example 1	87.3	86.4	87.1	86.3	88.3
Example 2	90.7	87.0	87.0	86.4	86.4
Example 3	87.4	87.1	87.3	86.0	85.3
Example 4	89.6	86.9	87.2	86.6	87.1
Example 5	87.9	86.8	86.7	86.6	86.8
Example 6	87.7	86.7	86.9	86.5	85.4
Example 7	88.8	86.8	87.2	86.7	86.6

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Example 8	88.6	86.9	87.0	86.9	87.2
Example 9	88.7	86.7	87.3	86.8	86.5
Comparative Example 1	88.9	87.0	86.6	86.7	86.2
Comparative Example 2	87.5	91.0	91.3	91.3	89.9

Referring to Table 14, after 4 weeks at 5±3° C. temperature condition, the charge variants (relative content of peak 4 to peak 11 among all 12 peaks) of all Examples 1-9 that set the range of the formulation of the present invention are compared. It can be seen that the pharmaceutical formulation is similar to the results of Example 1-2. In addition, even after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the charge variants of Examples 1-9 (relative content of peak 4 to peak 11 out of 12 peaks in total) of Comparative Example 1-2 It can be seen that the pharmaceutical preparation is at a level similar to that of

VEGF binding affinity (%)

division	5±3°C after 0 weeks	5±3°C 2 weeks later	5±3°C after 4 weeks	40±2°C, 75±5% 2 weeks later	40±2°C, 75±5% after 4 weeks
Example 1	82	-	86	82	59
Example 2	89	-	78	70	73
Example 3	91	-	79	70	74
Example 4	98	-	72	70	69
Example 5	90	-	82	75	75
Example 6	95	-	81	84	74
Example 7	89	-	83	76	63
Example 8	99	-	73	68	59
Example 9	96	-	81	82	60
Comparative Example 1	99	-	89	78	78
Comparative Example 2	97	-	92	79	78

Looking at Table 15, it can be seen that after 4 weeks at 5±3° C. temperature condition, the VEGF binding affinity of all Examples 1-9 setting the range of the formulation of the present invention is a pharmaceutical formulation similar to the results of Comparative Example 1-2. have. In addition, it can be seen that even after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the VEGF binding affinity of Examples 1-9 is a pharmaceutical formulation having a level similar to that of Comparative Example 1-2.

Experimental Example 2: Adjustment of target pH and pH range for improvement of low molecular weight content

With respect to the pharmaceutical formulation used in Experimental Example 2, each buffer was prepared to suit each pH, trehalose and sodium chloride were added thereto, a recombinant fusion protein was added thereto, and a surfactant was added to prepare the samples in Table 16 below. did The specific content of each component is as described in Table 16. In the following examples, aflibercept purified by Celltrion Labs was added, and Comparative Example 1 is a sample to which aflibercept manufactured by another company is added, which is a product of Regeneron's Ayila. The difference between Example 2 and Example 10 is the protein concentration, and the difference between Examples 10-14 is the pH. The target glass vial fill capacity for each sample was 0.278 ml.

The pharmaceutical formulations prepared according to Examples 2, 10 to 14 and Comparative Example 1 were stored at a temperature of 5±3° C. and 50±2° C., and stability after 0 days and 10 days at a temperature of 5±3° C. and 50 Stability after 5 days and 10 days at a temperature of ±2° C. was measured. The results are shown in Tables 17-30.

Configuration of Experimental Example 2

division	buffer	pH	sugar	isotonic agent	Surfactants	protein concentration
Example 2	Histidine 8.0 mM	6.0	Trehalose 10% (w/v)	sodium chloride 13.0 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL
Example 10	Histidine 8.0 mM	6.0	Trehalose 10% (w/v)	sodium chloride 13.0 mM	Polysorbate 20 0.03 % (w/v)	30 mg/mL
Example 11	Histidine 8.0 mM	5.5	Trehalose 10% (w/v)	sodium chloride 13.0 mM	Polysorbate 20 0.03 % (w/v)	30 mg/mL
Example 12	Histidine 8.0 mM	5.9	Trehalose 10% (w/v)	sodium chloride 13.0 mM	Polysorbate 20 0.03 % (w/v)	30 mg/mL
Example 13	Histidine 8.0 mM	6.2	Trehalose 10% (w/v)	sodium chloride 13.0 mM	Polysorbate 20 0.03 % (w/v)	30 mg/mL
Example 14	Histidine 8.0 mM	6.5	Trehalose 10% (w/v)	sodium chloride 13.0 mM	Polysorbate 20 0.03 % (w/v)	30 mg/mL
Comparative Example 1	Sodium Phosphate 10 mM	6.2	Sucrose 5% (w/v)	sodium chloride 40.0 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL

Intact main ingredient content (% of main peak)

division	5±3°C after 0 days	5±3°C after 10 days	50±2°C 5 days later	50±2°C after 10 days

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Example 2	99.13	99.05	49.23	28.78
Example 10	99.15	99.08	54.71	34.78
Example 11	99.16	99.05	53.66	34.20
Example 12	99.16	98.97	51.06	32.47
Example 13	97.26	98.98	53.02	34.74
Example 14	99.12	98.99	51.82	33.02
Comparative Example 1	98.00	97.82	36.13	21.64

Referring to Table 17, it can be seen that the main ingredient content of Examples 2 and 10 to 14, which sets the range of the formulation of the present invention after 10 days at 5±3° C. temperature condition, is a pharmaceutical formulation similar to the result of Comparative Example 1. On the other hand, after 10 days at a temperature of 50±2° C., since the main component content of Examples 2 and 10 to 14 is higher than that of Comparative Example 1, the formulation of the present invention allows the target recombinant fusion protein to be stored more stably, an improved drug It can be seen that it is a medical preparation.

High molecular weight component content (%)

division	5±3℃ after 0 days	5±3℃ after 10 days	50±2℃ 5 days later	50±2℃ after 10 days
Example 2	0.84	0.92	44.81	64.84
Example 10	0.75	0.90	39.99	59.85
Example 11	0.81	0.90	39.27	57.98
Example 12	0.83	0.99	43.80	62.17
Example 13	2.70	0.98	42.91	61.49
Example 14	0.86	0.99	45.23	63.92
Comparative Example 1	1.98	2.15	61.70	76.09

Referring to Table 18, it can be seen that after 10 days at a temperature of 5±3° C., the content of the high molecular weight component of Examples 2 and 10 to 14, which sets the range of the formulation of the present invention, is lower than that of Comparative Example 1. In addition, since the high molecular weight component content of Examples 2 and 10 to 14 is lower than that of Comparative Example 1 even after 10 days at 50±2° C. temperature condition, the formulation of the present invention allows the target recombinant fusion protein to be stored more stably, an improved drug It can be seen that it is a medical preparation.

Low molecular weight component content (%)

division	5±3℃ after 0 days	5±3℃ after 10 days	50±2℃ 5 days later	50±2℃ after 10 days
Example 2	0.04	0.03	5.96	6.38
Example 10	0.10	0.02	5.29	5.38
Example 11	0.03	0.05	7.06	7.82
Example 12	0.01	0.04	5.14	5.36
Example 13	0.03	0.04	4.07	3.77
Example 14	0.02	0.02	2.95	3.06
Comparative Example 1	0.02	0.02	2.17	2.28

Looking at Table 19, it can be seen that after 10 days at 5 ± 3 ° C., the low molecular weight component content of Examples 2 and 10 to 14, which sets the range of the formulation of the present invention, is a pharmaceutical formulation similar to the result of Comparative Example 1. . On the other hand, it can be seen that after 10 days at a temperature of 50±2° C., the content of low molecular weight components of Examples 2 and 10 to 14 is somewhat higher than that of Comparative Example 1. Looking at the low molecular weight component content of Examples 11 to 14, it can be seen that the lower the pH, the higher the low molecular weight content. It can be seen that the low molecular weight content is rapidly increased by about 4% or more. Therefore, it can be confirmed that the pH range of the formulation of the present invention is more excellent to set to 5.9 to 6.5.

Content (%) of one recombinant human VEGF receptor extracellular region 1, one recombined human VEGF receptor extracellular region 2, and a portion of recombinant human IgG Fc (1VEGF1 + 1VEGF2 + Fc)

division	5±3℃ after 0 days	5±3℃ after 10 days	50±2℃ 5 days later	50±2℃ after 10 days
Example 2	96.58	96.32	88.31	82.11
Example 10	96.66	96.62	88.58	84.32
Example 11	96.01	96.14	86.38	81.27
Example 12	96.17	96.18	89.43	85.43
Example 13	96.52	96.35	92.50	91.52
Example 14	96.48	96.34	95.90	92.85
Comparative Example 1	97.37	96.11	95.77	94.11

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Referring to Table 20, after 10 days at 5±3° C. temperature conditions, Examples 2, 10 to 14, which set the scope of the formulation of the present invention, one recombined human VEGF receptor extracellular region 1, one recombined human VEGF It can be seen that the content of the receptor extracellular region 2 and the recombinant human IgG Fc part (1VEGF1 + 1VEGF2 + Fc) is a pharmaceutical preparation similar to the results of Comparative Example 1. On the other hand, after 10 days at 50±2° C. temperature conditions, one recombined human VEGF receptor extracellular region 1, one recombined human VEGF receptor extracellular region 2 and recombined human IgG of Examples 2, 10 to 14 It can be seen that the content of some of the Fc (1VEGF1 + 1VEGF2 + Fc) is somewhat lower than that of Comparative Example 1. Looking at the content of one recombinant human VEGF receptor extracellular region 1, one recombined human VEGF receptor extracellular region 2 and recombinant human IgG Fc part (1VEGF1 + 1VEGF2 + Fc) of Examples 11 to 14, the pH was As it is lower, it can be seen that the content of one recombinant human VEGF receptor extracellular region 1, one recombined human VEGF receptor extracellular region 2, and a portion of the recombinant human IgG Fc (1VEGF1 + 1VEGF2 + Fc) is low, Referring to Example 11, it can be seen that the low molecular weight content is rapidly increased by about 10% or more in the pH 5.5 formulation beyond the level of variation in the assay method compared to Example 13, which is the target pH pharmaceutical formulation. Therefore, it was confirmed that the pH range of the formulation of the present invention is better to set to 5.9 to 6.5.

Number of insoluble foreign matter particles measured by MFI (1.00㎛≤, <100.00㎛, particles/mL)

division	5±3°C after 0 days	5±3°C after 10 days	50±2°C 5 days later	50±2°C after 10 days
Example 2	222	530	324	1201
Example 10	546	84	234	430
Example 11	198	245	1112	704
Example 12	146	234	153	290
Example 13	198	143	125	14709
Example 14	157	205	231	430
Comparative Example 1	-	-	-	-

Referring to Table 21, after 10 days at 5±3° C. temperature conditions, the number of insoluble foreign particles measured by MFI of Examples 2 and 10 to 14, which sets the range of the formulation of the present invention (1.00 μm≤, <100.00 μm) It can be seen that it is an absolutely low, very clean pharmaceutical preparation. In addition, the number of insoluble foreign particles (1.00㎛≤, <100.00㎛) measured by the MFI of Examples 2 and 10 to 14 even after 10 days at 50±2°C temperature condition is absolutely low, it is a very clean pharmaceutical preparation Able to know.

Number of insoluble foreign matter particles measured by MFI (10.00 μm≤, <100.00 μm, particles/mL)

division	5±3°C after 0 days	5±3°C after 10 days	50±2°C 5 days later	50±2°C after 10 days
Example 2	0	26	2	4
Example 10	4	4	9	7
Example 11	0	8	10	29
Example 12	6	6	4	2
Example 13	0	6	5	97
Example 14	11	37	4	14
Comparative Example 1	-	-	-	-

Referring to Table 22, after 10 days at 5±3° C., the number of insoluble foreign particles measured by MFI of Examples 2 and 10 to 14, which sets the range of the formulation of the present invention (10.00 μm≤, <100.00 μm) It can be seen that it is an absolutely low, very clean pharmaceutical preparation. In addition, even after 10 days at 50±2° C. temperature and conditions, the number of insoluble foreign particles measured by MFI of Examples 2 and 10 to 14 (10.00 μm≤, <100.00 μm) is absolutely low, very clean pharmaceutical formulation it can be seen that

Number of insoluble foreign matter particles measured by MFI (25.00㎛≤, <100.00㎛, particles/mL)

division	5±3°C after 0 days	5±3°C after 10 days	50±2°C 5 days later	50±2°C after 10 days
Example 2	0	2	0	0
Example 10	0	0	0	6
Example 11	0	4	2	8
Example 12	4	0	0	0
Example 13	0	0	0	10
Example 14	3	12	2	0
Comparative Example 1	-	-	-	-

Referring to Table 23, after 10 days at 5±3° C. temperature condition, the number of insoluble foreign particles measured by MFI of Examples 2 and 10 to 14, which sets the range of the formulation of the present invention (25.00 μm≤, <100.00 μm) It can be seen that it is an absolutely low, very clean pharmaceutical preparation. In addition, even after 10 days at 50±2° C. temperature and conditions, the number of insoluble foreign particles (25.00 μm≤, <100.00 μm) measured by the MFI of Examples 2 and 10 to 14 is absolutely low, very clean pharmaceutical formulation it can be seen that

VEGF binding affinity (%)

division	5±3°C after 0 days	5±3°C after 10 days	50±2°C 5 days later	50±2°C after 10 days

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Example 2	94	106	45	31
Example 10	104	110	56	34
Example 11	103	98	38	20
Example 12	102	91	67	39
Example 13	99	117	66	58
Example 14	113	83	78	59
Comparative Example 1	110	86	73	55

Looking at Table 24, it can be seen that after 10 days at 5±3° C. temperature conditions, the VEGF binding affinity of Examples 2 and 10 to 14, which sets the range of the formulation of the present invention, is a pharmaceutical formulation similar to the results of Comparative Example 1. . On the other hand, it can be seen that after 10 days at 50±2° C. temperature conditions, the VEGF binding affinity of some of Examples 2 and 10 to 14 is somewhat higher than that of Comparative Example 1. Looking at the VEGF binding affinity of Examples 11 to 14, it can be seen that the lower the pH, the lower the VEGF binding affinity. It can be confirmed that the VEGF binding affinity is rapidly decreased by about 38% or more. Therefore, it can be confirmed that the pH range of the formulation of the present invention is more excellent to set to 5.9 to 6.5.

Experimental Example 3: Additional comparison for reinforcing the concentration range of sodium chloride isotonic agent and confirmation of superiority of the formulation of the present invention

With respect to the pharmaceutical formulation used in Experimental Example 2, each buffer was prepared according to each sodium chloride concentration, the recombinant fusion protein was added, and a surfactant was added to prepare the samples in Table 25. The specific content of each component is as described in Table 25. Examples 15-16 and Comparative Example 1 are samples to which aflibercept purified by Celltrion Labs was added. The target glass vial fill capacity for each sample was 0.276 ml.

The pharmaceutical formulations prepared according to Examples 15-16 and Comparative Example 1 were stored at a temperature of 5±3° C., a temperature of 40±2° C., and a relative humidity of 75±5%, and stability after 4 weeks at a temperature of 5±3° C. And stability was measured after 2 weeks and 4 weeks at 40±2℃ temperature and 75±5% relative humidity. The results are shown in Tables 26-31.

Configuration of Experimental Example 3

division	buffer	pH	sugar	isotonic agent	Surfactants	protein concentration
Example 15	Histidine 8.0 mM	6.2	Trehalose 10% (w/v)	sodium chloride 0.0 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL
Example 16	Histidine 8.0 mM	6.2	Trehalose 10% (w/v)	sodium chloride 7.2 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL
Comparative Example 1	Sodium Phosphate 10 mM	6.2	Sucrose 5% (w/v)	sodium chloride 40.0 mM	Polysorbate 20 0.03 % (w/v)	40 mg/mL

turbidity

division	5±3℃ after 4 weeks	40±2℃, 75±5% 2 weeks later	40±2℃, 75±5% after 4 weeks
Example 15	0.0048	0.0065	0.0052
Example 16	0.0034	0.0064	0.0127
Comparative Example 1	0.0078	0.0095	0.0103

Referring to Table 26, it can be seen that the turbidity of Examples 15-16 under the low concentration sodium chloride condition is very low even after 4 weeks at the temperature condition of 5±3° C., so that it is a transparent pharmaceutical formulation. In particular, it can be seen that the absorbance is 0.0400 or less even after 4 weeks at a temperature of 40±2℃ and a relative humidity of 75±5%. It can be seen that the results are similar to those of the existing formulation (Comparative Example 1).

Charge variant (relative content of peak 4 to peak 11 out of 12 peaks in total, %)

division	5±3℃ after 4 weeks	40±2℃, 75±5% 2 weeks later	40±2℃, 75±5% after 4 weeks
Example 15	89.9	-	86.9
Example 16	89.6	-	87.7
Comparative Example 1	90.3	-	86.2

Referring to Table 27, after 4 weeks at 5±3° C. temperature conditions, the charge variants (relative content of peak 4 to peak 11 among all 12 peaks) of Examples 15-16 under low-concentration sodium chloride conditions were similar to those of Comparative Example 1 It can be seen that it is a pharmaceutical preparation. In addition, even after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the charge variants of Examples 15-16 (relative content of peak 4 to peak 11 among all 12 peaks) were similar to those of Comparative Example 1. It can be seen that it is a level pharmaceutical preparation.

Number of insoluble foreign matter particles measured by MFI (1.00㎛≤, <100.00㎛, particles/mL)

division	5±3℃ after 4 weeks	40±2℃, 75±5% 2 weeks later	40±2℃, 75±5% after 4 weeks
Example 15	2770	1935	2184

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Example 16	2504	985	1355
Comparative Example 1	1399	6004	9586

Referring to Table 28, the number of insoluble foreign particles (1.00㎛≤, <100.00㎛) measured by MFI of Example 15-16 of Example 15-16 under low concentration sodium chloride after 4 weeks at 5±3°C temperature condition is the result of Comparative Example 1 and it can be seen that it is a similar, very clean pharmaceutical formulation. In addition, even after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the number of insoluble foreign particles measured by MFI of Examples 15-16 (1.00 μm≤, <100.00 μm) was higher than that of Comparative Example 1. It can be seen that it is an absolutely low, very clean pharmaceutical formulation.

Number of insoluble foreign matter particles measured by MFI (10.00 μm≤, <100.00 μm, particles/mL)

division	5±3°C after 4 weeks	40±2°C, 75±5% 2 weeks later	40±2°C, 75±5% after 4 weeks
Example 15	77	28	61
Example 16	64	0	22
Comparative Example 1	24	216	731

Referring to Table 29, after 4 weeks at 5±3° C. temperature condition, the number of insoluble foreign particles (10.00 μm≤, <100.00 μm) measured by MFI of Example 15-16 under low-concentration sodium chloride condition was the result of Comparative Example 1 and it can be seen that it is a similar, very clean pharmaceutical formulation. In addition, even after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the number of insoluble foreign particles measured by MFI of Examples 15-16 (10.00 μm≤, <100.00 μm) was higher than that of Comparative Example 1. It can be seen that it is an absolutely low, very clean pharmaceutical formulation.

Number of insoluble foreign matter particles measured by MFI (25.00㎛≤, <100.00㎛, particles/mL)

division	5±3°C after 4 weeks	40±2°C, 75±5% 2 weeks later	40±2°C, 75±5% after 4 weeks
Example 15	9	7	8
Example 16	6	0	0
Comparative Example 1	2	4	7

Referring to Table 30, the number of insoluble foreign particles (25.00㎛≤, <100.00㎛) measured by MFI of Example 15-16 of Example 15-16 under low concentration sodium chloride after 4 weeks at 5±3°C temperature condition is the result of Comparative Example 1 and it can be seen that it is a similar, very clean pharmaceutical formulation. In addition, even after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the number of insoluble foreign particles measured by MFI of Examples 15-16 (25.00 μm≤, <100.00 μm) was the result of Comparative Example 1 It can be seen that, similar to that, it is a very clean pharmaceutical preparation.

VEGF binding affinity (%)

division	5±3°C after 4 weeks	40±2°C, 75±5% 2 weeks later	40±2°C, 75±5% after 4 weeks
Example 15	105	95	87
Example 16	101	96	68
Comparative Example 1	99	85	72

Referring to Table 31, it can be seen that after 4 weeks at 5±3° C. temperature conditions, the VEGF binding affinity of Examples 15-16 under low sodium chloride conditions is a pharmaceutical formulation similar to the results of Comparative Example 1. In addition, it can be seen that even after 4 weeks at a temperature of 40±2° C. and a relative humidity of 75±5%, the VEGF binding affinity of Examples 15-16 is a pharmaceutical formulation similar to that of Comparative Example 1.

Experimental Example 4: Confirmation of superiority of the formulation of the present invention according to the long-term stability evaluation of Example 13

Guideline Q5C Quality of Biotechnological Products: Stability Testing of Biotechnological or Biological Products and ICH Guideline Q1A (R2): Stability Testing of New Drug Substances and Drug Products).

0.278 mL of the stable liquid pharmaceutical formulation of Example 13 prepared by the method of Experimental Example 1 was stored in an airtight container at 5±3° C./ambient relative humidity. Stability was confirmed after 0 months, 3 months, 6 months and 9 months at the above temperature and humidity. However, the protein concentration condition was 40 mg/mL.

In order to confirm the long-term stability of Example 13, appearance analysis, protein concentration measurement using SoloVPE, main component, high molecular weight, low molecular weight content measurement using SEC-HPLC, one recombinant human VEGF receptor cell using reduced CE-SDS Measurement of the content of extra-region 1, one recombined human VEGF receptor extracellular region 2 and a portion of recombinant human IgG Fc (1VEGF + 1VEGF2 + Fc), charge variants using cIEF (peak 4 to peak 11 in total 12 peaks) The relative content) was measured, the VEGF binding affinity, and the number of insoluble foreign particles (sub-visible particles) were measured, and the results are shown in Tables 32 to 41.

Exterior

division	5±3°C 0 months	5±3°C after 3 months	5±3°C 6 months later	5±3°C after 9 months
Example 13	milk white	milk white	milk white	milk white

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Referring to Table 32, it can be seen that Example 13 is a stable pharmaceutical formulation without change in appearance even after 9 months under long-term storage temperature conditions.

Protein concentration (mg/ml)

division	5±3℃ 0 months	5±3℃ after 3 months	5±3℃ 6 months later	5±3℃ after 9 months
Example 13	39.1	39.9	39.7	42.4

Referring to Table 33, it can be seen that Example 13 is a stable pharmaceutical formulation without a change in protein concentration even after 9 months under long-term storage temperature conditions.

High molecular weight component content (%)

division	5±3℃ 0 months	5±3℃ after 3 months	5±3℃ 6 months later	5±3℃ after 9 months
Example 13	0.42	0.72	0.48	0.63

Referring to Table 34, it can be seen that Example 13 is a stable pharmaceutical formulation with no change in the content of high molecular weight components even after 9 months under long-term storage temperature conditions.

Low molecular weight component content (%)

division	5±3℃ 0 months	5±3℃ after 3 months	5±3℃ 6 months later	5±3℃ after 9 months
Example 13	0.24	0.00	0.00	0.00

Referring to Table 35, it can be seen that Example 13 is a stable pharmaceutical formulation with no change in the content of low molecular weight components even after 9 months under long-term storage temperature conditions.

Content (%) of one recombinant human VEGF receptor extracellular region 1, one recombined human VEGF receptor extracellular region 2, and a portion of recombinant human IgG Fc (1VEGF1 + 1VEGF2 + Fc)

division	5±3℃ 0 months	5±3℃ after 3 months	5±3℃ 6 months later	5±3℃ after 9 months
Example 13	97.42	97.48	97.40	97.37

Referring to Table 36, Example 13 shows one recombinant human VEGF receptor extracellular region 1, one recombinant human VEGF receptor extracellular region 2, and a part of recombinant human IgG Fc (1VEGF1 even after 9 months at long-term storage temperature conditions) It can be seen that the content of + 1VEGF2 + Fc) is a stable pharmaceutical formulation without change.

Number of insoluble foreign matter particles measured by HIAC (2.00㎛≤, <400.00㎛, particles/mL)

division	5±3℃ 0 months	5±3℃ after 3 months	5±3℃ 6 months later	5±3℃ after 9 months
Example 13	-	185	172	297

Referring to Table 37, it can be seen that Example 13 is a very clean pharmaceutical formulation with a low number of insoluble foreign particles (2.00 μm≤, <400.00 μm) even after 9 months under long-term storage temperature conditions.

Number of insoluble foreign particles as measured by HIAC (10.00 μm≤, <400.00 μm, particles/mL)

division	5±3℃ 0 months	5±3℃ after 3 months	5±3℃ 6 months later	5±3℃ after 9 months
Example 13	8	3	8	33

Referring to Table 38, it can be seen that Example 13 is a very clean pharmaceutical formulation with a low number of insoluble foreign particles (10.00 μm≤, <400.00 μm) even after 9 months under long-term storage temperature conditions.

Number of insoluble foreign matter particles measured by HIAC (25.00㎛≤, <400.00㎛, particles/mL)

division	5±3℃ 0 months	5±3℃ after 3 months	5±3℃ 6 months later	5±3℃ after 9 months
Example 13	0	0	0	5

Referring to Table 39, it can be seen that Example 13 is a very clean pharmaceutical preparation with a low number of insoluble foreign particles (25.00 μm≤, <400.00 μm) even after 9 months under long-term storage temperature conditions.

Charge variants (relative content of peak 4 to peak 11 out of 12 peaks in total, %)

division	5±3℃ 0 months	5±3℃ after 3 months	5±3℃ 6 months later	5±3℃ after 9 months
Example 13	87.9	87.6	88.0	87.5

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Referring to Table 40, it can be seen that Example 13 is a stable pharmaceutical formulation with no change in the content of charge variants (relative content of peak 4 to peak 11 among all 12 peaks) even after 9 months under long-term storage temperature conditions.

VEGF binding affinity (%)

division	5±3℃ 0 months	5±3℃ after 3 months	5±3℃ 6 months later	5±3℃ after 9 months
Example 13	112	106	98	92

Referring to Table 41, it can be seen that Example 13 is a stable pharmaceutical formulation with no change in VEGF binding affinity even after 9 months under long-term storage temperature conditions.

Patent Citations (8)

Publication number	Priority date	Publication date	Assignee	Title
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US6331174B1	1994-10-27	2001-12-18	Schott Glaswerke	Prefilled, low particle, sterile disposable syringe for injecting preparations and procedure for manufacture
US6833349B2 *	1999-06-08	2004-12-21	Regeneron Pharmaceuticals, Inc.	Methods of treating inflammatory skin diseases
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KR20200029374A *	2018-09-10	2020-03-18	삼성바이오에피스 주식회사	Liquid Composition Comprising Protein
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Family To Family Citations

* Cited by examiner, † Cited by third party

Similar Documents

Publication	Publication Date	Title
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WO2017116205A1	2017-07-06	Persistent conjugate of triple activator activating glucagon, gip-1 and gip receptor
WO2020017901A1	2020-01-23	Stable liquid pharmaceutical preparation
WO2022035197A1	2022-02-17	Stable pharmaceutical formulation
WO2014079842A1	2014-05-15	Liquid formulation of protein conjugate comprising the oxyntomodulin and an immunoglobulin fragment
WO2012173422A9	2013-03-14	A conjugate comprising oxyntomodulin and an immunoglobulin fragment, and use thereof
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WO2023277620A1	2023-01-05	Therapeutic use of combination comprising triple activator having activity on all of glucagon, glp-1 and gip receptor
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Priority And Related Applications

Priority Applications (4)

Application	Priority date	Filing date	Title
CN202180058213.6A	2020-07-31	2021-07-29	Stable pharmaceutical formulation
EP21851412.3A	2020-07-31	2021-07-29	Stable pharmaceutical preparation
AU2021315381A	2020-07-31	2021-07-29	Stable pharmaceutical preparation
CA3190325A	2020-07-31	2021-07-29	Stable pharmaceutical preparation

Applications Claiming Priority (2)

Application	Filing date	Title
KR10-2020-0096434	2020-07-31	
KR20200096434	2020-07-31	

Legal Events

Date	Code	Title	Description
2022-03-16	121	Ep: the epo has been informed by wipo that ep was designated in this application	**Ref document number:** 21851412 **Country of ref document:** EP **Kind code of ref document:** A1
2023-01-27	ENP	Entry into the national phase	**Ref document number:** 3190325 **Country of ref document:** CA
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			Kind code of ref document: A
2023-03-01	NENP	Non-entry into the national phase	Ref country code: DE
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2023-03-12	ENP	Entry into the national phase	Ref document number: 2021851412 Country of ref document: EP Effective date: 20230228

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(54) Title: EXTENDED, HIGH DOSE VEGF ANTAGONIST REGIMENS FOR TREATMENT OF ANGIOGENIC EYE DISORDERS

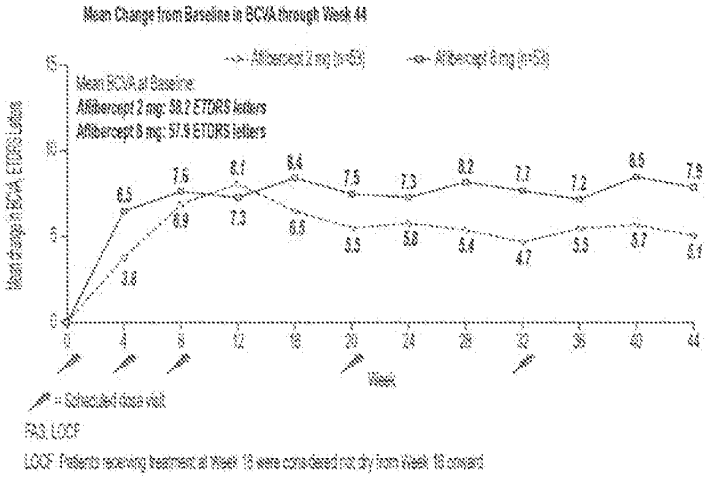


FIG. 56

(57) Abstract: The present invention relates to treatment regimens characterized by high doses of aflibercept (e.g., 8 mg) and extended intervals between doses (e.g., 12 weeks) with improved visual and anatomic outcomes relative to treatment with lower doses such as 2 mg.

[Continued on next page]

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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))*

Published:

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Extended, High Dose VEGF Antagonist Regimens for Treatment of Angiogenic Eye Disorders

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 63/189,541, filed May 17, 2021; U.S. Provisional Patent Application No. 63/235,398, filed August 20, 2021; U.S. Provisional Patent Application No. 63/297,420, filed January 7, 2022; and U.S. Provisional Patent Application No. 63/306,315, filed February 3, 2022; each of which is herein incorporated by reference for all purposes.

REFERENCE TO A SEQUENCE LISTING

[0002] The sequence listing of the present application is submitted electronically as an ASCII formatted sequence listing with a file name "10861WO01-Sequence", creation date of May 16, 2022, and a size of 6,006 bytes. This sequence listing submitted is part of the specification and is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0003] The field of the present invention relates to methods for treating or preventing angiogenic eye disorders by administering a VEGF antagonist.

BACKGROUND OF THE INVENTION

[0004] Neovascular (wet) AMD (nAMD) is a major health issue in aging populations globally. Vision loss in nAMD results from the abnormal growth and leakage of blood vessels in the macula. In elderly patients affected by nAMD, vision loss frequently has an even greater impact, as it substantially reduces the visual compensation of functional impairment by other age-related comorbidities, such as arthritis and osteoporosis.

[0005] Intravitreally (IVT) administered anti-vascular endothelial growth factor (VEGF) therapies like EYLEA® inhibit neovascular vessel growth and leakage in the retina, and they are currently the standard-of-care for patients with nAMD. They not only maintain visual function but also provide clinically meaningful visual gains. Treatment of nAMD is chronic and life-long in most patients to suppress retinal edema and recurrences of choroidal neovascularization (CNV). Although the currently approved IVT anti-VEGF therapies are efficacious and well-tolerated, the need for IVT injections every 4 to 8 weeks, specifically in the initial phase and during maintenance of treatment, represents a significant

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burden to physicians, patients, and caregivers. While the procedure is straightforward and relatively easy to perform, capacity issues for ensuring an appropriate injection frequency in order to achieve patient outcomes similar to those seen in the pivotal studies represent an increasing challenge to individual practices and the healthcare system, overall. Moreover, high frequency dosing leads to increased burdens on patients, *e.g.*, to find transportation and miss work. A secondary effect of this burden is a lower probability of non-compliance with the prescribed treatment regimen.

[0006] While the efficacy and safety of currently approved VEGF antagonist therapies have been established for the treatment of nAMD, there remains an unmet medical need for the development of therapies with the potential to reduce treatment burden while providing at least similar or even improved visual outcomes over currently available standard-of-care.

[0007] EYLEA (2 mg dose, administered at a concentration of 40 mg/mL, also called intravitreal aflibercept injection [IAI]) is currently approved in the United States (US) for the treatment of nAMD, and is also approved for the treatment of macular edema following retinal vein occlusion (RVO), diabetic macular edema (DME), and diabetic retinopathy (DR).

SUMMARY OF THE INVENTION

[0008] The present invention provides a method for treating or preventing an angiogenic eye disorder (*e.g.*, neovascular age-related macular edema (nAMD), diabetic macular edema (DME), diabetic retinopathy (DR), macular edema (ME) secondary to retinal vein occlusion (RVO) (ME-RVO), in a subject in need thereof, comprising administering to an eye of the subject (*e.g.*, by intravitreal injection), *e.g.*, in about 70 μl, a single initial dose of about 8 mg or more of a VEGF antagonist (*e.g.*, aflibercept) to an eye of the subject, followed by one or more secondary doses of the VEGF antagonist, followed by one or more tertiary doses of the VEGF antagonist; wherein each secondary dose is administered about 2 to 4 or 3 to 5 weeks after the immediately preceding dose; and wherein each tertiary dose is administered about 4, 8 or 12 weeks after the immediately preceding dose.

[0009] In an embodiment of the invention, wherein, *e.g.*, while receiving such a regimen: (i) with respect to visual acuity or best corrected visual acuity (BCVA), the subject achieves: no loss in visual acuity or BCVA; a gain in visual acuity or BCVA; no loss of visual acuity or BCVA by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44, following the initial dose wherein visual acuity or BCVA is according to ETDRS or the Snellen equivalent; no loss of

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visual acuity or BCVA of about 5 or more, about 10 or more, or about 15 or more letters by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44, following the initial dose wherein visual acuity or BCVA is according to ETDRS or the Snellen equivalent; a gain in visual acuity or BCVA, of about 5 or more, about 10 or more or about 15 or more letters, by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44 following the initial dose, wherein visual acuity or BCVA is according to ETDRS or the Snellen equivalent; and/or a gain in visual acuity or BCVA of about 6 or 7 or 8 letters by about week 8 and maintaining a gain of about 6 or 7 or 8 letters until at least about week 44 wherein visual acuity or BCVA is according to ETDRS or the Snellen equivalent; (ii) with respect to central retinal thickness (CRT), the subject achieves: a decrease in central retinal thickness; a decrease in central retinal thickness by at least about 123, 125, 131, 142, 147, 149, 150, 151, 156, 157, 158, 159, 161, 162, 166, 167, 168, 172, 173, 175, 177, 178 or 183 micrometers (μm) by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44 following the initial dose; a decrease in central retinal thickness of about 47 micrometers from about week 12 to about week 20 following the initial dose; a decrease in central retinal thickness of about 17 micrometers from about week 24 to about week 32 following the initial dose; a decrease in central retinal thickness of about 18 micrometers from about week 36 to about week 44 following the initial dose; a decrease in central retinal thickness of about 123, 131 161 micrometers by about week 4, 8, 12, 16 or 20 following the initial dose and maintaining the decrease until at least about week 44 following the initial dose; and/or a reduction in CRT of about 159, 160, 161 or 162 micrometers by about week 4 or 8 or 12 and maintaining a reduction of about 159, 160, 161 or 162 micrometers until at least about week 44; (iii) with respect to retinal fluid, the subject achieves: a dry retina having no intraretinal fluid and no subretinal fluid; or no intraretinal fluid; or no subretinal fluid; in the center subfield or macula as measured by spectral domain optical coherence tomography; a dry retina having no intraretinal fluid and no subretinal fluid; or no intraretinal fluid; or no subretinal fluid; in the center subfield as measured by spectral domain optical coherence tomography by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44, following the initial dose; no SRF and IRF in the macula as measured by SD-OCT by week 16 or week 44 following the initial dose; no sub-retinal pigment epithelium (RPE) fluid until at least about week 44 following the initial dose as measured by spectral domain optical coherence tomography; and/or maintenance of a dry retina once achieved until at least about week 44 following the initial dose as measured by spectral domain

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optical coherence tomography; and/or (iv) the subject achieves: a reduction in total choroidal neovascularization (CNV) lesion size by at least about 3.2 or 3.3 μm by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44 following the initial dose; no significant increase in intraocular pressure from baseline by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44 or later following the initial dose; and/or no significant increase in systolic (S) and/or diastolic (D) blood pressure from baseline by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44 or later following the initial dose. The scope of the present invention also encompasses methods for achieving any of such achievements in a subject in need thereof who is suffering from an angiogenic eye disorder, by administering the dosing regimen set forth above. For example, in an embodiment of the invention, the method for treating or preventing an angiogenic eye disorder (*e.g.*, neovascular age-related macular edema, diabetic retinopathy, diabetic macular edema or macular edema following retinal vein occlusion (RVO)) includes administering a single initial dose of VEGF antagonist (*e.g.*, aflibercept), followed by one or more secondary doses of the VEGF antagonist, followed by one or more tertiary doses of the VEGF antagonist; wherein each secondary dose is administered about 4 weeks after the immediately preceding dose; and wherein each tertiary dose is administered about 4, 8 or 12 weeks after the immediately preceding dose. In an embodiment of the invention, relative to a subject receiving such a dosing regimen, except that only 2 mg of VEGF antagonist is administered, the subject receiving 8 mg of VEGF antagonist exhibits a greater decrease in central retinal thickness after 4, 8, 12, 16 or more weeks; a greater improvement in best corrected visual acuity after 4, 8, 12, 16 or more weeks; and/or a greater likelihood of having a dry retina (*e.g.*, lacks intraretinal fluid and/or subretinal fluid) after 4, 8, 12, 16 or more weeks. In an embodiment of the invention, 2-4 weeks is 2, 3 or 4 weeks. The present invention further provides a method for improving best corrected visual acuity, decreasing central retinal thickness and/or achieving a dry retina, in the eye of a subject (*e.g.*, suffering from neovascular age-related macular edema, diabetic retinopathy, diabetic macular edema or macular edema following retinal vein occlusion (RVO)) in need thereof, comprising administering to the eye of the subject, a single initial dose of about 8 mg or more of a VEGF antagonist (*e.g.*, aflibercept) to an eye of the subject, followed by one or more secondary doses of the VEGF antagonist, followed by one or more tertiary doses of the VEGF antagonist; wherein each secondary dose is administered about 2 to 4 weeks after the immediately preceding dose; and wherein each

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tertiary dose is administered about 4, 8 or 12 weeks after the immediately preceding dose. Also provided by the present invention is a method for promoting retinal drying, in the eye of a subject suffering from an angiogenic eye disorder (*e.g.*, neovascular age-related macular edema, diabetic retinopathy, diabetic macular edema or macular edema following retinal vein occlusion (RVO)), comprising administering to the eye of the subject, a single initial dose of about 8 mg or more of a VEGF antagonist, followed by one or more secondary doses of about 8 mg or more of the VEGF antagonist, followed by one or more tertiary doses of about 8 mg or more of the VEGF antagonist; wherein each secondary dose is administered about 2 to 4 weeks after the immediately preceding dose; and wherein each tertiary dose is administered about 4, 8 or 12 weeks after the immediately preceding dose; for example, wherein retinal drying is characterized by no intraretinal fluid (IRF) and/or no subretinal fluid (SRF) in the eye of the subject, *e.g.*, after the subject has received the three monthly doses of the VEGF antagonist. In an embodiment of the invention, the VEGF antagonist is:

(i) a VEGF receptor fusion protein, for example, comprising two polypeptides that comprise (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) a VEGF receptor fusion protein comprising two polypeptides that comprise immunoglobin-like (Ig) domain 2 of a VEGFR1 and Ig domain 3 of VEGFR2, and a multimerizing component;

(iii) a VEGF receptor fusion protein comprising two polypeptides that comprise an immunoglobin-like (Ig) domain 2 of VEGFR1, an Ig domain 3 of VEGFR2, an Ig domain 4 of said VEGFR2; and a multimerizing component;

(iv) a VEGF receptor fusion protein comprising two VEGFR1R2-FcΔC1(a) polypeptides encoded by the nucleic acid sequence of SEQ ID NO: 1; or

(v) selected from the group consisting of: aflibercept, conbercept, bevacizumab, ranibizumab, pegaptanib, brolucizumab, an anti-VEGF DARPin, abicipar pegol, faricimab, an anti-VEGF antibody or antigen-binding fragment thereof or biopolymer conjugate thereof (*e.g.*, KSI-301), bevacizumab, ranibizumab and a bispecific anti-VEGF/ANG2 antibody.

[0010] In an embodiment of the invention, the VEGF antagonist is administered to the eye of the subject in a pharmaceutical formulation, for example, which is selected from the group consisting of A-KKKK as so designated herein. In an embodiment of the invention,

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the VEGF antagonist is administered to the eye from a syringe, e.g., a pre-filled syringe, (*e.g.*, which is glass, plastic and/or sterile). In an embodiment of the invention, the syringe is characterized by the ornamental design as set forth in International Design Registration No. DM/212 509; which is herein incorporated by reference.

[0011] In an embodiment of the invention, a dosage of about 8 mg or more is a dose of about 9, 9.3, 9.33, 9.7, 9.8, 9.9, 9.7-9.9 mg or more and such a dosage amount may vary within a given range, *e.g.*, ± about 0.5, or ± about 0.51 mg. The volume in which a dose is delivered can be, for example, about 70, 81, 82, 81.7, 85, 86, 87, 85-87 microliters and the volume may vary within a given range, *e.g.*, ± about 4, 4.45, 4.5, or 5 microliters. Doses may be delivered with a dose delivery device (DDD) which is a syringe.

[0012] Highly precise doses of VEGF antagonist (*e.g.*, aflibercept) may be delivered, for example, in a volume that is device-determined. In an embodiment of the invention, a dose is delivered with a syringe by a method that includes the steps: (a) withdrawing a plunger rod of the syringe to fill the syringe with the formulation; (b) priming the syringe, thereby removing air from the syringe and, thus avoiding injection of air into the eye, by advancing the plunger rod by a predetermined distance into the syringe body until advancement of the plunger rod is resisted by a stop; (c) rotating the plunger rod about a longitudinal axis; and (d) actuating the plunger rod to dispense a predetermined (device-determined) volume (*e.g.*, about 70, 81, 82, 81.7, 85, 86, 87, 85-87 microliters, ± about 4, 4.45, 4.5, or 5 microliters) of the formulation.

BRIEF DESCRIPTION OF THE FIGURES

[0013] **Figure 1**: Summary of CANDELA Clinical Trial Plan and Dosing Schedule.

[0014] **Figure 2**: Baseline Demographics of Patients in CANDELA trial (IAI dosing group, HD dosing group and All patients (all patients with data reported as of data cut-off (IAI n=27; HD n=28))) at week 16.

[0015] **Figure 3**: Continued Baseline Demographics of Patients in CANDELA Trial at (all patients with data reported as of data cut-off (IAI n=27; HD n=28))) at week 16.

[0016] **Figure 4**: Baseline Blood Pressures of IAI and HD Dosing Groups in CANDELA trial (all patients with data reported as of data cut-off (IAI n=27; HD n=28))) at week 16.

[0017] **Figure 5**: Mean Change in Central Retinal Thickness (CRT) from Baseline (BL) in IAI and HD CANDELA Dosing Groups in Patients Completing Week 16. Completers are patients with OCT values at both weeks 12 and 16 (IAI n=22; HD n=27).

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[0018] **Figure 6**: Mean change in Best Corrected Visual Acuity (BCVA) (ETDRS letters) from Baseline (BL) in IAI and HD CANDELA Dosing Groups in Patients Completing to Week 16 (IAI n=22; HD n=27).

[0019] **Figure 7**: Proportion of HD and IAI patients in CANDELA Completing to Week 16 with a Dry Retina (no intraretinal or subretinal fluid) at Baseline and Weeks 4, 8, 12 and 16.

[0020] **Figure 8**: Proportion of HD and IAI patients in CANDELA Completing to week 16 with a Retina that is Not Dry (intraretinal and/or subretinal fluid) at Baseline and Weeks 4, 8, 12 and 16.

[0021] **Figure 9**: Proportion of HD and IAI Patients in CANDELA Completing to Week 16 with Intraretinal Fluid (IRF) at Baseline and Weeks 4, 8, 12 and 16.

[0022] **Figure 10**: Proportion of HD and IAI Patients in CANDELA Completing to Week 16 with Subretinal fluid (SRF) at Baseline and Weeks 4, 8, 12 and 16.

[0023] **Figure 11**: Retinal Fluid status of IAI patients (n=22) in CANDELA Completing to Week 16 at Baseline and Weeks 4, 8, 12 and 16.

[0024] **Figure 12**: Retinal Fluid Status of HD Patients in CANDELA Completing to Week 16 at Baseline and Weeks 4, 8, 12 and 16. N=27 at Baseline, Week 8, 12 and 16; N=26 at Week 4.

[0025] **Figure 13**: Summary of Treatment Exposure of IAI and HD Patients in CANDELA Trial. IAI n=27; HD n=28.

[0026] **Figure 14**: Summary of Treatment Emergent Adverse Events (TEAE) of IAI and HD Patient Study Eye in CANDELA Trial. IAI n=27; HD n=27.

[0027] **Figure 15**: Continued Summary of Treatment Emergent Adverse Events (TEAE) of IAI and HD Patients in CANDELA Trial. IAI n=27; HD n=27.

[0028] **Figure 16**: Intraocular Pressure (IOP) of HD and IAI Patients in CANDELA trial. IAI n=27; HD n=28.

[0029] **Figure 17**: Summary of Non-Ocular Treatment Emergent Adverse Events (TEAE) of IAI and HD Patients in CANDELA trial. IAI n=27; HD n=28.

[0030] **Figure 18**: Continued Summary of Non-Ocular Treatment Emergent Adverse Events (TEAE) of IAI and HD Patients in CANDELA Trial. IAI n=27; HD n=28.

[0031] **Figure 19**: Summary of Non-Ocular Serious Adverse Events (SAES) of IAI and HD Patients in CANDELA Trial. TIA-transient ischemic attack. IAI n=27; HD n=28.

[0032] **Figure 20**: Summary of Hypertension Adverse Events of IAI and HD patients in CANDELA Trial. IAI n=27; HD n=28.

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[0033] **Figure 21**: Mean Systolic Blood Pressure of IAI and HD Patients in CANDELA trial.

[0034] **Figure 22**: Mean Diastolic Blood Pressure of IAI and HD Patients in CANDELA Trial.

[0035] **Figure 23**: Mean Intraocular Pressure of IAI and HD Patients in CANDELA Trial.

[0036] **Figure 24**: Change in Intraocular Pressure from Baseline of IAI and HD Patients in CANDELA Trial.

[0037] **Figures 25A, 25B** and **25C:** Precision Dose Delivery Device Analyses. (Fig. 25A) Delivered dose summary for REGN3-PFS-0.5mL; (Fig. 25B) Delivered dose summary for 1mL BD Luer Lok syringe; (Fig. 25C) Individual value plot of doses delivered with REGN3-PFS-0.5mL and 1mL BD Luer Lok syringe.

[0038] **Figure 26**: Disposition and Exposure through week 16 in Patients treated with 8 mg Aflibercept High-Dose Regimen (HD) or 2 mg Aflibercept Regimen (IAI) or All Patients (Total) (analysis of additional patients reaching week 16: HD n=53; IAI n=53; All n=106).

[0039] **Figure 27**: Baseline Demographics of in Patients treated with HD, IAI or All Patients (Total) (analysis of additional patients reaching week 16). HD n=53; IAI n=53; All n=106.

[0040] **Figure 28**: Baseline Characteristics in Patients treated with HD, IAI or All Patients (analysis of additional patients reaching week 16). HD n=53; IAI n=53; All n=106.

[0041] **Figure 29**: Baseline Blood Pressure and Medical History of Hypertension in All Patients treated with HD or IAI (analysis of additional patients reaching week 16; HD n=53; IAI n=53).

[0042] **Figure 30**: Primary Efficacy Endpoint - Proportion of Patients Treated with HD or IAI Regimen with Dry Retina at Week 16 (analysis of additional patients reaching week 16; number of patients analyzed is shown).

[0043] **Figure 31**: Proportion of Patients Treated with HD or IAI Regimen without Intraretinal Fluid (IRF) and without Subretinal Fluid (SRF) at Week 16 (analysis of additional patients reaching week 16; number of patients analyzed is shown).

[0044] **Figure 32**: Median Change from Baseline in Central Retinal Thickness of Patients Treated with HD or IAI Regimen through Week 16 (analysis of additional patients reaching week 16; number of patients analyzed is shown).

[0045] **Figure 33**: Mean Change from Baseline in Central Retinal Thickness of Patients Treated with HD or IAI Regimen through Week 16 (analysis of additional patients reaching week 16; number of patients analyzed is shown).

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[0046] **Figure 34**: Mean Change from Baseline to Week 12 in Choroidal Neovascular Size and Lesion Size of Patients Treated with HD or IAI Regimen. IAI n=44; HD n=46.

[0047] **Figure 35**: Mean Change from Baseline in Best Corrected Visual Acuity (BCVA) through Week 16 in Patients Treated with HD or IAI Regimen (analysis of additional patients reaching week 16; number of patients analyzed is shown).

[0048] **Figure 36**: Changes in Vision at Week 16 in Patients Treated with HD or IAI Regimen (analysis of additional patients reaching week 16; number of patients analyzed is shown).

[0049] **Figure 37**: Proportion of Patients Treated with HD or IAI Regimen Receiving Additional Treatment at Week 16 (analysis of additional patients reaching week 16; HD n=53; IAI n=53).

[0050] **Figures 38A, 38B** and **38C**: Summary of Treatment Emergent Adverse Events (TEAEs) in Patients Treated with HD or IAI Regimen. (Fig. 38A) Ocular TEAEs in Study Eye Occurring in ≥2% of Patients through Week 16, (Fig. 38B) Ocular Serious TEAEs in the Study Eye through Week 16, (Fig. 38C) Intraocular Inflammation TEAEs in the Study Eye through Week 16 (analysis of additional patients reaching week 16; HD n=53; IAI n=53).

[0051] **Figure 39**: Mean Intraocular Pressure Change from Baseline in Patients Treated with HD or IAI Regimen through Week 16 (analysis of additional patients reaching week 16; number of patients analyzed is shown).

[0052] **Figure 40**: Summary of Intraocular Pressure Events in Patients Treated with HD or IAI Regimen through Week 16 (analysis of additional patients reaching week 16; HD n=53; IAI n=53).

[0053] **Figure 41**: Anti-Platelet Trialists' Collaboration (APTC) Events or Deaths in Patients Treated with HD or IAI Regimen through Week 16 (analysis of additional patients reaching week 16; HD n=53; IAI n=53).

[0054] **Figure 42**: Hypertension Adverse Events in Patients Treated with HD or IAI Regimen through Week 16 (analysis of additional patients reaching week 16; HD n=53; IAI n=53).

[0055] **Figures 43A** and **43B**: Mean Change from Baseline in Blood Pressure (Fig. 43A: Systolic; Fig. 43B: Diastolic) in Patients Treated with HD or IAI Regimen through Week 16-All Patients (analysis of additional patients reaching week 16; HD n=53; IAI n=53).

[0056] **Figures 44A and 44B**: Mean Change from Baseline in Blood Pressure (Fig. 44A: Systolic; Fig. 44B: Diastolic) in Patients Treated with HD or IAI Regimen through Week 16-

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Dense Pharmacokinetic (PK) Sub-study Patients (analysis of additional patients reaching week 16; HD n=15; IAI n=15).

[0057] **Figure 45**: Patient Disposition as of study start, week 16 and week 44 of HD and IAI patient study groups and combined groups (All).

[0058] **Figure 46**: Baseline Demographics (sex, ethnicity, race, age) of HD and IAI patient study groups and combined groups (All).

[0059] **Figure 47**: Baseline Characteristics of the Study Eye of HD and IAI patient study groups and combined groups (All). 58 ETDRS letters is about equivalent to 20/60-20/70 Snellen visual acuity. SD=standard deviation; FA=fluorescein angiography.

[0060] **Figure 48**: Treatment Exposure summary through Week 44 of HD and IAI patient study groups.

[0061] **Figure 49**: Proportion of Eyes without Fluid in the Center Subfield (no intraretinal fluid (IRF) and no subretinal fluid (SRF) in the center subfield by SD-OCT (Spectral Domain Optical Coherence Tomography)) at Weeks 16 & 44. Percentages and number of patients out of 53 (n/53) are shown. LOCF=last observation carried forward. FAS=full analysis set. At baseline, 9/53 (17.0%) and 10/53 (18.9%) of eyes in the aflibercept 2 mg and 8 mg groups, respectively, had no IRF or SRF in the center subfield. These eyes met criteria for inclusion due to presence of pigment epithelial detachment (PED) due to nAMD

[0062] **Figure 50**: Proportion of Eyes without Fluid in the Macula (no IRF and no SRF in the macula on SD-OCT) at week 16 (difference of 17 (95% confidence interval: -0.9, 34.8)) and week 44 (difference of 17 (95% confidence interval: 1.1, 32.8)). Percentages and number of patients out of 53 (n/53) are shown.

[0063] **Figure 51**: Proportion of Eyes without Fluid in the Center Subfield (without fluid in the center subfield refers to no intraretinal fluid and no subretinal fluid in the center subfield on SD-OCT (Spectral Domain Optical Coherence Tomography)) at weeks 16, 20, 24 28, 32, 36, 40 and 44. Percentages and number of patients out of 53 (n/53) are shown. Syringes on the X-axis indicate scheduled dose visits.

[0064] **Figure 52**: Proportion of Eyes without Intraretinal Fluid (without intraretinal fluid is dry or with subretinal fluid only) in the Center Subfield at Weeks 16 and 44. Percentages and number of patients out of 53 (n/53) are shown.

[0065] **Figure 53**: Proportion of Eyes without Subretinal Fluid (without subretinal fluid is dry or with intraretinal fluid only) in the Center Subfield at Weeks 16 and 44. The week 16

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and 44 treatment effect was 18.9%. Percentages and number of patients out of 53 (n/53) are shown.

[0066] **Figure 54**: Mean Change from Baseline (BL) in Central Retinal Thickness (CRT; micrometers) over time through Week 44. Syringes on the X-axis indicate scheduled dose visits. Week 16 data for patients receiving treatment at week 16 are carried forward. LOCF: Patients receiving treatment at week 16 were considered not dry from week 16 onward. Inset box shows changes in CRT between weeks 12 and 20, 24 and 32; or 36 and 44 in the HD and IAI groups.

[0067] **Figure 55**: Median Change from Baseline (BL) in Central Retinal Thickness (CRT; micrometers) over time through Week 44. Syringes on the X-axis indicate scheduled dose visit. LOCF: Patients receiving treatment at week 16 were considered not dry from week 16 onward.

[0068] **Figure 56**: Mean Change from Baseline (BL) in Best Corrected Visual Acuity (BCVA; ETDRS letters) over time through Week 44. Syringes on the X-axis indicate scheduled dose visit. LOCF: Patients receiving treatment at week 16 were considered not dry from week 16 onward.

[0069] **Figure 57**: Proportion of Patients Losing and Gaining Vision (ETDRS letters) from Baseline (BL) at Week 44. Percentages and number of patients out of 53 (n/53) are shown.

[0070] **Figure 58**: Ocular Treatment Emergent Adverse Events (TEAEs) in the Study Eye Occurring in $\geq$ 2% of Patients (Pts). Percentages and number of patients out of 53 (n/53) in HD and IAI groups are shown.

[0071] **Figure 59**: Ocular Serious Treatment Emergent Adverse Events (TEAEs) in the Study Eye. Percentages and number of patients out of 53 (n/53) in HD and IAI groups are shown.

[0072] **Figure 60**: Intraocular Inflammation Treatment Emergent Adverse Events (TEAEs) in the Study Eye. Percentages and number of patients out of 53 (n/53) in HD and IAI groups are shown.

[0073] **Figure 61**: Summary of Intraocular Pressure Events in the HD and IAI Study Groups. Safety analysis set. Percentages are calculated based on N. Percentages and number of patients out of 53 (n/53) in HD and IAI groups are shown.

[0074] **Figure 62**: Mean Pre-Dose Intraocular Pressure, Change from Baseline in the HD and IAI study groups over time (mmHg). Safety analysis set.

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[0075] **Figure 63**: Non-Ocular Treatment Emergent Adverse Events (TEAEs) Occurring in ≥ 2% of Patients. Percentages and number of patients out of 53 (n/53) in HD and IAI groups are shown.

[0076] **Figure 64**: Non-Ocular Serious Treatment Emergent Adverse Events (TEAEs) in the HD and IAI study groups. Percentages and number of patients out of 53 (n/53) are shown.

[0077] **Figure 65**: Hypertension Treatment Emergent Adverse Events (TEAEs) in the HD and IAI study groups. Percentages and number of patients out of 53 (n/53) in HD and IAI groups are shown.

[0078] **Figure 66**: Adjudicated Anti-Platelet Trialists' Collaboration (APTC) Events and Deaths in the HD and IAI study groups.

DETAILED DESCRIPTION OF THE INVENTION

[0079] Increasing the molar fraction of VEGF antagonist therapeutic protein in the dosing formulation is a potential way to bring further benefits to patients with chorioretinal vascular diseases, including nAMD. A higher dose of aflibercept administered IVT has the potential to prolong the drug's therapeutic effects and for improvement in pharmacodynamics such as better drying. The resulting extension of treatment intervals early after the initiation of treatment to every 12 weeks would reduce the number of injections in the first treatment year. A potential decrease in injection-related treatment burden and safety events with fewer injections could be a significant contribution to patient care and healthcare services. The present invention provides, in part, a safe and effective method for treating angiogenic eye disorders with an 8 mg dose of aflibercept in a regimen calling for monthly loading doses before quarterly maintenance doses. First, patients receiving an 8 mg dose, which is four times the dose approved for Eylea, (2 mg), were not observed in the CANDELA clinical trial (discussed herein) to develop any more hypertension treatment-emergent adverse events than patients receiving 2 mg (Figure 65). Systemic exposure to VEGF inhibitor is known to be associated with the risk of systemic blood pressure (BP) increases. Moreover, intravitreal injection of VEGF inhibitors has been associated with blood pressure effects. See, for example, Hanna *et al.*, (2019) "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 and Raiser *et al.*, (2009) "The effect of intravitreal bevacizumab (Avastin) administration on systemic hypertension", Eye

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23, 1714–1718. In addition, in the CANDELA clinical trial, subjects receiving the 8 mg dose (HD) achieved numerically superior anatomical improvements in the eye as well as numerically superior improvements in vision relative to subjects receiving a 2 mg dose (IAI). A higher proportion of eyes treated with aflibercept 8 mg (HD) were dry (without intraretinal or subretinal fluid on OCT) in the center subfield versus aflibercept 2 mg (IAI). Treatment groups followed identical dosing regimens with the 8 mg group receiving slightly fewer PRN doses. A change from baseline in central retinal thickness (CRT) suggested better anatomic outcomes with aflibercept 8 mg versus aflibercept 2 mg; and a change from baseline in BCVA favored aflibercept 8 mg (+7.9 vs +5.1 letters).

[0080] The anatomical and visual improvements for the HD patients that was observed was also comparable to those of subjects in the VIEW1 and VIEW2 trials (VIEW1/2) that received a 2 mg dose every 8 weeks (following three monthly loading doses (2q8)). See *e.g.*, Heier *et al.*, Intravitreal Aflibercept (VEGF Trap-Eye) in Wet Age-related Macular Degeneration, Ophthalmology 2012;119:2537-2548. The VIEW1/2 2q8 subjects achieved 8.1 letters of BCVA mean improvement at 44 weeks following the initial dose (Heier *et al.* (2012), Figure 3); whereas the CANDELA HD subjects herein were observed to achieve a mean improvement of 7.9 letters at 44 weeks (Figure 56). Moreover, the improvements in the central retinal thickness (CRT) that were observed to be achieved by CANDELA HD subjects were comparable to that observed in the VIEW1/2 2q8 subjects. The VIEW1 2q8 subjects achieved a mean reduction of about 125 micrometers in CRT and VIEW2 2q8 subjects achieved about 150 micrometers mean reduction in CRT, at 44 weeks (Heier *et al.* (2012), Figure 4). The CANDELA HD subjects were observed to achieve a mean reduction of 159 micrometers and a median reduction of 162 micrometers at 44 weeks (Figure 54 & Figure 55; and Heier *et al.* (2012), Figure 4). While the 2q8 VIEW1/2 subjects and the CANDELA HD subjects were not evaluated side-by-side in the same clinical trial and the VIEW trials had a greater number of participants, these data suggest that subjects can be administered 8 mg doses of aflibercept as infrequently as every 12 weeks yet achieve anatomic and visual outcomes comparable to that of patients dosed every 8 weeks (following three monthly loading doses) with 2 mg. While the VIEW1/2 subjects received only the scheduled doses within the first year, some of the CANDELA subjects received additional doses *pro re nata* after week 16 (Figure 1). Twenty eight out of 53 of the CANDELA HD subjects did not receive any additional doses (Figure 48). Moreover, time-domain optical coherence tomography was performed in VIEW1/2 to evaluated CRT

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whereas, a more sensitive spectral domain optical coherence tomography (SD-OCT) was used in CANDELA.

[0081] "Isolated" VEGF antagonists and VEGF receptor fusion proteins (*e.g.*, aflibercept), polypeptides, polynucleotides and vectors, are at least partially free of other biological molecules from the cells or cell culture from which they are produced. Such biological molecules include nucleic acids, proteins, other VEGF antagonists and VEGF receptor fusion proteins, lipids, carbohydrates, or other material such as cellular debris and growth medium. An isolated VEGF antagonist or VEGF receptor fusion protein may further be at least partially free of expression system components such as biological molecules from a host cell or of the growth medium thereof. Generally, the term "isolated" is not intended to refer to a complete absence of such biological molecules (*e.g.*, minor or insignificant amounts of impurity may remain) or to an absence of water, buffers, or salts or to components of a pharmaceutical formulation that includes the VEGF antagonists or VEGF receptor fusion proteins.

[0082] A "subject" or "patient" is a mammal, for example a human, mouse, rabbit, monkey or non-human primate. A subject or patient may be said to be "suffering from" an angiogenic eye disorder such as nAMD, DR or DME. Such a subject has the disorder in one or both eyes. In an embodiment of the invention, a subject or patient has one or more of the following characteristics (at or before the start of treatment):

1. ≥50 years of age
2. Subfoveal CNV secondary to nAMD, *e.g.*, including juxtafoveal lesions that affect the fovea in an eye.
3. Best Corrected Visual Acuity (BCVA) Early Treatment Diabetic Retinopathy Study (ETDRS) letter score of 78 to 24 (Snellen equivalent of 20/32 to 20/320) in an eye; or about 58±10, 58 ±14 or 58±12 ETDRS letters (or Snellen equivalent).
4. A central retinal thickness in an eye greater than normal, *e.g.*, greater than approximately 130, 140, 150, 160, 170 or 180 micrometers, *e.g.*, as determined manually or by optical coherence tomography (OCT) mapping software, for example with a central retinal thickness of about 300, 400, 500 or 600 micrometers or more; or about 488.1±204.9, 516.2±175.64 or 502.1±190.6 micrometers.
5. Intraocular pressure of about 14.8±3.4 or 14.9±3.4 mmHg.
6. nAMD lesion size of about 7.9±6.21, 7.7±6.84 or 7.8±6.50 mm^2.
7. Choroidal neovascularization lesion size of about 7.9±6.20, 7.5±6.86 or 7.7±6.51 mm^2.

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8. As per fluorescein angiography (FA), has occult choroidal neovascularization, minimally classic choroidal neovascularization or predominantly classic choroidal neovascularization. and/or, has or lacks any one or more of the following characteristics:

1. CNV (choroidal neovascularization) due to any cause other than nAMD in either eye.
2. Subretinal hemorrhage in an eye that is ≥50% of the total lesion area.
3. Intraocular pressure ≥25 mm Hg in an eye.
4. Evidence of infectious blepharitis, keratitis, scleritis, or conjunctivitis in an eye.
5. Any intraocular inflammation and/or ocular infection in an eye.
6. Any history of macular hole of stage 2 and above in an eye.
7. Iris neovascularization, vitreous hemorrhage, or tractional retinal detachment visible in an eye.
8. Uncontrolled blood-pressure (BP) (defined as systolic >140 mm Hg or diastolic >90 mm Hg).
9. Variation by more than 10% in the 3 pre-randomization BP measurements.
10. History of cerebrovascular accident/ transient ischemic attack or myocardial infarction/ acute coronary syndrome.
11. Renal failure, dialysis, or history of renal transplant.
12. Known sensitivity to aflibercept.
13. Any other intraocular surgery within 12 weeks (84 days).

VEGF Antagonists

[0083] The present invention includes methods for using a VEGF antagonist for treating or preventing angiogenic eye disorders. 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, anti-VEGF receptor antibodies, and VEGF receptor fusion proteins.

[0084] For purposes herein, a "VEGF receptor fusion protein" refers to a molecule that comprises one or more VEGF receptors or domains thereof, fused to another polypeptide, which interferes with the interaction between VEGF and a natural VEGF receptor, *e.g.*, wherein two of such fusion polypeptides are associated thereby forming a homodimer or other multimer. Such VEGF receptor fusion proteins may be referred to as a "VEGF-Trap" or "VEGF Trap". VEGF receptor fusion proteins within the context of the present disclosure

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that fall within this definition include chimeric polypeptides which comprise two or more immunoglobulin (Ig)-like domains of a VEGF receptor such as VEGFR1 (also known as Flt1) and/or VEGFR2 (also known as Flk1 or KDR), and may also contain a multimerizing domain (for example, an Fc domain).

[0085] An exemplary VEGF receptor fusion protein is a molecule referred to as VEGF1R2-FcΔC1(a) which is encoded by the nucleic acid sequence of SEQ ID NO:1 or nucleotides 79-1374 or 79-1371 thereof.

[0086] VEGF1R2-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 acids of SEQ ID NO:2, *i.e.*, K458, may or may not be included in the VEGF receptor fusion proteins, see U.S. Patent No. 7,396,664 or 7,354,579, incorporated herein for all purposes). Note that amino acids 1 to 26 of SEQ ID NO:2 are the signal sequence.

[0087] If the multimerizing component (MC) of a VEGF receptor fusion protein is derived from an IgG (*e.g.*, IgG1) Fc domain, then the MC has no fewer amino acids than are in amino acids 232 to 457 of SEQ ID NO:2. Thus, the IgG of the MC cannot be truncated to be shorter than 226 amino acids.

[0088] In an embodiment of the invention, the VEGF receptor fusion protein comprises amino acids 27-458 or 27-457 of SEQ ID NO: 2.

atggtcagctactgggacaccggggtcctgctgtgcgcgctgctcagctgtctgcttctcacaggatctagttccgg
aagtgataccggtagacctttcgtagagatgtacagtgaaatccccgaaattatacacatgactgaaggaagggagc
tcgtcatttcctgccgggttacgtcaacctaacatcactgttacttttaaaaaagtttccacttgacactttgatcct
gatggaaaacgcataatctgggacagtagaaagggcttcatcatatcaaatgcaacgtacaaagaaatagggcttct
gacctgtgaagcaacagtcaatgggcatttgtataagacaaactatctcacacatcgacaaaccaatacaatcatag
atgtggttctgagtccgtctcatggaattgaactatctgttggagaaaagcttgtcttaaattgtacagcaagaact
gaactaaatgtggggattgacttcaactgggaataccattcttcgaagcatcagcataagaaacttgtaaaccgaga
cctaaaaaccagtcrgggagtgagatgaagaaattttttgagcaccttaactatagatggtgtaaccccggagtgacc
aaggattgtacacctgtgcagcatccagtgggctgatgaccaagaagaacagcacatttgtcagggtccatgaaaag
gacaaaactcacacatgcccaccgtgcccagcacctgaactcctgggggggaccgtcagtcttcctcttccccccaaa
acccaaggacaccctcatgatctcccggacccctgaggtcacatgcgtggtggtggacgtgagccacgaagaccctg
aggtcaagttcaactggtacgtggacggcgtggaggtgcataatgccaagacaaagccgcgcgggaggagcagtacaac

agcacgtaccgtgtggtcagcgtcctcaccgtcctgcaccaggactggctgaatggcaaggagtacaagtgcaaggt
ctccaacaaagccctcccagcccccatcgagaaaaccatctccaaagccaaagggcagccccgagaaccacaggtgt
acaccctgcccccatcccgggatgagctgaccaagaaccaggtcagcctgacctgcctggtcaaaggcttctatccc
agcgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaagaccacgcctcccgtgctggactc
cgacggctccttcttcctctacagcaagctcaccgtggacaagagcaggtggcagcaggggaacgtcttctcatgct
ccgtgatgcatgaggctctgcacaaccactacacgcagaagagcctctccctgtctccgggtaaatga
(SEQ ID NO: 1)

MVSYWDTGVLLCALLSCLLLTGSSSGSDTGRPFVEMYSEIPEIIHMTEGRELVIPCRVTS
PNITVTLKKFPLDTLIPDGKRIIWDSRKGFIISNATYKEIGLLTCEATVNGHLYKTNYLT
HRQTNTIIDVVLSPSHGIELSVGEKLVLNCTARTELNVGIDFNWEYPSSKHQHKKLVNRD
LKTQSGSEMKKFLSTLTIDGVTRSDQGLYTCAASSGLMTKKNSTFVRVHEKDKTHTCPPC
PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKT
KPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY
TLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK
LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
(SEQ ID NO: 2; signal sequence underscored-missing in mature, processed aflibercept; optionally, lacking C-terminal Lysine)

[0089] In an embodiment of the invention, aflibercept is N-glycosylated at any one or more of Asparagines 62, 94, 149, 222 and 308.

[0090] In an embodiment of the invention, the VEGF receptor fusion protein comprises
(1) an immunoglobin-like (Ig) domain 2 of a first VEGF receptor (*e.g.*, VEGFR1), and
(2) an Ig domain 3 of a second VEGF receptor (*e.g.*, VEGFR2),
(3) and, optionally, further including an Ig domain 4 of the second VEGF receptor (*e.g.*, VEGFR2) and
(4) a multimerizing component (*e.g.*, Fc domain of IgG including the hinge, CH2 and CH3 domains).

[0091] For example, in an embodiment of the invention, the VEGF receptor fusion protein has the following arrangement of said domains:

- [VEGFR1 Ig domain 2]-[VEGFR2 Ig domain 3]-[MC] (*e.g.*, a homodimer thereof) or
- [VEGFR1 Ig domain 2]-[VEGFR2 Ig domain 3]-[VEGFR2 Ig domain 4]-[MC] (*e.g.*, a homodimer thereof).

[0092] Note that the present disclosure also includes, within its scope, high concentration formulations including, instead of a VEGF receptor fusion protein, a VEGF binding molecule

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or anti-VEGF antibody or antigen-binding fragments thereof or biopolymer conjugate thereof (*e.g.*, KSI-301) and uses thereof as discussed, *e.g.*,

- bevacizumab (*e.g.*, at a concentration of about 80-90 or 88 mg/ml),
- ranibizumab (*e.g.*, at a concentration of about 20-40 mg/ml, *e.g.*, 21-35, 21 or 35 mg/ml),
- an anti-VEGF aptamer such as pegaptanib (*e.g.*, pegaptanib sodium),
- a single chain (*e.g.*, V_L-V_H) anti-VEGF antibody such as brolucizumab (*e.g.*, at a concentration of about 200-400 or 200, 210, 400 or 420 mg/ml),
- an anti-VEGF DARPin such as the Abicipar Pegol DARPin (*e.g.*, at a concentration of about 70-140, 70 or 140 mg/ml), or
- a bispecific anti-VEGF antibody, *e.g.*, which also binds to ANG2, such as RG7716 (faricimab) (*e.g.*, at a concentration of about 100-400, 100, 105, 400 or 420 mg/ml).

[0093] In order to minimize the repetitiveness of the embodiments discussed herein, it is contemplated that the scope of the present invention includes embodiments wherein any of the formulations discussed herein include, in place of a VEGF receptor fusion protein, an anti-VEGF antibody or antibody fragment or other VEGF binding molecule as discussed herein (*e.g.*, substituted with an anti-VEGF DARPin) at any of the concentrations discussed herein. For example, the present invention includes a formulation having 35 or 80 mg/ml ranibizumab, a buffer, a thermal stabilizer, a viscosity reducing agent and a surfactant.

[0094] DARPins are Designed Ankyrin Repeat Proteins. DARPins generally contain three to four tightly packed repeats of approximately 33 amino acid residues, with each repeat containing a β-turn and two anti-parallel α-helices. This rigid framework provides protein stability whilst enabling the presentation of variable regions, normally comprising six amino acid residues per repeat, for target recognition.

[0095] An "anti-VEGF" antibody or antigen-binding fragment of an antibody refers to an antibody or fragment that specifically binds to VEGF.

[0096] Illustrative VEGF receptor fusion proteins include aflibercept (EYLEA®, Regeneron Pharmaceuticals, Inc.) or conbercept (sold commercially by Chengdu Kanghong Biotechnology Co., Ltd.). See International patent application publication no. WO2005/121176 or WO2007/112675. The terms "aflibercept" and "conbercept" include biosimilar versions thereof. A biosimilar version of a reference product (*e.g.*, aflibercept) generally refers to a product comprising the identical amino acid sequence but includes

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products which are biosimilar under the U.S. Biologics Price Competition and Innovation Act.

Pharmaceutical Formulations

[0097] The present invention includes methods in which the VEGF antagonist (e.g., aflibercept) that is administered to the patient's eye is contained within a pharmaceutical formulation. The pharmaceutical formulation includes a VEGF antagonist along with a pharmaceutically acceptable carrier. Other agents may be incorporated into the pharmaceutical formulation 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 VEGF antagonist 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), *e.g.*, Chapter 87 by Blaug, Seymour, therein.

[0098] Pharmaceutical formulations for use in a method of the present invention can be "high concentration". High concentration pharmaceutical formulations of the present invention include VEGF antagonist, *e.g.*, VEGF receptor fusion protein (e.g., aflibercept), at a concentration of greater than 40 mg/ml, at least 41 mg/ml, of at least 80 mg/ml, of at least 100 mg/ml, of at least 125 mg/ml, of at least 140 mg/ml, of at least 150 mg/ml, of at least 175 mg/ml, of at least 200 mg/ml, of at least 225 mg/ml, of at least 250 mg/ml, or of at least 275 mg/ml. "High concentration" can refer to formulations that include a concentration of VEGF antagonist of from about 140 mg/ml to about 160 mg/ml, at least about 140 mg/ml but less than 160 mg/ml, from about 41 mg/ml to about 275 mg/ml, from about 70 mg/ml to about 75 mg/ml or from about 80 mg/ml to about 250 mg/ml. In some aspects, the VEGF antagonist concentration in the formulation is about any of the following concentrations: 41 mg/ml; 42 mg/ml; 43 mg/ml; 44 mg/ml; 45 mg/ml; 46 mg/ml; 47 mg/ml; 48 mg/ml; 49 mg/ml; 50mg/ml; 51 mg/ml; 52 mg/ml; 53 mg/ml; 54 mg/ml; 55 mg/ml; 56 mg/ml; 57 mg/ml; 58 mg/ml; 59 mg/ml; 60 mg/ml; 61 mg/ml; 62 mg/ml; 63 mg/ml; 64 mg/ml; 65 mg/ml; 66 mg/ml; 67 mg/ml; 68 mg/ml; 69 mg/ml; 70 mg/ml; 71 mg/ml; 72 mg/ml; 73 mg/ml; 74 mg/ml; 75 mg/ml; 76 mg/ml; 77 mg/ml; 78 mg/ml; 79 mg/ml; 80 mg/ml; 81 mg/ml; 82mg/ml; 83 mg/ml; 84 mg/ml; 85 mg/ml; 86 mg/ml; 87mg/ml; 88 mg/ml; 89

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mg/ml; 90 mg/ml; 91 mg/ml; 92 mg/ml; 93 mg/ml; 94 mg/ml; 95mg/ml; 96 mg/ml; 97 mg/ml; 98 mg/ml; 99 mg/ml; 100 mg/ml; 101 mg/ml; 102 mg/ml; 103 mg/ml; 104 mg/ml; 105 mg/ml; 106mg/ml; 107 mg/ml; 108 mg/ml; 109 mg/ml; 110 mg/ml; 111 mg/ml; 112 mg/ml; 113 mg/ml; 113.3 mg/ml; 114 mg/ml; 114.1 mg/ml; 114.2 mg/ml; 114.3 mg/ml; 114.4 mg/ml; 114.5 mg/ml; 114.6 mg/ml, 114.7 mg/ml, 114.8 mg/ml; 114.9 mg/ml; 115 mg/ml; 116 mg/ml; 117 mg/ml; 118 mg/ml; 119 mg/ml; 120 mg/ml; 121 mg/ml; 122 mg/ml; 123 mg/ml; 124 mg/ml; 125 mg/ml; 126 mg/ml; 127mg/ml; 128 mg/ml; 129 mg/ml; 130 mg/ml; 131 mg/ml; 132 mg/ml; 133 mg/ml; 133.3 mg/ml; 133.4 mg/ml, 134 mg/ml; 135 mg/ml; 136 mg/ml; 137 mg/ml; 138 mg/ml; 139 mg/ml; 140 mg/ml; 141 mg/ml; 142 mg/ml; 143 mg/ml; 144 mg/ml; 145 mg/ml; 146 mg/ml; 147 mg/ml; 148 mg/ml; 149 mg/ml; 150 mg/ml; 151 mg/ml; 152 mg/ml; 153 mg/ml; 154mg/ml; 155 mg/ml; 156 mg/ml; 157mg/ml; 158 mg/ml; 159 mg/ml; 160 mg/ml; 161 mg/ml; 162 mg/ml; 163 mg/ml; 164 mg/ml; 165 mg/ml; 166 mg/ml; 167 mg/ml; 168 mg/ml; 169 mg/ml; 170 mg/ml; 171 mg/ml; 172 mg/ml; 173 mg/ml; 174 mg/ml; 175 mg/ml; 176 mg/ml; 177 mg/ml; 178 mg/ml; 179 mg/ml; 180 mg/ml; 181 mg/ml; 182 mg/ml; 183 mg/ml; 184 mg/ml; 185 mg/ml; 186 mg/ml; 187 mg/ml; 188 mg/ml; 189 mg/ml; 190 mg/ml; 191 mg/ml; 192 mg/ml; 193 mg/ml; 194 mg/ml; 195 mg/ml; 196 mg/ml; 197 mg/ml; 198 mg/ml; 199 mg/ml; 200 mg/ml; 201 mg/ml; 202 mg/ml; 203 mg/ml; 204 mg/ml; 205 mg/ml; 206 mg/ml; 207 mg/ml; 208 mg/ml; 209 mg/ml; 210 mg/ml; 211 mg/ml; 212 mg/ml; 213 mg/ml; 214 mg/ml; 215 mg/ml; 216 mg/ml; 217 mg/ml; 218 mg/ml; 219 mg/ml; 220 mg/ml; 221 mg/ml; 222 mg/ml; 223 mg/ml; 224 mg/ml; 225 mg/ml; 226 mg/ml; 227 mg/ml; 228 mg/ml; 229 mg/ml; 230 mg/ml; 231 mg/ml; 232 mg/ml; 233 mg/ml; 234 mg/ml; 235 mg/ml; 236 mg/ml; 237mg/ml; 238 mg/ml; 239 mg/ml; 240 mg/ml; 241 mg/ml; 242 mg/ml; 243 mg/ml; 244 mg/ml; 245 mg/ml; 246 mg/ml; 247 mg/ml; 248 mg/ml; 249 mg/ml; 250 mg/ml; 251 mg/ml; 252 mg/ml; 253 mg/ml; 254 mg/ml; 255 mg/ml; 256 mg/ml; 257 mg/ml; 258 mg/ml; 259 mg/ml; 260 mg/ml; 261 mg/ml; 262 mg/ml; 263 mg/ml; 264 mg/ml; 265 mg/ml; 266 mg/ml; 267 mg/ml; 268 mg/ml; 269 mg/ml; 270 mg/ml; 271 mg/ml; 272 mg/ml; 273 mg/ml; 274 mg/ml; or 275 mg/ml. Other VEGF antagonist concentrations are contemplated herein, as long as the concentration functions in accordance with embodiments herein.

[0099] In an embodiment of the invention, a pharmaceutical formulation for use in a method of the present invention is of such a concentration as to contain about 4, 6, 8, 10, 12, 14, 16, 18 or 20 mg VEGF receptor fusion protein (*e.g.*, aflibercept), or the amount of

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such protein in any of the acceptable doses thereof which are discussed herein, in about 100 µl or less, about 75 µl or less or about 70 µl or less, e.g., about 50 µl; 51 µl; 52 µl; 53 µl; 54 µl; 55 µl; 56 µl; 57 µl; 58 µl; 59 µl; 60 µl; 61 µl; 62 µl; 63 µl; 64 µl; 65 µl; 66 µl; 67 µl; 68 µl; 69 µl; 70 µl; 71 µl; 72 µl; 73 µl; 74 µl; 75 µl; 76 µl; 77 µl; 78 µl; 79 µl; 80 µl; 81 µl; 82 µl; 83 µl; 84 µl; 85 µl; 86 µl; 87 µl; 88 µl; 89 µl; 90 µl; 91 µl; 92 µl; 93 µl; 94 µl; 95 µl; 96 µl; 97 µl; 98 µl; 99 µl; or 100 µl.

[0100] The present invention includes methods of using (as discussed herein) any of the formulations set forth under "*Illustrative Formulations*" herein, but wherein the concentration of the VEGF receptor fusion protein (*e.g.*, aflibercept) is substituted with a concentration which is set forth in this section ("VEGF Receptor Fusion Proteins and Other VEGF Inhibitors").

[0101] Buffers for use in pharmaceutical formulations herein that may be used in a method of the present invention refer to solutions that resist pH change by use of acid-base conjugates. Buffers are capable of maintaining pH in the range of from about 5.0 to about 6.8, and more typically, from about 5.8 to about 6.5, and most typically, from about 6.0 to about 6.5. In some cases, the pH of the formulation of the present invention is about 5.0, about 5.1, about 5.2, about 5.3, about 5.4, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, about 6.6, about 6.7, or about 6.8. Example buffers for inclusion in formulations herein include histidine-based buffers, for example, histidine, histidine hydrochloride, and histidine acetate. Buffers for inclusion in formulations herein can alternatively be phosphate-based buffers, for example, sodium phosphate, acetate-based buffers, for example, sodium acetate or acetic acid, or can be citrate-based, for example, sodium citrate or citric acid. It is also recognized that buffers can be a mix of the above, as long as the buffer functions to buffer the formulations in the above-described pH ranges. In some cases, the buffer is from about 5 mM to about 25 mM, or more typically, about 5 mM to about 15 mM. Buffers can be about 5 mM, about 6 mM, about 7 mM, about 8 mM, about 9 mM, about 10 mM, about 11 mM, about 12 mM, about 13 mM, about 14 mM, about 15 mM, about 16 mM, about 17 mM, about 18 mM, about 19 mM, about 20 mM, about 21 mM, about 22 mM, about 23 mM, about 24 mM, or about 25 mM.

[0102] In an embodiment of the invention, a histidine-based buffer is prepared using histidine and histidine monohydrochloride.

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[0103] Surfactant for use herein refers to ingredients that protect the higher concentration of VEGF antagonist, *e.g.*, VEGF receptor fusion protein (e.g., aflibercept), from various surface and interfacial induced stresses. As such, surfactants can be used to limit or minimize VEGF receptor fusion protein aggregation and promote protein solubility. Suitable surfactants herein have been shown to be non-ionic, and can include surfactants that have a polyoxyethylene moiety. Illustrative surfactants in this category include: polysorbate 20, polysorbate 80, poloxamer 188, polyethylene glycol 3350, and mixtures thereof. Surfactants in the formulations can be present at from about 0.02% to about 0.1% weight per volume (w/v), and more typically, about 0.02% to about 0.04% (w/v). In some cases, the surfactant is about 0.02% (w/v), about 0.03% (w/v), about 0.04% (w/v), about 0.05% (w/v), about 0.06% (w/v), about 0.07% (w/v), about 0.08% (w/v), about 0.09% (w/v), or about 0.1% (w/v).

[0104] Thermal stabilizers for use in pharmaceutical formulations that may be used in methods set forth herein refers to ingredients that provide thermal stability against thermal denaturation of the VEGF antagonist, *e.g.*, VEGF receptor fusion protein (e.g., aflibercept), as well as protect against loss of VEGF receptor fusion protein potency or activity. Suitable thermal stabilizers include sugars, and can be sucrose, trehalose, sorbitol or mannitol, or can be amino acids, for example L-proline, L-arginine (*e.g.*, L-arginine monohydrochloride), or taurine. Additionally, thermal stabilizers may also include substituted acrylamides or propane sulfonic acid, or may be compounds like glycerol.

[0105] In some cases, the pharmaceutical formulations for use in a method herein include both a sugar and taurine, a sugar and an amino acid, a sugar and propane sulfonic acid, a sugar and taurine, glycerol and taurine, glycerol and propane sulfonic acid, an amino acid and taurine, or an amino acid and propane sulfonic acid. In addition, formulations can include a sugar, taurine and propane sulfonic acid, glycerol, taurine and propane sulfonic acid, as well as L-proline, taurine and propane sulfonic acid.

[0106] Embodiments herein may have thermal stabilizers present alone, each independently present at a concentration of, or present in combination at a total concentration of, from about 2% (w/v) to about 10% (w/v) or 4% (w/v) to about 10% (w/v), or about 4% (w/v) to about 9% (w/v), or about 5% (w/v) to about 8% (w/v). Thermal stabilizers in the formulation can be at a concentration of about 2% (w/v), about 2.5% (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), about 10% (w/v) or about 20% (w/v).

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[0107] With respect to taurine and propane sulfonic acid, in an embodiment of the invention, these thermal stabilizers can be present in the formulations at about from 25 mM to about 100 mM, and more typically from about 50 mM to about 75 mM (as compared to the other thermal stabilizers).

[0108] Viscosity reducing agents typically are used to reduce or prevent protein aggregation. Viscosity reducing agents for inclusion herein include: sodium chloride, magnesium chloride, D- or L-arginine (*e.g.*, L-arginine monohydrochloride), lysine, or mixtures thereof. When present herein, viscosity reducing agents can be present at from about 10 mM to about 100 mM, and more typically from about 30 mM to about 75 mM, and even more typically from about 40 mM to about 70 mM. In some cases, the viscosity reducing agent is present at about 10 mM, about 15 mM, about 20 mM, about 25 mM, about 30 mM, about 35 mM, about 40 mM, about 45 mM, about 50 mM, about 55 mM, about 60 mM, about 65 mM, about 70 mM, about 75 mM, about 80 mM, about 85 mM, about 90 mM, about 95 mM or about 100 mM.

[0109] Pharmaceutical formulations for use in a method as set forth herein can also have a pharmaceutically acceptable viscosity for ocular administration, for example, intravitreal injection. Viscosity generally refers to the measure of resistance of a fluid which is being deformed by either shear stress or tensile stress (typically measured by techniques known in the art, viscometer or rheometer, for example). Typical viscosities of formulations for use in a method set forth herein are from about 5.0 cP (centipoise) to about 15 cP, from about 11 cP to about 14 cP, from about 12 cP to about 15 cP or from about 11 cP to about 12 cP. As such, formulation viscosity herein can be about 5.0 cP, about 6.0, about 7.1 cP, about 7.2 cP, about 7.3 cP, about 7.4 cP, about 7.5 cP, about 7.6 cP, about 10 cP, about 10.5 cP, about 11.0 cP, about 11.5 cP, about 12.0 cP, about 12.5 cP, about 13.0 cP, about 13.5 cP, about 14.0 cP, about 14.5 cP, or about 15.0 cP (*e.g.*, when measured at 20°C).

[0110] Various embodiments herein do not require inclusion of an inorganic salt, or other viscosity reducing agent, to maintain these highly useful viscosities. Typically, high concentration protein solutions require viscosity reducing agents to avoid protein aggregation and higher viscosity, making the formulations difficult for intravitreal injection and reducing the potency of the VEGF receptor fusion protein. As such, embodiments herein include methods of using formulations that have had substantially no, or no added, sodium chloride (NaCl), magnesium chloride ($MgCl_2$), D- or L-arginine hydrochloride, lysine or other viscosity reducing agent.

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[0111] Osmolality is a critical attribute for injectable pharmaceutical formulations for use in a method of the present invention. It is desirable to have products match physiological osmotic conditions. Furthermore, osmolality provides confirmation of soluble content in solution. In an embodiment of the invention, the osmolality of a formulation for use in a method of the present invention is less than or equal to about 506 mmol/Kg or from about 250 to about 506 mmol/Kg., *e.g.*, about 250, 260, 270, 280, 290, 299, 300, 310, 314, 315, 316, 324, 343, 346, 349, 369, 384, 403, 426, 430 or 506 mmol/Kg. In an embodiment of the invention, the osmolality is lower than about 250 mmol/Kg.

[0112] Illustrative pharmaceutical formulations for use in the methods of the present invention include the following:

Formulation A: 80 mg/ml aflibercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

Formulation B: 80 mg/ml aflibercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

Formulation C: 80 mg/ml aflibercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

Formulation D: 80 mg/ml aflibercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 6.2.

Formulation E: 80 mg/ml aflibercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

Formulation F: 80 mg/ml aflibercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

Formulation G: 80 mg/ml aflibercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

Formulation H: 80 mg/ml aflibercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

Formulation I: 80 mg/ml aflibercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.03% (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

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Formulation J: 80 mg/ml aflibercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

Formulation K: 80 mg/ml aflibercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

Formulation L: 80 mg/ml aflibercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

Formulation M: 150 mg/ml aflibercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

Formulation N: 150 mg/ml aflibercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

Formulation O: 150 mg/ml aflibercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

Formulation P: 150 mg/ml aflibercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 6.2.

Formulation Q: 150 mg/ml aflibercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

Formulation R: 150 mg/ml aflibercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

Formulation S: 150 mg/ml aflibercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

Formulation T: 150 mg/ml aflibercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2 (*e.g.*, 6.2), and, optionally, specifically excluding a viscosity reducing agent.

Formulation U: 150 mg/ml aflibercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

Formulation V: 150 mg/ml aflibercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

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Formulation W: 150 mg/ml aflibercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.
Formulation X: 150 mg/ml aflibercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.
Formulation Y: 80 mg/ml conbercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.
Formulation Z: 80 mg/ml conbercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.
Formulation AA: 80 mg/ml conbercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.
Formulation BB: 80 mg/ml conbercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 6.2.
Formulation CC: 80 mg/ml conbercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.
Formulation DD: 80 mg/ml conbercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.
Formulation EE: 80 mg/ml conbercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.
Formulation FF: 80 mg/ml conbercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.
Formulation GG: 80 mg/ml conbercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.
Formulation HH: 80 mg/ml conbercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.
Formulation II: 80 mg/ml conbercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

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Formulation JJ: 80 mg/ml conbercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.
Formulation KK: 150 mg/ml conbercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.
Formulation LL: 150 mg/ml conbercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.
Formulation MM: 150 mg/ml conbercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.
Formulation NN: 150 mg/ml conbercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 6.2.
Formulation OO: 150 mg/ml conbercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.
Formulation PP: 150 mg/ml conbercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.
Formulation QQ: 150 mg/ml conbercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.
Formulation RR: 150 mg/ml conbercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.
Formulation SS: 150 mg/ml conbercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.
Formulation TT: 150 mg/ml conbercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.
Formulation UU: 150 mg/ml conbercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.
Formulation VV: 150 mg/ml conbercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

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Formulation WW: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 50 mM taurine, with a pH of 5.8.

Formulation XX: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 mM histidine-based buffer, 4 % (w/v) proline, 0.03 % (w/v) polysorbate 20, and 50 mM arginine hydrochloride, with a pH of 5.8.

Formulation YY: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 mM histidine-based buffer, 2.5 % (w/v) sucrose, 2.0 % (w/v) proline, 0.03 % (w/v) polysorbate 20, and 50 mM taurine, with a pH of 5.8.

Formulation ZZ: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 mM histidine-based buffer, 2.5 % (w/v) sucrose, 2.0 % (w/v) proline, 0.03 % (w/v) polysorbate 20, and 50 mM arginine hydrochloride, with a pH of 5.8.

Formulation AAA: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 50 mM PSA, with a pH of 5.8.

Formulation BBB: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 mM histidine-based buffer, 2.5 % (w/v) sucrose, 2.0 % (w/v) proline, 0.03 % (w/v) polysorbate 20, and 50 mM PSA, with a pH of 5.8.

Formulation CCC: 80, 100, 120 or 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 50 mM arginine hydrochloride, with a pH of 5.8.

Formulation DDD: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 mM histidine-based buffer, 4 % (w/v) proline, 0.03 % (w/v) polysorbate 20, and 50 mM PSA, with a pH of 5.8.

Formulation EEE: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 mM histidine-based buffer, 5 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20 and, optionally, no thermal stabilizer, with a pH of 5.8.

Formulation FFF: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10mM sodium phosphate, 5 % (w/v) sucrose and 0.03 % polysorbate 20 with a pH of 6.2.

Formulation GGG: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5% sucrose; 0.03 % polysorbate 20; 50 mM sodium sulfate

Formulation HHH: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5% sucrose; 0.03 % polysorbate 20; 50 mM sodium thiocyanate

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Formulation III: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose, 0.03 % polysorbate 20; 40 mM sodium citrate
Formulation JJJ: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5% Sucrose, 0.03 % polysorbate 20; 50 mM glycine
Formulation KKK: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose, 0.03 % polysorbate 20; 50 mM sodium chloride
Formulation LLL: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose; 0.03 % polysorbate 20; 50 mM lysine
Formulation MMM: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose; 0.03% polysorbate 20; 50 mM sodium aspartate
Formulation NNN: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose; 0.03 % polysorbate 20; 50 mM sodium glutamate
Formulation OOO: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose; 0.03% polysorbate 20; 50 mM sodium citrate; 50 mM arginine hydrochloride
Formulation PPP: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose; 0.03% polysorbate 20; 50 mM glycine; 50 mM arginine hydrochloride
Formulation QQQ: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose; 0.03% polysorbate 20; 50 mM sodium aspartate; 50 mM arginine hydrochloride
Formulation RRR: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose; 0.03% polysorbate 20; 50 mM sodium glutamate; 50 mM arginine hydrochloride
Formulation SSS: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM His, pH 5.8; 5 % sucrose; 0.03 % polysorbate 20; 10 mM L-arginine hydrochloride
Formulation TTT: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM His, pH 5.8; 5 % sucrose; 0.03 % polysorbate 20; 100 mM L-arginine hydrochloride
Formulation UUU: 30 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, pH 6.2
Formulation VVV: 30 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, pH 6.2

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Formulation WWW: 60 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, pH 6.2
Formulation XXX: 60 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, pH 6.2
Formulation YYY: 120 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, pH 6.2
Formulation ZZZ: 120 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, pH 6.2
Formulation AAAA: 120 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, 50 mM NaCl, pH 6.2
Formulation BBBB: 120 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, 50 mM NaCl, pH 6.2
Formulation CCCC: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 mM sodium phosphate, 5 % sucrose, 40 mM sodium chloride, 0.03 % PS20, pH 6.2
Formulation DDDD: 80 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 50 mM L-arginine monohydrochloride, with a pH of 5.8.
Formulation EEEE: 120.0 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept) (*e.g.*, ± 12 mg/ml), 20 mM histidine-based buffer (*e.g.*, ± 2 mM), 5 % (w/v) sucrose (*e.g.*, ± 0.5%), 0.03 % (w/v) polysorbate 20 (*e.g.*, 0.02-0.04%), and 50 mM L-arginine monohydrochloride (*e.g.*, ± 5 mM), with a pH of 5.8 (*e.g.*, 5.6-6.0 or 5.5-6.1).
Formulation FFFF: 113.3 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept) (*e.g.*, 102-125 mg/ml), 20 mM histidine-based buffer (*e.g.*, ± 2 mM), 5 % (w/v) sucrose (*e.g.*, ± 0.5%), 0.03 % (w/v) polysorbate 20 (*e.g.*, 0.02-0.04%), and 50 mM L-arginine monohydrochloride (*e.g.*, ± 5 mM), with a pH of 5.8 (*e.g.*, 5.6-6.0 or 5.5-6.1).
Formulation GGGG: 114.3 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept) (*e.g.*, 103-126 mg/ml), 10 mM histidine-based buffer (*e.g.*, ± 1 mM), 5 % (w/v) sucrose (*e.g.*, ± 0.5%), 0.03 % (w/v) polysorbate 20 (*e.g.*, 0.02-0.04%), and 50 mM L-arginine (*e.g.*, L-arginine monohydrochloride) (*e.g.*, ± 5 mM), with a pH of 5.8 (*e.g.*, 5.6-6.0 or 5.5-6.1).
Formulation HHHH: 100.0 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept) (*e.g.*, ± 10 mg/ml), 20 mM histidine-based buffer (*e.g.*, ± 2 mM), 5 % (w/v) sucrose (*e.g.*, ± 0.5%), 0.03 % (w/v) polysorbate 20 (*e.g.*, 0.02-0.04%), and 50 mM L-arginine monohydrochloride (*e.g.*, ± 5 mM), with a pH of 5.8 (*e.g.*, 5.6-6.0 or 5.5-6.1).

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Formulation IIII: 133.3 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept) (*e.g.*, ± 13 mg/ml), 20 mM histidine-based buffer (*e.g.*, ± 2 mM), 5 % (w/v) sucrose (*e.g.*, ± 0.5%), 0.03 % (w/v) polysorbate 20 (*e.g.*, 0.02-0.04%), and 50 mM L-arginine monohydrochloride (*e.g.*, ± 5 mM), with a pH of 5.8 (*e.g.*, 5.6-6.0 or 5.5-6.1).

Formulation JJJJ: 150 mg/ml aflibercept (*e.g.*, aflibercept) (*e.g.*, ± 15 mg/ml), 10 mM sodium phosphate, 8% (w/v) sucrose (*e.g.*, ± 0.8%), 0.03% (w/v) polysorbate 20 (*e.g.*, 0.02-0.04%) and 50 mM L-arginine hydrochloride, pH 6.2 (*e.g.*, 6.0-6.4 or 5.9-6.5).

Formulation KKKK: 114.3 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept) (*e.g.*, ± 14 mg/ml), 20 mM histidine-based buffer (*e.g.*, ± 2 mM), 5% (w/v) sucrose (*e.g.*, ± 0.5%), 0.03% (w/v) polysorbate 20 (*e.g.*, 0.02-0.04%), and 50 mM L-arginine monohydrochloride (*e.g.*, ± 5 mM), with a pH of 5.8 (*e.g.*, 5.6-6.0 or 5.5-6.1).

[0113] In an embodiment of the invention the formulation that can be administered, *e.g.*, intravitreally, to a subject is an aqueous pharmaceutical formulation comprising:

at least about 100 mg/ml of a VEGF receptor fusion protein comprising two polypeptides that each comprises an immunoglobin-like (Ig) domain 2 of VEGFR1, an Ig domain 3 of VEGFR2, and a multimerizing component (*e.g.*, aflibercept);

about L-arginine (e.g., at a concentration of about 10-100 mM);

sucrose;

a histidine-based buffer; and

a surfactant;

wherein the formulation has a pH of about 5.0 to about 6.8; wherein the VEGF receptor fusion protein has less than about 3.5% high molecular weight species immediately after manufacture and purification and/or less than or equal to about 6% high molecular weight species after storage for about 24 months at about 2-8°C.

[0114] See International Patent Application Publication No. WO2019/217927 and US Patent No. 11103552.

Treatment and Administration

[0115] The present invention provides methods for treating angiogenic eye disorders by sequentially administering initial loading doses of greater than or equal to (≥) about 8 mg (*e.g.*, about every 2-4 or 3-5 weeks) of VEGF antagonist (*e.g.*, aflibercept) followed by additional doses of ≥ about 8 mg every 12 weeks of VEGF antagonist (*e.g.*, aflibercept). For example, in an embodiment of the invention, the methods of the present invention

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include treating or preventing angiogenic eye disorders, such as diabetic retinopathy, diabetic macular edema or neovascular AMD, by administering, sequentially, one or more (*e.g.*, 3, 4 or 5) doses (*e.g.*, ≥ about 8 mg) about every month (or about every 28 days, 28 ± 5 days or about every 4 weeks), followed by one or more doses (*e.g.*, ≥ about 8 mg) about every 12 weeks. For example, in an embodiment of the invention, about 8 mg is 7.2 mg, 8.8 mg or 7.2-8.8 mg or 8 mg ± about 10%.

[0116] The terms "initial dose," "secondary doses," and "tertiary doses," refer to the temporal sequence of administration of the VEGF antagonist (e.g., aflibercept). Thus, the "initial dose" is the dose which is administered at the beginning of the treatment regimen (also referred to as the "baseline dose"); the "secondary doses" are the doses which are administered after the initial dose; and the "tertiary doses" are the doses which are administered after the secondary doses. The initial dose occurs on day 1 for the purposes of counting or numbering days thereafter (see *e.g.*, Tables 1-1 and 1-2 herein). The initial, secondary, 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. Thus, a dosing regimen of the present invention may be expressed as follows:

a single initial dose (*e.g.*, ≥ about 8 mg) of a VEGF antagonist (*e.g.*, aflibercept), followed by one or more (*e.g.*, 2, or 3 or 4) secondary doses of the VEGF antagonist, followed by one or more tertiary doses of the VEGF antagonist;

wherein each secondary dose is administered 2 to 4 weeks after the immediately preceding dose; and

wherein each tertiary dose is administered about 12 weeks after the immediately preceding dose. The initial and secondary doses administered before the tertiary doses may be referred to, generally, as "loading" doses. The tertiary doses may be referred to as "maintenance" doses.

[0117] The present invention includes methods wherein one or more additional, non-scheduled, *pro re nata* (PRN) doses, in addition to any of the scheduled initial, secondary and/or tertiary doses of VEGF antagonist (*e.g.*, aflibercept) are administered to a subject. Such PRN doses are typically administered at the discretion of the treating physician depending on the particular needs of the subject.

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[0118] In an embodiment of the invention,

- the method comprises administering the secondary doses to a subject who has received the initial dose
- the method comprises administering the remaining secondary doses to a subject who has already received one or more secondary doses;
- the method comprises administering one or more tertiary doses to a subject who has already received the secondary doses
- the method comprises administering one or more tertiary doses to a subject who has already received one or more tertiary doses;

optionally wherein the subject receives doses, earlier in the regimen, in one pharmaceutical formulation and additional doses, later in the regimen, in a different pharmaceutical formulation, for example, comprising a different buffer (*e.g.*, wherein one or more of the secondary doses are in one pharmaceutical formulation and the tertiary doses are in a different pharmaceutical formulation).

[0119] Dosing every "month" refers to dosing about every 28 days, about every 4 weeks, or about every 28 $\pm$ 5 days and may encompass up to every 5 weeks. Dosing every "4 weeks" refers to dosing about every 28 days, about every month or about every 28 $\pm$ 5 days, and may encompass up to every 5 weeks.

[0120] Dosing every "2-4 weeks" refers to dosing about every 2 weeks, 3 weeks or 4 weeks. Dosing every "8 weeks" refers to dosing about every 2 months, about every 56 days, 56 $\pm$ 5 days.

[0121] Dosing every "12 weeks" refers to dosing about every 3 months, about every quarter year, about every 84, 90 days, 84 $\pm$ 5 days, or 90 $\pm$ 5 days.

[0122] A dose of greater than or equal to about 8 mg or $\geq$ about 8 mg VEGF antagonist (e.g., aflibercept) includes 7.2 mg; 7.2-8.8 mg; 8.0 mg; 8.01 mg; 8.1 mg; 8.2 mg; 8.3 mg; 8.4 mg; 8.5 mg; 8.6 mg; 8.7 mg; 8.8 mg; 8.9 mg; 9 mg; 9.1 mg; 9.2 mg; 9.3 mg; 9.4 mg; 9.5 mg; 9.6 mg; 9.7 mg; 9.8 mg; 9.9 mg, 10.0 mg, 10.1 mg; 10.2 mg; 10.3 mg; 10.4 mg; 10.5 mg; 10.6 mg; 10.7 mg; 10.8 mg; 10.9 mg; 11 mg; 11.1 mg; 11.2 mg; 11.3 mg; 11.4 mg; 11.5 mg; 11.6 mg; 11.7 mg; 11.8 mg; 11.9 mg; 12 mg; 12.1 mg; 12.2 mg; 12.3 mg; 12.4 mg; 12.5 mg; 12.6 mg; 12.7 mg; 12.8 mg; 12.9 mg; 13 mg; 13.1 mg; 13.2 mg; 13.3 mg; 13.4 mg; 13.5 mg; 13.6 mg; 13.7 mg; 13.8 mg; 13.9 mg; 14 mg; 14.1 mg; 14.2 mg; 14.3 mg; 14.4 mg; 14.5 mg; 14.6 mg; 14.7 mg; 14.8 mg; 14.9 mg; 15 mg; 15.1 mg; 15.2 mg; 15.3 mg; 15.4 mg; 15.5 mg; 15.6 mg; 15.7 mg; 15.8 mg; 15.9 mg; 16 mg; 16.1 mg; 16.2 mg; 16.3 mg; 16.4 mg; 16.5

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mg; 16.6 mg; 16.7 mg; 16.8 mg; 16.9 mg; 17 mg; 17.1 mg; 17.2 mg; 17.3 mg; 17.4 mg; 17.5 mg; 17.6 mg; 17.7 mg; 17.8 mg; 17.9 mg; 18 mg; 18.1 mg; 18.2 mg; 18.3 mg; 18.4 mg; 18.5 mg; 18.6 mg; 18.7 mg; 18.8 mg; 18.9 mg; 19 mg; 19.1 mg; 19.2 mg; 19.3 mg; 19.4 mg; 19.5 mg; 19.6 mg; 19.7 mg; 19.8 mg; 19.9 mg; or 20 mg (± about 10%, ± about 0.5, or ± about 0.51 mg of any of the foregoing). In an embodiment of the invention, a dosage of ≥ 8 mg VEGF antagonist is administered in a dose having a volume of about 100 μl or less, about 75 μl or less or about 70 μl or less, *e.g.*, about 50 μl; 51 μl; 52 μl; 53 μl; 54 μl; 55 μl; 56 μl; 57 μl; 58 μl; 59 μl; 60 μl; 61 μl; 62 μl; 63 μl; 64 μl; 65 μl; 66 μl; 67 μl; 68 μl; 69 μl; 70 μl; 71 μl; 72 μl; 73 μl; 74 μl; 75 μl; 76 μl; 77 μl; 78 μl; 79 μl; 80 μl; 81 μl; 82 μl; 83 μl; 84 μl; 85 μl; 85-87μl; 86 μl; 87 μl; 88 μl; 89 μl; 90 μl; 91 μl; 92 μl; 93 μl; 94 μl; 95 μl; 96 μl; 97 μl; 98 μl; 99 μl; or 100 μl (± about 4, 4.45, 4.5, or 5 microliters).

[0123] Any dosing frequency specified herein may, in an embodiment of the invention, be expressed as the specific frequency "± 5 days" (*e.g.*, where "4 weeks" is stated, the present invention also includes embodiments such as 4 weeks ± 5 days).

[0124] "Sequentially administering" means that each dose of VEGF antagonist (e.g., aflibercept) 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). The present invention includes methods which comprise sequentially administering, to the eye of a patient, a single initial dose of a VEGF antagonist, followed by one or more secondary doses of the VEGF antagonist, followed by one or more tertiary doses of the VEGF antagonist.

[0125] An effective or therapeutically effective dose of VEGF antagonist, *e.g.*, aflibercept, for treating or preventing an angiogenic eye disorder refers to the amount of VEGF antagonist sufficient to alleviate one or more signs and/or symptoms of the disease or condition in the treated subject, whether by inducing the regression or elimination of such signs and/or symptoms or by inhibiting the progression of such signs and/or symptoms. In an embodiment of the invention, an effective or therapeutically effective dose of VEGF antagonist is ≥ about 8 mg every month followed by once every 12 weeks.

[0126] An "angiogenic eye disorder" 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 or preventable using the methods of the present invention include:

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- age-related macular degeneration (neovascular (nAMD)),
- macular edema (ME),
- macular edema following retinal vein occlusion (ME-RVO),
- retinal vein occlusion (RVO),
- central retinal vein occlusion (CRVO),
- branch retinal vein occlusion (BRVO),
- diabetic macular edema (DME),
- choroidal neovascularization (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,
- diabetic retinopathies (DR) (*e.g.*, non-proliferative diabetic retinopathy (*e.g.*, characterized by a Diabetic Retinopathy Severity Scale (DRSS) level of about 47 or 53) or proliferative diabetic retinopathy; *e.g.*, in a subject that does not suffer from DME), and
- diabetic retinopathy in a patient who has diabetic macular edema (DME).

[0127] In an embodiment of the invention, a subject receiving a treatment for an angiogenic eye disorder as set forth herein (*e.g.*, three monthly doses of about 8 mg aflibercept followed by doses of about 8 mg aflibercept every 12 weeks) achieves one or more of the following:

- with respect to visual acuity (VA) or best corrected visual acuity (BCVA), achieving:
  - No loss in visual acuity or BCVA or a gain in visual acuity or BCVA;
  - no loss of visual acuity or BCVA, for example, by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44, following the initial dose *e.g.*, according to ETDRS (Early

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Treatment Diabetic Retinopathy Study) chart or Snellen equivalent (*e.g.*, no loss of 5, 10, or 15 or more ETDRS letters (*e.g.*, no loss of 5 (or more), 6 (or more), 7 (or more), 8 (or more), 9 (or more), 10 (or more), 11 (or more), 12 (or more), 13 (or more), 14 (or more) or 15 (or more) letters) or Snellen equivalent),

- a gain in visual acuity or BCVA, for example, by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44 following the initial dose, *e.g.*, according to ETDRS chart or Snellen equivalent (*e.g.*, gaining 5 or more, 10 or more or 15 or more ETDRS letters (*e.g.*, a gain of 5 (or more), 6 (or more), 7 (or more), 8 (or more), 9 (or more), 10 (or more), 11 (or more), 12 (or more), 13 (or more), 14 (or more) or 15 (or more) letters)); and/or
- a gain in BCVA of about 6 or 7 or 8 letters (or more) (*e.g.*, according to ETDRS chart or Snellen equivalent) by week 8 and maintaining a gain of about 6 or 7 or 8 letters until at least about week 44;

- with respect to central retinal thickness (CRT), achieving:
  - a decrease in central retinal thickness;
  - a decrease in central retinal thickness by at least about 123, 125, 131, 142, 147, 149, 150, 151, 156, 157, 158, 159, 161, 162, 166, 167, 168, 172, 173, 175, 177, 178 or 183 micrometers (or more), for example, by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44 following the initial dose, for example, wherein the baseline (prior to treatment) CRT, is about 488, 492, 497 or 516 micrometers;
  - a decrease in CRT of about 47 micrometers (or more) from about week 12 to about week 20 following the initial dose, *e.g.*, wherein the baseline CRT is about 516 micrometers;
  - a decrease in CRT of about 17 micrometers (or more) from about week 24 to about week 32 following the initial dose, *e.g.*, wherein the baseline CRT is about 516 micrometers;
  - a decrease in CRT of about 18 micrometers (or more) from about week 36 to about week 44 following the initial dose, *e.g.*, wherein the baseline CRT is about 516 micrometers;
  - a decrease in central retinal thickness by at least about 4 or 24.3 micrometers (or more) from about week 12 to about week 16, for example, wherein the baseline CRT, prior to treatment, is about 516 micrometers;

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- a decrease in CRT of about 123, 131 161 micrometers (or more) (*e.g.*, by about week 4, 8, 12, 16 or 20) and maintaining the decrease until at least about week 44 following the initial dose; and/or
- a reduction in CRT of about 159, 160, 161 or 162 micrometers (or more) by about week 4 or 8 or 12 and maintaining a reduction of about 159, 160, 161 or 162 micrometers (or more) until at least about week 44;

- with respect to retinal fluid, achieving:
  - a dry retina (*e.g.*, no IRF and no SRF; or no IRF; or no SRF, *e.g.*, in the center subfield or in the macula, *e.g.*, on SD-OCT);
  - no fluid in the center subfield (*e.g.*, no IRF and no SRF; or no IRF; or no SRF) by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44 following the initial dose (*e.g.*, as measured by SD-OCT);
  - no sub-retinal pigment epithelium (RPE) fluid; *e.g.*, by about week 16, *e.g.*, until at least about week 44 following the initial dose (*e.g.*, as measured by SD-OCT);
  - no SRF and IRF in the macula, *e.g.*, by SD-OCT, *e.g.*, by week 16 or week 44 following the initial dose, and/or
  - maintenance of a dry retina, *e.g.*, once achieved (*e.g.*, at 16 weeks following the initial dose); until at least about week 44 (*e.g.*, as measured by SD-OCT);
- achieving:
  - reduction in total lesion size by at least about 3.3 μm; and/or choroidal neovascularization (CNV) size of at least about 3.2 μm from baseline by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44, *e.g.*, wherein baseline total lesion size is about 7.7 μm and/or baseline CNV size is about 7.5 μm;
  - no significant increase in intraocular pressure from baseline by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44 or later (*e.g.*, by no more than about 0.2 or 0.5 mmHg); and/or
  - no significant increase in blood pressure (*e.g.*, systolic (S) or diastolic (D)) from baseline by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44 or later (*e.g.*, by no more than about 0.1 (S) or 0.9 (S), 1.0 (D) or 1.4 (D) mmHg); *e.g.*, wherein baseline systolic pressure is about 125 mmHg or 129 mmHg and/or baseline diastolic pressure is about 72 mmHg or 74 mmHg;

and/or

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- with respect to efficacy or safety:
  - Efficacy and/or safety, in a subject suffering from DR or DME, similar to or greater than that of aflibercept which is intravitreally dosed at 2 mg approximately every 4 weeks for the first 5 injections followed by 2 mg approximately once every 8 weeks or once every 2 months, *e.g.*, wherein efficacy is measured as an increase in visual acuity or BCVA and/or a reduction in central retinal thickness, achievement of dry retina (*e.g.*, no IRF and/or SRF), *e.g.*, wherein safety is as measured as the incidence of adverse events (treatment-emergent adverse events occurring anytime within 30 days of any injection) such as intraocular inflammation, clinically significant blood pressure increase, clinically significant intraocular pressure increase, visual impairment, vitreous floaters, vitreous detachment, iris neovascularization and/or vitreous hemorrhage; and/or
  - Efficacy and/or safety, in a subject suffering from nAMD, similar to or greater than that of aflibercept which is intravitreally dosed at 2 mg approximately every 4 weeks for the first 3 injections followed by 2 mg approximately once every 8 weeks or once every 2 months, *e.g.*, wherein efficacy is measured as an increase in visual acuity or BCVA and/or a reduction in central retinal thickness, achievement of dry retina (*e.g.*, no IRF and/or SRF), *e.g.*, wherein safety is as measured as the incidence of adverse events (treatment-emergent adverse events occurring anytime within 30 days of any injection) such as intraocular inflammation, clinically significant blood pressure increase, clinically significant intraocular pressure increase, visual impairment, vitreous floaters, vitreous detachment, iris neovascularization and/or vitreous hemorrhage;

for example, wherein such an effect (*e.g.*, improvement of BCVA, CRT and/or retinal fluid) are achieved and/or maintained for as long as the subject is receiving the treatment regimen.

[0128] The center subfield of the retina is a 1 mm diameter area around the macula. The macula itself is about 6 mm in diameter.

[0129] The present invention also includes methods for achieving any one or more of the foregoing in a subject (*e.g.*, increase in VA or BCVA, or decrease in CRT) suffering from an angiogenic eye disorder, *e.g.*, nAMD, DR or DME, comprising administering to an eye of the subject, a single initial dose of about 8 mg or more of a VEGF antagonist (e.g., aflibercept),

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followed by one or more (*e.g.*, 3, 4 or 5) secondary doses of about 8 mg or more of the VEGF antagonist, followed by one or more tertiary doses of about 8 mg or more of the VEGF antagonist; wherein each secondary dose is administered about 2 to 4 weeks after the immediately preceding dose; and wherein each tertiary dose is administered about 12 weeks after the immediately preceding dose.

[0130] The present invention includes methods for treating or an angiogenic eye disorder (*e.g.*, nAMD, DR, DME or ME-RVO), in a subject in need thereof, comprising administering to an eye of the subject, a single initial dose of about 8 mg or more of a VEGF antagonist (e.g., aflibercept), followed by one or more secondary doses of about 8 mg or more of the VEGF antagonist, followed by one or more tertiary doses of about 8 mg or more of the VEGF antagonist; wherein each secondary dose is administered about 2 to 4 weeks after the immediately preceding dose; and wherein each tertiary dose is administered about 12 weeks after the immediately preceding dose;

wherein the subject achieves a change in central retinal thickness, from baseline at the initiation of treatment, as depicted in Figure 54 or Figure 55 (or a greater reduction), *e.g.*, by the timepoint shown for such a change; and/or

wherein the subject achieves a change in central retinal thickness, during the time period as set forth in the inset box of Figure 54 (or a greater reduction),

and/or

wherein the subject achieves a change in BCVA, from baseline at the initiation of treatment, as depicted in Figure 56 (or a greater increase), *e.g.*, by the timepoint shown for such a change (such change in BCVA can be in terms of ETDRS letters gained or in the Snellen equivalent); and/or

wherein the subject does not experience any one or more of the ocular TEAEs set forth in Figure 58, ocular serious TEAEs set forth in Figure 59, intraocular inflammation, any of the intraocular pressure events set forth in Figure 61, non-ocular TEAEs set forth in Figure 63, non-ocular serious TEAEs set forth in Figure 64, hypertension TEAEs set forth in Figure 65 and/or an APTC event.

Best Corrected Visual Acuity (BCVA)

[0131] Best corrected visual acuity (BCVA) can be measured in various methods known in the art. First, determining the proper level of lens refraction needed to best correct the

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visual acuity (VA) of a subject is determined before testing best corrected visual acuity (BCVA) with a visual acuity chart.

[0132] Two separate VA charts used for testing the right and left eye (*e.g.*, Sloan Letter ETDRS Chart 1 and Sloan Letter ETDRS Chart 2, respectively), and a third refraction chart is used for testing appropriate refraction (*e.g.*, Sloan Letter ETDRS Chart R). The features of the Sloan charts are high-contrast Sloan letters of equal difficulty, 5 letters in each of 14 rows, and a geometric progression of letter size (and, thus, an arithmetic progression of the logarithm of minimum angle of resolution [LogMAR]) from row to row. The charts have different letter sequences.

[0133] There are three basic components to determining refraction according to ETDRS protocol-determining spherical power, determining cylindrical axis and determining cylindrical power using methods known by practitioners in the art. For assessing refraction, if the subject wears contact lenses and has glasses, he or she should be told not to wear the contact lenses on the day of the examination or remove them 30-60 minutes before refraction is done. A trial frame is placed and adjusted on the subject's face so that lens cells placed in the frame are parallel to the anterior plane of the orbits and centered in front of the pupils. (It is permissible to use a phoroptor for subjective refraction. However, for testing visual acuity, the lenses from the final phoroptor refraction must be placed in a frame, and the final sphere must be rechecked).

[0134] BCVA can be measured first in one eye with a visual acuity chart, and then in the other eye with another visual acuity chart (*e.g.*, Charts 1 and 2 as discussed herein), wherein each chart remains hidden from view until the eye in question is ready for testing. The distance from the subject's eyes to the visual acuity chart is typically 4 meters (13 feet and 1.5 inches, or 157.5 inches). The subject should be asked to read slowly (*e.g.*, at a rate not faster than about one letter per second). Eyes reading 19 or fewer letters correctly at 4 meters can be tested at 1 meter.

[0135] Two commonly used tools for testing visual acuity (VA) or BCVA include the Snellen and the Early Treatment Diabetic Retinopathy Study (ETDRS) VA charts (Early Treatment Diabetic Retinopathy Study research group. Photocoagulation for diabetic macular edema. Early Treatment Diabetic Retinopathy Study report number 1, Arch Ophthalmol. 1985 Dec;103(12):1796-806; Chen *et al.*, Comparison of visual acuity estimates using three different letter charts under two ambient room illuminations. 2012;60(2):101-104; Bailey & Lovie, New design principles for visual acuity letter charts,

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1976;53(11):740-745; Shamir *et al.*, Comparison of Snellen and Early Treatment Diabetic Retinopathy Study charts using a computer simulation, Int. J. Opthamology 9(1): 119-123 (2016); Kaiser, Prospective Evaluation of Visual Acuity Assessment: A Comparison of Snellen Versus ETDRS Charts in Clinical Practice (An AOS Thesis), Trans Am Ophthalmol Soc 2009;107:311-324). A version of the Bailey-Lovie chart (Bailey & Lovie, New design principles for visual acuity letter charts. Am J Optometry Physiol Opt 1976;53:740-745) was modified in 1982 based on the recommendations of the Committee on Vision of the National Academy of Sciences, National Research Council, and Working Group 39, and by Dr. Rick Ferris for use in the Early Treatment Diabetic Retinopathy Study (ETDRS). The "ETDRS chart" and the protocol to test vision with the chart is commonly used in clinical trials. The ETDRS chart is typically tested from a shorter distance (13 feet (or 4 meters) rather than 20 feet) than Snellen, but does not allow the use of mirrors to simulate the correct distance, has the same amount of letters in every row (five letters each), and has an equal spacing of both the letters and the rows on a logarithmic scale. The Snellen Chart uses a geometric scale to measure visual acuity, with normal vision at a distance being set at 20/20. In an embodiment of the invention, VA or BCVA can be expressed in terms of ETDRS or Snellen. ETDRS VA values can be converted to a corresponding Snellen equivalent using methods known in the art. In an embodiment of the invention, VA or BCVA is measured with an ETDRS chart or with a Snellen chart.

Precision Dose Drug Delivery

[0136] The present invention provides methods as set forth herein wherein a VEGF antagonist (*e.g.*, aflibercept) is delivered with a high amount of precision, *e.g.*, with a drug delivery device (DDD) (*e.g.*, with a 0.5 mL volume), whether pre-filled or capable of being filled from a vial, and delivering a volume of between 70 and 100 microliter with an average volume of about 81 or 82 or 81-82 microliters, *e.g.*, with a standard deviation of about 4 or 5 or 4-5 microliters (*e.g.*, about 4.5 or 4.46 microliters) or less. In an embodiment of the invention, the DDD is a syringe, *e.g.*, with a 30 gauge, ½ inch needle.

[0137] One means for ensuring precision of a dose to be delivered with a device, such as a syringe, is by employing a syringe wherein the dose volume is device-determined. If the dose volume is device-determined, the device is designed only to deliver a single volume (*e.g.*, 87 microliters) or a single volume with a limited amount of acceptable error (± 4-5

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microliters). Thus, if used properly, the user cannot deliver the wrong dose (*e.g.*, cannot deliver more than the intended volume from the device).

[0138] The present invention includes embodiments wherein, a precise dosage of about 8 mg or more is a dose of about 9, 9.3, 9.33, 9.7, 9.8, 9.9, 9.7-9.9 mg or more ± about 0.5, or ± about 0.51 mg is delivered to a subject's eye. The volume in which a dose is delivered can be, for example, about 70, 81, 82, 81.7, 85, 86, 87, 85-87 microliters ± about 4, 4.45, 4.5, or 5 microliters. Doses may be delivered with a dose delivery device (DDD) which is a syringe.

[0139] Highly precise doses of VEGF antagonist (*e.g.*, aflibercept) may be delivered, for example, in a volume that is device-determined (wherein the device is a syringe), by a method that includes the steps: (a) priming the syringe (*e.g.*, a pre-filled syringe), thereby removing air from the syringe and, thus avoiding injection of air into the eye, by advancing the plunger rod by a predetermined distance into the syringe body until advancement of the plunger rod is resisted by a stop; (b) rotating the plunger rod about a longitudinal axis; and (c) actuating the plunger rod to dispense a predetermined (device-determined) volume (*e.g.*, about 70, 81, 82, 81.7, 85, 86, 87, 85-87 microliters, ± about 4, 4.45, 4.5, or 5 microliters) of the formulation.

[0140] In an embodiment of the invention, the drug delivery device (DDD), comprises:

- a barrel including a longitudinal axis, a proximal end region, and a distal end region, the proximal end region including an opening, wherein the barrel is configured to receive a drug therein;
- a plunger rod disposed at least partially inside the barrel and protruding from the opening, wherein the plunger rod includes a rack having a plurality of teeth; and
- a pinion having a plurality of teeth configured to engage with the plurality of teeth of the rack,

wherein rotation of the pinion against the rack moves at least a part of the plunger rod along the longitudinal axis of the barrel; for example, which further comprises a shaft affixed to the pinion, wherein rotation of the shaft rotates the pinion against the rack which may include a knob affixed to the shaft. In an embodiment of the invention, the DDD further includes a magnifier disposed on the distal end region of the barrel. In an embodiment of the invention, the DDD further includes a stopper inside the barrel, wherein the stopper is affixed to a distal end of the plunger rod. In an embodiment of the invention, the DDD

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further includes a circular ratchet disposed coaxially with the pinion, wherein the circular ratchet has a diameter smaller than a diameter of the pinion; a spring-loaded pawl disposed on an internal circumference of the pinion, wherein the pawl is configured to engage the ratchet; and a shaft affixed to the ratchet, wherein rotation of the shaft in one direction causes rotation of the pinion, and rotation of the shaft in a second direction does not cause rotation of the pinion for example wherein the ratchet is disposed inside the pinion. In an embodiment of the invention, the pinion includes a plurality of teeth having a first height, and a stopper tooth having a second height greater than the first height, for example, wherein the second height of the stopper tooth prevents the pinion from engaging the plurality of teeth of the rack, and/or wherein the second height of the stopper tooth is configured to contact one of the plunger rod and the rack to stop rotation of the pinion. In an embodiment of the invention, the plunger rod includes an inner column and an outer lumen, and wherein the rack is disposed on the inner column, *e.g.*, wherein rotation of the pinion against the rack moves the inner column of the plunger rod independently of the outer lumen, and/or further including a shaft removably affixed to the pinion, wherein the shaft prevents movement of the outer lumen of the plunger rod relative to the barrel, and wherein removal of the shaft allows for movement of the outer lumen of the plunger rod relative to the barrel. In an embodiment of the invention, the plunger rod further includes a body and a flange, the flange extending partially along a longitudinal length of the body and having a width greater than a width of the body; wherein the barrel further comprises a plunger lock, the plunger lock including a through hole configured to allow the flange to pass through the second plunger lock in a specific orientation.

[0141] In an embodiment of the invention, the drug delivery device (DDD), comprises:

- a barrel including a longitudinal axis, a proximal end region, a distal end region, and an interior, the proximal end region including an opening and the interior including a threaded region; and
- a plunger rod disposed at least partially inside the barrel and protruding from the opening, the plunger rod including a threaded region configured to engage the threaded region of the barrel interior,

wherein rotation of the plunger rod about the longitudinal axis of the drug delivery device moves the plunger rod along the longitudinal axis. In an embodiment of the invention, the plunger rod further includes a tab protruding from the plunger rod in a first direction and

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located proximally from the threaded region of the plunger rod, and wherein the threaded region in the interior of the barrel further includes a slot sized and configured to allow for the tab to pass through the threaded region in the interior of the barrel, *e.g.*, wherein the slot includes a first segment parallel to the longitudinal axis of the drug delivery device and a second segment perpendicular to the longitudinal axis of the drug delivery device—the slot may include a third segment parallel to the longitudinal axis of the drug delivery device, wherein the second segment is in between the first segment and the third segment. In an embodiment of the invention, the tab is a first tab, and wherein the plunger rod further includes a second tab protruding from the plunger rod in a second direction opposite to the first direction, and wherein the threaded region in the interior of the barrel further includes a second slot sized and configured to allow for the second tab to pass through the threaded region in the interior of the barrel.

[0142] In an embodiment of the invention, the drug delivery device, includes:

- a barrel having a proximal end region, a distal end region, an opening in the proximal end region, an interior, and a threaded region in the interior;
- a sleeve disposed partly inside the barrel and protruding from the opening in the proximal end region of the barrel, the sleeve including a threaded region engaged with the threaded region of the barrel interior;
- a plunger rod disposed at least partially inside the sleeve; and
- a stopper inside the barrel and located distally from the sleeve, the stopper connected to a distal end of the plunger rod,

wherein rotation of the sleeve in a first direction around a longitudinal axis of the drug delivery device moves the sleeve towards the distal end region of the barrel. In an embodiment of the invention, rotation of the sleeve in the first direction moves the stopper towards the distal end region of the barrel. In an embodiment of the invention; the sleeve includes an inner passage, and the stopper has a diameter larger than a diameter of the inner passage; and/or the sleeve includes a tab disposed on an exterior of the sleeve, the tab located proximally from the threaded region of the barrel interior, and wherein the tab stops movement of the sleeve towards the distal end region of the barrel, *e.g.*, wherein the tab is configured to stop movement of the sleeve towards the distal end region of the barrel after the drug delivery device has been primed or wherein the tab is a first tab, and wherein the sleeve further includes a second tab disposed on an exterior of the sleeve, the second

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tab located distally from the threaded region of the barrel interior, wherein the second tab stops movement of the sleeve towards the proximal end region of the barrel.

[0143] In an embodiment of the invention, the drug delivery device, comprises:

- a barrel including a proximal end region and a distal end region, the proximal end region including an opening;
- a plunger rod including a body and a flange, the flange extending partially along a longitudinal length of the body and having a width greater than a width of the body, the plunger rod disposed at least partially inside the barrel and protruding from the opening;
- a first plunger lock disposed on the barrel, the first plunger lock configured to block the flange from entering the barrel; and
- a second plunger lock disposed in the barrel, the second plunger lock including a through hole configured to allow the flange to pass through the second plunger lock in a specific orientation.

For example, in an embodiment of the invention, the first plunger lock is removable and/or frangible. In an embodiment of the invention, a distance between the first plunger lock and the second plunger lock is equivalent to the distance that the stopper must travel to prime the drug delivery device; and/or the plunger rod is rotatable around a longitudinal axis of the drug delivery device.

[0144] Substances from such a DDD (*e.g.*, a formulation including aflibercept as described herein), having a plunger rod and a barrel, may be dispensed as follows:

- advancing the plunger rod by a predetermined distance into the barrel until advancement of the plunger rod is resisted by a stop;
- deactivating the stop; and
- actuating the plunger rod (*e.g.*, which includes a flange, wherein the stop includes a lock that prevents the flange from entering the barrel; or which includes a flange, wherein the stop comprises a lock that prevents the flange from entering the barrel) to deliver the substance.

Advancing the plunger rod may include the step of rotating a pinion against a rack disposed on the plunger rod, *e.g.*, wherein the stop comprises a shaft removably affixed to the pinion, and wherein deactivating the stop comprises removing the shaft from the pinion. Deactivating the stop may include the step of rotating the plunger rod. In an embodiment of

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the invention, deactivating the stop includes the step of removing the lock and/or breaking the lock.

[0145] In an embodiment of the invention, the drug delivery device, includes:

- a barrel including a longitudinal axis, a proximal end region, and a distal end region, the proximal end region including an opening and a rack disposed on the interior of the barrel, the rack having a plurality of teeth, wherein the barrel is configured to receive a drug therein;
- a plunger rod disposed at least partially inside the barrel and protruding from the opening, wherein the plunger rod includes a rack having a plurality of teeth; a pinion having a plurality of teeth configured to engage with the plurality of teeth of the plunger rod rack; and
- an inner plunger coupled to the pinion by a rod, wherein rotation of the pinion against the plunger rod rack results in movement of the inner plunger along the longitudinal axis of the barrel;

for example, wherein the teeth of the pinion are further configured to engage with the plurality of teeth of the rack disposed on the barrel. In an embodiment of the invention, the pinion is a first pinion, and further includes: a second pinion disposed coaxially with the first pinion, the second pinion having a diameter smaller than a diameter of the first pinion and a plurality of teeth configured to engage with the plurality of the teeth of the rack disposed on the barrel, wherein rotation of the first pinion results in rotation of the second pinion against the rack disposed on the barrel and in movement of the inner plunger along the longitudinal axis of the barrel.

[0146] See International patent application publication no. WO2019/118588.

[0147] In an embodiment of the invention, the drug delivery device (DDD), includes:

- a body;
- a plunger rod disposed partially inside the body;
- a protrusion extending from the plunger rod; and
- a blocking component coupled to a proximal end portion of the body, wherein the blocking component is a flange piece,

wherein, when the protrusion is in a first position relative to the blocking component, the blocking component restricts distal movement of the plunger rod to a first stopping point, and when the protrusion is in a second position relative to the blocking component, the

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blocking component restricts distal movement of the plunger rod to a second stopping point. In an embodiment of the invention, the DDD further includes: a stopper disposed in the body, wherein distal movement of the plunger rod distally moves the stopper; and a drug substance disposed in the body in between the stopper and a distal end of the body, wherein distal movement of the plunger rod to the first stopping point primes the drug delivery device, and distal movement of the plunger rod to the second stopping point dispenses a predetermined volume of the drug substance from a distal end of the device. In an embodiment of the invention, moving the protrusion from the first position to the second position includes twisting the plunger rod relative to the blocking component. In an embodiment of the invention, the DDD further includes: a cavity in a proximal side of the blocking component, the cavity sized and configured to receive a portion of the protrusion, wherein when the protrusion is in the second position relative to the blocking component, the protrusion is positioned proximally from the cavity, such that distal movement of the plunger rod moves the protrusion into the cavity; *e.g.*, wherein the cavity is a first cavity, and further includes: a second cavity in a proximal side of the blocking component, the second cavity sized and configured to receive a portion of the protrusion, wherein the first and second cavity are located on opposite sides of a central longitudinal axis of the drug delivery device. In an embodiment of the invention, the plunger rod passes through an opening in the blocking component. In an embodiment of the invention the DDD further includes an actuation portion at a proximal end portion of the plunger rod, wherein the protrusion extends from the actuation portion, *e.g.*, wherein the actuation portion includes a generally cylindrical shape having a diameter greater than a width of the remainder of the plunger rod, wherein the protrusion extends from a side of the generally cylindrical shape, and wherein the actuation portion further comprises: a thumb pad on a proximal end of the actuation portion, and a ring on an exterior surface on the side of the generally cylindrical shape; *e.g.*, further including a proximal collar on the blocking component, wherein the actuation portion partially fits inside the proximal collar; *e.g.*, wherein the plunger rod further includes a pair of extensions protruding distally from the actuation portion and the blocking component (*e.g.*, which includes one or more indents formed along a bottom wall of the blocking component; and wherein a portion of each extension is configured to be received by the one or more indents upon distal movement of the plunger rod relative to the blocking component to allow distal movement of the plunger rod to the second stopping point; or, which includes one or more indents formed along a bottom wall of the blocking component;

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and wherein a portion of each extension is configured to be received by the one or more indents upon distal movement of the plunger rod relative to the blocking component to allow distal movement of the plunger rod to the second stopping point; or, which includes a pair of internal grooves formed along a sidewall of the blocking component; and wherein a portion of each extension is configured to be received by at least one of the pair of internal grooves upon rotation of the plunger rod relative to the blocking component to expand the extensions radially-outward from a compressed state to a relaxed state) includes a pair of openings; and wherein a portion of each extension is configured to be received by one of the pair of openings in the first stopping point. In an embodiment of the invention, the protrusion is a first protrusion, and further includes a second protrusion extending from the plunger rod in a direction opposite to the first protrusion. In an embodiment of the invention, the blocking component is slidably coupled to the body and includes a third cavity and a pair of ribs that extend into the third cavity, wherein the body includes a top flange and the pair of ribs are configured to engage the top flange received in the third cavity; wherein the pair of internal ribs are configured to apply a distally-directed force onto the top flange. In an embodiment of the invention, the blocking component is slidably coupled to the body and includes a pair of movable tabs that are configured to engage the body; and the pair of movable tabs are laterally deflectable upon receiving the body in the blocking component and are configured to apply a radially-inward directed force onto the body. In an embodiment of the invention, the blocking component further includes a pair of finger flanges, and each of the finger flanges includes a textured surface having a predefined pattern that increases a grip of the blocking component.

[0148] In an embodiment of the invention, the drug delivery device (DDD), includes:

- a body;
- a plunger rod having a distal end contacting a stopper inside the body, and a proximal end including an actuation portion with a thumb pad;
- a plurality of protrusions extending from the actuation portion; and
- a blocking component disposed on the body, the blocking component including a proximal collar having a plurality of slots,

wherein, when the protrusions and the slots are in a first configuration relative to one another, the blocking component restricts distal movement of the plunger rod to a first stopping point, and when the protrusions and the slots are in a second configuration, the

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blocking component restricts distal movement of the plunger rod to a second stopping point, wherein, in the second configuration, the slots are configured to receive the protrusions upon distal movement of the plunger rod. In an embodiment of the invention, the protrusions and the slots are movable from the first configuration to the second configuration by rotation of the actuation portion about a longitudinal axis in relation to the blocking component, and wherein when the protrusions and the slots are in the second configuration, the protrusions and the slots are not movable to the first configuration; and/or a difference between the first stopping point and the second stopping point is equivalent to a distance that the stopper must travel to expel a predetermined volume of a drug product from a distal end of the body, and wherein the plunger rod is prevented from moving from the second stopping point to the first stopping point; and/or the plurality of protrusions includes two protrusions disposed symmetrically about the actuation portion; and/or the blocking component further comprises a pair of finger flanges; and/or the drug delivery device is a pre-filled syringe; and/or the drug delivery device is changeable: (a) from a pre-use state to a primed state, by longitudinally moving the plunger rod (*e.g.*, wherein the plunger rod includes a neck disposed distally from the actuation portion, wherein the neck interfaces with an opening in the blocking component to prevent proximal movement of the plunger rod, for example, wherein the neck further interfaces with the opening in the blocking component to prevent movement of the drug delivery device from the delivery state to the primed state) until the plunger rod reaches the first stopping point; (b) from the primed state to a delivery state by rotating the plunger rod in relation to the blocking component until the protrusions and the blocking component are in the second configuration; and (c) from a delivery state to a used state by longitudinally moving the plunger rod until the plunger reaches the second stopping point, wherein the drug delivery device is not changeable from the used state to the delivery state, from the delivery state to the primed state, or from the primed state to the pre-use state. In an embodiment of the invention, when the plunger rod is at the second stopping point, the stopper does not contact a distal end of the body.

[0149] In an embodiment of the invention, a drug delivery device, includes:

- a body;
- a plunger rod, including:
- a distal portion contacting a stopper inside the body;

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- a proximal end including a generally cylindrical actuation portion disposed outside of the body; and
- two protrusions extending from opposite sides of the actuation portion in a symmetrical configuration; and
- a blocking component coupled to the body, the blocking component including: a collar configured to accept a distal part of the actuation portion; and two cavities in the collar having proximally-facing openings, wherein each cavity is configured to accept a distal portion of one of the two protrusions;

wherein the plunger rod is longitudinally movable and rotatable about a longitudinal axis relative to the blocking component, and

wherein, when the drug delivery device is in a pre-use state, the protrusions and the cavity openings are not longitudinally aligned, and when the drug delivery device is in a delivery state, the protrusions and the cavity openings are longitudinally aligned. In an embodiment of the invention, the blocking component further includes a finger flange, and further includes a ribbed surface on a side of the actuation portion. In an embodiment of the invention, the plunger rod further includes: two extensions protruding distally from the actuation portion; and a plurality of openings in the collar of the blocking component, wherein a portion of each extension is configured to be received by one of the plurality of openings upon distal movement of the plunger rod relative to the blocking component.

[0150] In an embodiment of the invention, a drug delivery device includes:

- a body;
- a stopper disposed inside the body;
- a sleeve having a proximal end and a distal end, the distal end being disposed inside the body, proximally from the stopper; and
- a plunger rod disposed at least partially inside the sleeve;

wherein, when the stopper is in a ready position, distal advancement of one of (a) only the sleeve, (b) only the plunger rod, or (c) both the sleeve and the plunger rod together, relative to the body advances the stopper to a primed position, and wherein, when the stopper is in the primed position, distal advancement of another of (a) only the sleeve, (b) only the plunger rod, or (c) both the sleeve and the plunger rod together, relative to the body advances the stopper to a dose completion position. For example, in an embodiment of the invention, a DDD further includes a removable blocking component (*e.g.*, wherein the

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blocking component is a clip removably secured around at least a portion of the sleeve) disposed between a proximal portion of the sleeve and a proximal end of the body, the blocking component obstructing distal advancement of the sleeve relative to the body, wherein distal advancement of the sleeve relative to the body after removal of the blocking component advances the stopper to the primed position. In an embodiment of the invention, the DDD further includes a removable locking component (*e.g.*, a pin, a tab, or a bar) that couples the plunger rod to the sleeve, wherein distal advancement of both the sleeve and the plunger rod together relative to the body advances the stopper to the primed position, wherein distal advancement of only the plunger rod relative to the body after removal of the locking component advances the stopper to the dose completion position. In an embodiment of the invention, in the dose completion position, a proximal end of the plunger rod abuts against a distal end of the sleeve, such that the plunger rod is prevented from advancing distally any further relative to the body. In an embodiment of the invention, the DDD further includes a protrusion disposed on the plunger rod; and an inner protrusion disposed on an interior wall of the sleeve distally to the protrusion of the plunger rod, wherein distal advancement of only the plunger rod relative to the body advances the stopper to the primed position and causes the protrusion of the plunger rod to contact the inner protrusion of the sleeve, and wherein distal advancement of both the plunger rod and the sleeve relative to the body, after the protrusion of the plunger rod has contacted the inner protrusion of the sleeve, advances the stopper to the dose completion position. In an embodiment of the invention, the sleeve includes a finger flange. In an embodiment of the invention, the DDD further includes a stop disposed at a proximal end of the body, the stop sized to block distal advancement of the sleeve or the plunger rod once the stopper is in the completion position.

[0151] In an embodiment of the invention, a drug delivery device, includes:

- a body;
- a plunger rod having a distal portion disposed inside the body and a proximal portion disposed outside a proximal end of the body, the proximal portion having a width greater than a width of the distal portion; and
- an obstruction that, in an obstructing position relative to the plunger rod, prevents distal advancement of the plunger rod from a primed position to a dose completion position,

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wherein displacement of the obstruction from the obstructing position permits distal advancement of the plunger rod to the dose completion position, for example, further including a collar affixed to a proximal end portion of the body, the collar surrounding the proximal portion of the plunger rod; and a collar projection extending radially inward from the collar, wherein the proximal portion of the plunger rod includes a channel into which the collar projection protrudes, the channel including a circumferential path and an axial dose completion path, wherein the obstruction comprises the collar projection, which, when disposed in the circumferential path of the channel, prevents distal advancement of the plunger rod to the dose completion position, and wherein displacement of the obstruction from the obstructing position comprises twisting the plunger rod about a longitudinal axis to align the collar projection with the axial dose completion path. For example, in an embodiment of the invention, the channel further includes an axial priming path offset from the axial dose completion path, and connected to the axial dose completion path by the circumferential path, and distal movement of the plunger rod such that the collar projection travels on the axial priming path advances the plunger rod to the primed position. In an embodiment of the invention, the DDD further includes a finger flange. In an embodiment of the invention, the proximal portion of the plunger rod includes a projection extending radially outward, and the drug delivery device further includes: a rotatable alignment component disposed in between the proximal portion of the plunger rod and the body, the alignment component including a channel, the channel sized and configured to accommodate the plunger rod projection, wherein the obstruction comprises a wall of the channel that blocks a distal axial path of the plunger rod projection when the plunger rod is in the primed position, and wherein displacement of the obstruction from the obstructing position comprises rotating the alignment component to remove the wall of the channel from the distal axial path of the plunger rod projection, e.g., further including a finger flange coupled to a proximal end portion of the body, wherein the rotatable alignment component is disposed between the finger flange and the proximal portion of the plunger rod. In an embodiment of the invention, the DDD further includes a flange piece disposed at the proximal end of the body, wherein the obstruction includes a removable cap that, when in the obstructing position relative to the plunger rod, is disposed partially in between the proximal portion of the plunger rod and the flange piece. In an embodiment of the invention, removal of the cap allows the proximal portion of the plunger rod to advance to a dose completion position, wherein, in the dose completion position, the proximal portion of the plunger rod contacts

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the flange piece. In an embodiment of the invention, the removable cap covers the proximal portion of the plunger rod when in the obstructing position. In an embodiment of the invention, the DDD further includes a collar disposed between the proximal end of the body and the proximal portion of the plunger rod, the collar defining an opening sized to accommodate the proximal portion of the plunger rod upon distal advancement of the plunger rod beyond a primed position; wherein the obstruction comprises a tab protruding radially outward from the proximal portion of the plunger rod, the tab preventing the proximal portion of the plunger rod from fitting into the opening of the collar, and wherein a depth of the collar opening coincides with a distance the plunger rod must travel to advance distally to the dose completion position, *e.g.*, wherein displacement of the obstruction from the obstructing position comprises either removing the tab or compressing the tab into a side of the proximal portion of the plunger rod; and/or wherein the tab is a first tab, and wherein the obstruction further comprises a second tab protruding radially outward from the proximal portion of the plunger rod in a direction opposite the protruding direction of the first tab; and/or wherein the obstruction comprises a tab that, when in the obstructing position, is disposed between the body and the proximal portion of the plunger rod, and wherein the plunger rod includes a geometry disposed proximally from the tab, wherein the geometry cannot advance distally past the tab when the tab is in the obstructing position. For example, displacement of the obstruction may include removing the tab from the drug delivery device by pulling the tab. In an embodiment of the invention, the DDD further includes a flange piece, wherein a portion of the tab is disposed inside a cavity of the flange piece. In an embodiment of the invention, displacement of the obstruction comprises removing the tab from the drug delivery device by breaking the tab. In an embodiment of the invention, the obstruction includes a flange piece that, in the obstructing position, is disposed proximally from the proximal end of the body, between the proximal portion of the plunger rod and the body, and is spaced from the proximal end of the body by a removable blocking component, and wherein displacement of the obstruction from the obstructing position comprises: removing the blocking component; and shifting the flange piece distally towards the proximal end of the body. In an embodiment of the invention, the plunger rod includes a projection extending radially outward, wherein the obstruction includes a lever having an end that, in the obstructing position, is located distally from the projection and blocks distal movement of the projection and thereby distal movement of the plunger rod, and wherein displacement of the obstruction from the obstructing position comprises

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actuating the lever to remove the end of the lever from its location distal from the projection. In an embodiment of the invention, distal advancement of the plunger rod beyond the dose completion position is prevented by contact between the proximal portion of the plunger rod and a portion of a flange piece coupled to the body.

[0152] In an embodiment of the invention, the drug delivery device, includes:

- a body;
- a sleeve affixed to the body, the sleeve including a proximal end, a distal end, and an opening disposed in a circumferential wall of the sleeve;
- a plunger rod passing through the sleeve, the plunger rod including a distal end portion disposed inside the body, and a radially-extending protrusion;

wherein the plunger rod may be distally advanced into the body from a ready position to a primed position, wherein, in the primed position, the protrusion of the plunger rod is disposed inside the opening, and further distal advancement of the plunger rod is resisted by contact between the protrusion and a wall of the opening, and wherein pressure may be exerted on the protrusion to overcome the resistance to further distal advancement of the plunger rod. In an embodiment of the invention, the opening in the sleeve is a second opening, and the sleeve further includes a first opening disposed in the circumferential wall of the sleeve proximally from the second opening, and a third opening disposed in the circumferential wall of the sleeve distally from the second opening, wherein, in the ready position, the protrusion of the plunger rod is disposed in the first opening, and further distal advancement of the plunger rod is resisted by contact between the protrusion and a wall of the first opening, and wherein, after further distal advancement of the plunger rod past the primed position, the protrusion of the plunger rod is disposed in the third opening, and further distal advancement of the plunger rod is prevented. In an embodiment of the invention, the radially-extending protrusion is a first protrusion, and wherein the plunger rod further includes a second radially-extending protrusion opposite the first protrusion, and wherein squeezing the first and second protrusions towards one another while applying axial pressure in the distal direction on the plunger rod overcomes the resistance to further distal advancement of the plunger rod. In an embodiment of the invention, the protrusion includes a distally-tapering profile to aid in distal advancement of the plunger rod.

[0153] In an embodiment of the invention, a drug delivery device, includes:

- a body;

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- a plunger rod including a distal end portion disposed inside the body and a rotatable element; and
- a sleeve affixed to the body, the sleeve including a proximal opening into which the plunger rod may be advanced,

wherein rotating the rotatable element causes distal advancement of the plunger rod to a primed position, and wherein once the plunger rod is in the primed position, further rotation of the rotatable element is resisted. In an embodiment of the invention, the DDD further includes a collar disposed at a proximal end of the body, an interior of the collar including a proximal threaded portion forming a proximal helical path, wherein the rotatable element comprises a proximal portion of the plunger rod including a protrusion, wherein the proximal portion of the plunger rod may be rotated about a longitudinal axis to cause the protrusion to travel distally along the proximal helical path, and wherein once the protrusion reaches the end of the proximal threaded portion of the collar, the plunger rod is in the primed position, *e.g.*, wherein once the plunger rod is in the primed position, the plunger rod may be depressed axially into the body to distally advance the plunger rod to a dose completion position; and/or wherein the interior of the collar further includes a distal threaded portion, wherein threads of the distal threaded portion form a distal helical path offset from, and opposite to, the proximal helical path, wherein alignment of the protrusion with the distal helical path places the plunger rod in the primed position, and wherein rotation of the proximal portion of the plunger rod to cause the protrusion to travel distally along the distal helical path causes distal advancement of the plunger rod to a dose completion position.

[0154] A substance may be dispensed using such a DDD having a plunger rod and a body, may be done by a method including:

(a) advancing the plunger rod by a predetermined distance into the body until advancement of the plunger rod is resisted by a stop;

(b) rotating the plunger rod about a longitudinal axis; and

(c) actuating the plunger rod to dispense a predetermined volume of the substance,

wherein none of steps (a), (b), and (c) are reversible. In an embodiment of the invention, the DDD further includes a flange piece having a collar, and advancing the plunger rod and actuating the plunger rod comprise pressing an actuation portion of the plunger rod into the collar of the flange piece; for example, wherein the plunger rod comprises a protrusion, and wherein the collar of the flange piece abuts against the protrusion to resist advancement of

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the plunger rod. For example, in an embodiment of the invention, wherein rotating the plunger rod comprises twisting an actuation portion of the plunger rod relative to the flange piece, until a protrusion on the plunger rod becomes longitudinally aligned with a cavity in the collar of the flange piece, which may further include advancing the protrusion into the cavity until the protrusion abuts a distal side of the cavity, wherein the predetermined volume of the substance is dispensed when the protrusion abuts the distal side of the cavity.

[0155] See International patent application publication no. WO2020/247686.

EXAMPLES

[0156] Example 1: **A Randomized, Single-Masked, Active-Controlled Phase 2 Study of the Safety, Tolerability, and Efficacy of Repeated Doses of High-Dose Aflibercept in Patients with Neovascular Age-Related Macular Degeneration (CANDELA trial).**

[0157] This phase 2, multi-center, randomized, single-masked study in patients with nAMD investigated the efficacy, safety, and tolerability of HD (8 mg doses) versus IAI (2mg doses). The Study dosing regimen is summarized in Figure 1. The Study consisted of a screening/baseline period, a treatment period, and an end of study (EOS) visit at week 44. Patients were seen monthly through week 44. One hundred and six eligible patients were randomized into 2 groups in a 1:1 ratio. One group received IAI and the other received HD. The investigational product was administered intravitreally (IVT) monthly for 3 initial injections (baseline, week 4, and week 8), followed by additional doses at weeks 20 and 32. At weeks 24, 28, 36 and 40, patients were evaluated and given a dose (at their randomized dose level) if either of the following criteria are met (PRN criteria):

- Loss of ≥5 letters from week 20 BCVA due to disease progression; or
- Anatomical findings that are considered vision threatening, such as worsening or persistent retinal fluid, new or worsening PED (pigment epithelial detachment), new or persistent hemorrhage, *etc.*

[0158] The following are the portions of the protocol by which the CANDELA human clinical trial was conducted.

[0159] The study also includes a pharmacokinetic (PK) sub-study, with dense blood sampling (dense PK sub-study) for systemic drug concentrations and PK assessments for approximately 15 patients from each group from selected sites. Additional patients (up to

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approximately 50% more in each treatment group) may be enrolled in the dense PK sub-study to ensure adequate data is are captured.

Dosing schedule

[0160] The dosing schedule for the IAI and HD groups are set forth below in Table 1-1.

Table 1-1. Dosing schedule of IAI and HD groups in CANDELA study.

			Wk 4 Safety Analysis				Wk 16 Efficacy Analysis							Wk 44 Analysis POP	
	Screen 1 & 2	Day 1 (BL)	Wk 4	Wk 6	Wk 8	Wk 12	Wk 16*	Wk 20	Wk 24	Wk 28	Wk 32	Wk 36	Wk 40	Wk 44
IAI – 2 mg 50 µl		X	X	X	SP/ PK			X	PRN	PRN	X	PRN	PRN	
HD – 8 mg 70 µl		X	X	X	SP/ PK			X	PRN	PRN	X	PRN	PRN	

[0161] Additional visits for Dense PK Substudy:

- Days 2, 3, 5, 8, 15 and 22
- Blood pressure & pharmacokinetic draws at all visits
- Urinalysis (UA) at days 8 & 15

[0162] Week 16. Additional treatment allowed after discussion with sponsor

[0163] See also Table 1-2 herein. As discussed in this Example, IAI dosing regimens call for 2 mg doses given as defined in Table 1-1; and HD dosing regimens call for 8 mg doses given as defined in Table 1-1.

Primary Endpoints

[0164] The co-primary endpoints are:

- Safety, which will be evaluated by assessment of treatment-emergent adverse events (TEAEs) and serious adverse events (SAEs) through week 4; and
- The proportion of patients without retinal fluid in the center subfield at week 16.

Secondary Endpoints

[0165] There are no secondary endpoints in this study.

Exploratory Endpoints

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[0166] The exploratory endpoints are:

- The proportion of patients without retinal fluid in the center subfield at week 44;
- The proportion of patients without retinal fluid in the center subfield at week 16;
- Change in central retinal thickness (CRT) from baseline through week 16 and week 44;
- The proportion of patients without intraretinal fluid (IRF) at week 16 and week 44;
- The proportion of patients without subretinal fluid (SRF) at week 16 and week 44;
- The proportion of patients without sub-retinal pigment epithelium (RPE) fluid at week 16 and week 44;
- The proportion of patients able to maintain dry retina (total fluid, IRF, and/or SRF) through week 44;
- The proportion of patients able to maintain a 12-week dosing interval from week 8 through week 44;
- Change in CRT between dosing visits from week 8 through week 44;
- Change in Best Corrected Visual Acuity (BCVA) from baseline, and proportions of patients gaining and losing vision, through week 16 and week 44;
- Change in lesion size and choroidal neovascularization (CNV) size from baseline through week 20 and week 44;
- Other safety outcomes (*e.g.*, TEAEs, SAEs, vital signs, clinical laboratory values; and intraocular pressure [IOP]) from baseline through week 16 and week 44; and
- Systemic PK of free and bound aflibercept assessed from baseline through week 44.

Efficacy Variables

[0167] The efficacy variable relevant to the primary efficacy endpoint is the assessment of retinal fluid. The efficacy variables relevant to the exploratory endpoints are:

- Assessment of retinal fluid levels (total fluid, IRF, and SRF) and retinal thickness on spectral domain optical coherence tomography (SD-OCT, or just OCT)
- Dosing interval;
- Visual acuity;
- Lesion size.

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Safety Variables

[0168] The safety variable relevant to the primary safety endpoint is the proportion of patients with TEAEs and SAEs.

The safety variables relevant to the exploratory endpoints are:

- Ocular exams
- Vital signs
- Clinical laboratory values
- IOP

Pharmacokinetic Variables

[0169] The PK variables are the concentrations of free and bound aflibercept in plasma at each time point using both sparse sampling and dense sampling.

Number of Patients Planned

[0170] The study will enroll approximately 100 patients to be randomized in a 1:1 ratio.

Study Population

[0171] The study population consists of treatment-naïve patients with nAMD.

Inclusion Criteria

[0172] A patient must meet the following criteria at both the screening and/or the randomization visits to be eligible for inclusion in the study:

1. Men or women ≥50 years of age with active subfoveal CNV secondary to nAMD, including juxtafoveal lesions that affect the fovea in the study eye as assessed by an independent reading center.
2. Best Corrected Visual Acuity (BCVA) Early Treatment Diabetic Retinopathy Study (ETDRS) letter score of 78 to 24 (Snellen equivalent of 20/32 to 20/320) in the study eye.
3. Willing and able to comply with clinic visits and study-related procedures.
4. Provide informed consent signed by study patient or legally acceptable representative.

Exclusion Criteria

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[0173] A patient who meets any of the following criteria at either the screening or randomization visits will be excluded from the study:

1. Evidence of CNV due to any cause other than nAMD in either eye.
2. Subretinal hemorrhage in the study eye that is ≥50% of the total lesion area.
3. Evidence of DME or diabetic retinopathy (defined as more than 1 microaneurysm) in either eye in diabetic patients.
4. Prior use of IVT anti-VEGF agents (aflibercept, ranibizumab, bevacizumab, brolucizumab, pegaptanib sodium) in the study eye.
5. Prior IVT investigational agents in either eye (*e.g.*, anti-ang-2/anti-VEGF bispecific monoclonal antibodies, gene therapy).
6. Previous use of intraocular or periocular corticosteroids within 120 days of screening or treatment with an IVT steroid implant at any time in the study eye.
7. Treatment with ocriplasmin in the study eye at any time.
8. Yttrium-aluminium-garnet capsulotomy in the study eye within 14 days of the screening Visits.
9. History of vitreoretinal surgery (including scleral buckling) in the study eye.
10. Intraocular pressure ≥25 mm Hg in the study eye.
11. Evidence of infectious blepharitis, keratitis, scleritis, or conjunctivitis in either eye.
12. Any intraocular inflammation/infection in either eye within 90 days of the screening visit.
13. Any history of macular hole of stage 2 and above in the study eye.
14. Current iris neovascularization, vitreous hemorrhage, or tractional retinal detachment visible at the screening assessments in the study eye.
15. Only 1 functional eye, even if that eye was otherwise eligible for the study (*e.g.*, BCVA of counting fingers or less in the eye with worse vision).
16. Ocular conditions with poorer prognosis in the fellow eye.
17. Inability to obtain fundus photographs, fluorescein angiography (FA), or OCT (*e.g.*, due to media opacity, allergy to fluorescein dye or lack of venous access) in the study eye.
18. Any prior systemic anti-VEGF administration.
19. Uncontrolled diabetes mellitus in the opinion of the investigator.
20. Uncontrolled BP (defined as systolic >140 mm Hg or diastolic >90 mm Hg). Patients may be treated with up to 3 agents known to have anti-hypertensive effects for arterial hypertension to achieve adequate BP control. This limit applies to drugs that could be used to treat hypertension even if their primary indication in the patient was not for BP

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control. Any recent changes in medications known to affect BP need to be stable for 90 days prior to the screening visit.

21. Variation by more than 10% in the 3 pre-randomization BP measures recorded at the screening 1, screening 2, and randomization visits.

22. History of cerebrovascular accident/ transient ischemic attack or myocardial infarction/ acute coronary syndrome within 180 days of screening visit.

23. Renal failure, dialysis, or history of renal transplant.

24. Known sensitivity to any of the compounds of the study formulation.

25. Members of the clinical site study team and/or his/her immediate family, unless prior approval granted by the sponsor.

26. Pregnant or breastfeeding women

27. Women of childbearing potential* who are unwilling to practice highly effective contraception prior to the initial dose/start of the first treatment, during the study, and for at least 90 days after the last dose. Highly effective contraceptive measures include:

a. stable use of combined (estrogen and progestogen containing) hormonal contraception (oral, intravaginal, transdermal) or progestogen-only hormonal contraception (oral, injectable, implantable) associated with inhibition of ovulation initiated 2 or more menstrual cycles prior to screening.

b. intrauterine device (IUD); intrauterine hormone-releasing system (IUS)

c. bilateral tubal ligation

d. vasectomized partner

e. and or sexual abstinence†, ‡

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

†Sexual abstinence is considered a highly effective method only if defined as refraining from heterosexual intercourse during the entire period of risk associated with the study treatments.

‡Periodic abstinence (calendar, symptothermal, post-ovulation methods), withdrawal (coitus interruptus), spermicides only, and lactational amenorrhea method (LAM) are not acceptable methods of contraception. Female condom and male condom should not be used together.

28. Participation in an investigational study within 30 days prior to screening visit that

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involved treatment with any drug (excluding vitamins and minerals) or device.
29. Any other intraocular surgery within 12 weeks (84 days) before the screening visit (see Exclusion Criterion #9).
30. History of corneal transplant or corneal dystrophy in study eye.
31. 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.
32. 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.

[0174] Additional Exclusion Criteria for the Dense PK Sub-study
1. Prior IAI in the fellow eye
2. Patients on more than 2 anti-hypertensive medications
3. Patients with known cardiac arrhythmia
4. Patients who, in the opinion of the investigator, are unlikely to have stable BP over the course of the study (*e.g.*, due to known non-compliance with medication)

Investigational and Reference Treatments

[0175] The HD will be provided as a liquid formulation in a vial. The target concentration of aflibercept is 114.3 mg/mL. The dose will be delivered in an injection volume of 70 microliters. The IAI will be provided as a liquid formulation in a vial. The target concentration of aflibercept is 40 mg/mL. The dose will be delivered in an injection volume of 50 microliters.

Additional Treatment

[0176] Deviation from the treatment schedule defined in the protocol is discouraged. Efforts should be made to ensure adherence to the protocol-specified dosing intervals. If, however, in the investigator's judgement, a patient cannot adhere to the protocol-specified dosing interval due to persistent or worsening disease and requires an interim injection, the patient may receive additional treatment at week 16. The investigator must make

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reasonable efforts to consult with the study director or sponsor designee prior to additional treatment being allowed.

[0177] Patients will receive their randomized dose of aflibercept if it is determined that additional treatment will be administered. Patients who receive additional treatment will continue to receive their randomized treatment at future visits and will remain masked to treatment assignment. Data from patients receiving additional treatment will be censored from the time additional treatment is administered.

Dose Modification

[0178] Dose modification for an individual patient is not allowed.

Concomitant Medications

[0179] Any treatment administered from the time of informed consent to the final study visit will be considered concomitant medication. This includes medications that were started before the study and are ongoing during the study.

[0180] If a pretreatment concomitant medication is administered in the study eye before injection (*e.g.*, antibiotic or anesthetic), it must be administered for fellow eye treatment as well.

Prohibited Medications

[0181] Patients are not allowed to receive any standard or investigational treatment for nAMD in the study eye other than their assigned study treatment with HD or IAI, as specified in the protocol. This includes medications administered locally (*e.g.*, IVT, topical, juxtascleral, or periorbital routes), as well as those administered systemically, with the intent of treating nAMD in the study eye or fellow eye.

[0182] If the fellow eye has nAMD, or any other approved indication, IAI (2 mg) will be allowed and supplied through the IWRS (Interactive web response system). Patients are not allowed to receive any other anti-VEGF agent in the fellow eye. Patients enrolled in the dense PK sub-study cannot receive IAI (2 mg) in the fellow eye before week 12.

[0183] Non-ocular (systemic) standard or investigational treatments for nAMD of the study or fellow eye are not permitted. Systemic anti-angiogenic agents and anti-Ang2 inhibitors are not permitted during the study.

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Permitted Medications

[0184] Any other medications that are considered necessary for the patient's welfare, and that are not expected to interfere with the evaluation of the study drug, are allowed.

Table 1-2. Clinical Study Schedule of Events

Study Procedure	Screening 1 Visit 1	Screening 2 Visit 2	Baseline Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 8	Visit 9	Visit 10	Visit 11	Visit 12	Visit 13	Visit 14	EOS Visit 15
Month			0	1	2	3	4	5	6	7	8	9	10	11	12
Week			0	4	8	12	16	20	24	28	32	36	40	44	48
Day	-21 to -1	-20 to -1	1	28	57	85	113	141	169	197	225	253	281	309	
Window (Day)				±2	±2	±2	±2	±2	±2	±2	±2	±2	±2	±2	±2
Screening Procedures															
Informed consent	X														
Urine PK sampling informed consent	X														
Genomic samples PRN informed consent	X														
Inclusion/Exclusion	X		X												
Medical history	X														
Demographics	X														
Concomitant medications	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Randomization			X												
Administered Study Drug															
Study drug			X	X	X				X	X PRN[9]	X PRN[9]	X	X PRN[9]	X PRN[9]	
Ocular Efficacy and Safety Evaluations															
Refraction and BCVA (ETDRS)[1]	X		X	X	X		X	X	X	X	X	X	X	X	X
IOP[2]	X		X	X	X		X	X	X	X	X	X	X	X	X
Slit lamp examination	X		X	X	X		X	X	X	X	X	X	X	X	X
Indirect ophthalmoscopy[3]	X		X	X	X		X	X	X	X	X	X	X	X	X
FA, CFP[10]	X						X		X						X
OCT[4,10]	X		X	X	X		X	X	X	X	X	X	X	X	X
Non-ocular Safety															
Physical examination	X														
Vital signs[5, 11, 12]	X	X[11]	X	X	X	X[11]	X	X	X	X	X	X	X	X	X
ECG	X														X
Adverse events	X		X	X	X	X	X	X	X	X	X	X	X	X	X
Laboratory Testing[11]															
Hematology	X						X								X

Study Procedure	Screening 1 Visit 1	Screening 2 Visit 2	Baseline Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 9	Visit 9	Visit 10	Visit 11	Visit 12	Visit 13	Visit 14	EOS Visit 15
Month			0	1	2	3	4	5	6	7	8	9	10	11	12
Week			0	4	8	12	16	20	24	28	32	36	40	44	48
Day	-21 to -1	-20 to -1	1	29	57	85	113	141	169	197	225	253	281	309	
Blood chemistry	X					X			X						X
Pregnancy test (women of childbearing potential)[6]	X Serum		X Urine	X Urine	X Urine				X Urine	X Urine	X Urine	X Urine	X Urine	X Urine	
Urinalysis[7, 11]	X			X[11]		X									X
Pharmacokinetic and Research Sampling															
PK samples (sparse)[8]			Pre-infusion at Infusion	X	X	X	X	X	X	X		X			X
PK samples (urine)[13]			X		X	X	X			X		X			X
Research samples[14]			X												X
Genomic DNA samples (optional)[15]			X												

[0185] BCVA=Best Corrected Visual Acuity, ECG=electrocardiogram, EOS=end of study, ETDRS=Early Treatment Diabetic Retinopathy Study, FA=fluorescein

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angiography, FBR=future biomedical research, FP=fundus photography, IOP=Intraocular pressure, PK=pharmacokinetics, PRN=*pro re nata* (as needed), SDOCT= spectral domain optical coherence tomography, UPCR=urine protein:creatinine ratio.

[0186] Footnotes:

1. For patients in the dense PK sub-study, the visit window is ± 0 days.
2. For patients in the dense PK sub-study, the visit window is ± 2 days.
3. Signed only by patients participating in the dense PK sub-study and in addition to the study ICF.
4. The optional genomic and FBR sub-study ICF (informed consent form) should be presented to patients at the screening visit and may be signed at any subsequent visit at which the patient chooses to participate after screening. The genomic DNA sample should be collected on day 1/baseline (pre-dose) or at any study visit from patients who have signed the sub-study ICF.
5. Refer to pharmacy manual for study drug injection guidelines. Following study drug injection, patients will be observed for approximately 30 minutes.
6. Patients will be dosed as needed per criteria herein.
7. Patients enrolled at sites participating in the optional visual function sub-study may undergo additional visual function tests. See study procedure manual for details.
8. Intraocular pressure will be measured bilaterally at all study visits. On days when study drug is administered, IOP should also be measured approximately 30 minutes after administration of study drug, in the study eye only. Intraocular pressure will be measured using Goldman applanation tonometry or Tono-pen™ and the same method of measurement must be used in each patient throughout the study.
9. Indirect ophthalmoscopy should be performed bilaterally at all visits. On days when study drug is administered, it should also be performed immediately after administration of study drug (study eye only).
10. The same SD-OCT/FA/FP imaging system used at screening and day 1 must be used at all subsequent visits in each patient. Images will be taken in both eyes before dosing at each required visit.
11. Vital signs (temperature, BP, heart rate) should be measured pre-injection, per the procedure outlined in the study procedure manual. Blood pressure assessments will be taken using automated office blood pressure (AOBP) with the Omron Model HEM

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907XL (or comparable). Measures will be taken in triplicate and a mean measure as displayed by the device will be recorded in the EDC. Detailed instructions can be found in the study procedure manual.

12. Timing of BP assessment at all visits must be within 2 hours of planned time of dosing on day 1 for patients in the dense PK sub-study. For all other patients, this window should be adhered to as closely as possible.

13. For patients participating in the dense PK sub-study, HR and BP also will be collected according to the schedule in Table 1-2.

14. Only BP and heart rate will be measured at these visits. No temperature measures are required.

15. All samples collected for laboratory assessments should be obtained prior to administration of fluorescein and prior to administration of study drug.

16. For women of childbearing potential, a negative serum pregnancy test at screening is required for eligibility. A negative urine pregnancy test is required before treatment is administered at subsequent visits.

17. For patients participating in the dense PK sub-study, urinalysis/UPCR will also be collected according to the schedule in Table 1-2.

18. Week 4 collection of urinalysis/UPCR only for patients in the dense PK sub-study.

19. Dense PK sampling will be performed in approximately 30 patients (15 in each group) drawn according to the schedule in Table 1-2. On dosing days, BP (blood pressure) and PK samples must be collected prior to study drug administration. Additional patients (up to approximately 50% more in each treatment group) may be enrolled in the dense PK sub-study to ensure adequate data is are captured.

20. Sparse PK sampling will be performed in all patients not enrolled in the dense PK sub-study according to the schedule defined in Table 1-2. On dosing days, PK samples should be collected prior to study drug administration.

21. Exploratory research serum sample should be drawn prior to the administration of study drug at baseline (visit 3, day 1) and week 44.

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Table 1-3. Dense PK Study Schedule of Events

Visit	Dose	Assessment Day and Time (h)		Dense PK Sample Collection	Heart Rate and Blood Pressure[1,2]	Urinalysis / UPCR
Visit 3 (Baseline)	X	1	Time of first dose	X (pre-dose)	X	
			4h post-dose (±30 min)[3]	X		
			8h post-dose (±2 h)[4]	X		
		2	±2h[5]	X	X	
		3	±2h[5]	X	X	
		5	±2h[5]	X	X	
		8	±2h[5]	X	X	X[6]
		15	±2h[5]	X	X	X[6]
		22	±2h[5]	X	X	

[0187] Footnotes:

1. Timing of all BP assessments must be within ±2 hours of the time of dosing on day 1. This may be done at the clinical study site or by the site personnel or another healthcare professional at a remote location (*e.g.*, the patient's home or other appropriate location). Regardless of where BP measurements are taken, the procedures described herein must be followed.

2. Blood pressure assessments will be taken using automated office blood pressure (AOBP) with the Omron Model HEM 907XL (or comparable). Measures will be taken in triplicate and a mean measure as displayed by the device will be recorded in the EDC. Detailed instructions can be found in the study procedure manual.

3. Intraocular pressure will be measured at approximately 4 hours post-dose only if the IOP measurement from approximately 30 minutes to 60 minutes post-dose remains clinically significantly higher than the pre-injection reading.

4. Intraocular pressure will be measured at approximately 8 hours post-dose only if the IOP measurements from approximately 30 minutes to 60 minutes and approximately 4 hours post-dose remain clinically significantly higher than the pre-injection reading.

5. PK draw for all assessment days are to be performed within ±2 hours of the time of dosing on day 1.

6. This may be done at the clinical study site or by the site personnel or another healthcare professional at a remote location (*e.g.*, the patient's home or other appropriate location).

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Ocular Procedures

[0188] Intraocular Pressure. Intraocular pressure will be measured in both eyes at every visit using Goldmann applanation tonometry or Tono pen™, as specified in Table 1-2. The same method of IOP measurement must be used throughout the study for each individual patient. On dosing visits, IOP will also be measured approximately 30 minutes post-dose (study eye).

[0189] For patients in the dense PK sub-study, IOP will also be measured 4 hours post-dose if the reading from approximately 30 minutes to 60 minutes post-dose remains clinically significantly higher than the pre-dose reading, and again at approximately 8 hours post-dose if the reading from approximately 4 hours post-dose remains clinically significantly higher than the pre-dose reading.

[0190] Slit Lamp Examination. Patients' anterior eye structure and ocular adnexa will be examined bilaterally pre-dose at each study visit using a slit lamp (see study procedure manual) by the investigator, as specified herein

[0191] Indirect Ophthalmoscopy. Patients' posterior pole and peripheral retina will be examined by indirect ophthalmoscopy at each study visit pre-dose (bilateral) and post-dose (study eye) by the investigator, as specified herein. Post-dose evaluation must be performed immediately after injection.

[0192] Fundus Photography/Fluorescein Angiography. The anatomical state of the retinal vasculature will be evaluated by FP and FA as specified herein. Fundus photography and FA will be captured and transmitted to an independent reading center for both eyes. For FA, the study eye will be the transit eye.

[0193] Fundus and angiographic images will be sent to an independent reading center where images will be read by masked readers. All FPs and FAs will be archived at the site as part of the source documentation. Photographers must be certified by the reading center to ensure consistency and quality in image acquisition. A detailed protocol for image acquisition and transmission can be found in the study procedure manual. Imaging technicians should remain masked to treatment assignment.

[0194] Spectral Domain Optical Coherence Tomography. Retinal characteristics will be evaluated at every visit using SD-OCT. Images will be captured and transmitted for both eyes. Images will be sent to an independent reading center where they will be read by masked readers. All OCTs will be electronically archived at the study site as part of the source documentation. Optical coherence tomography technicians must be certified by the

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reading center to ensure consistency and quality in image acquisition. A detailed protocol for acceptable OCT machines and OCT image acquisition/transmission can be found in the study procedure manual. Imaging technicians should remain masked to treatment assignment.

[0195] Best Corrected Visual Acuity. Visual function of the study eye and the fellow eye will be assessed using the ETDRS protocol (Early Treatment Diabetic Retinopathy Study Research Group, 1985) at 4 meters at each study visit, as specified in Table 1-2. Visual acuity examiners must be certified to ensure consistent measurement of BCVA, and must remain masked to treatment assignment, treatment schedule and study eye. Best corrected visual acuity should be done before any other ocular procedures are performed. Patients enrolled at sites participating in the optional visual function sub-study may undergo additional visual function tests.

Definitions

[0196] *Adverse Event.* An AE is any untoward medical occurrence in a patient administered a study drug which may or may not have a causal relationship with the study drug. Therefore, an AE is any unfavorable an unintended sign (including abnormal laboratory finding), symptom, or disease which is temporally associated with the use of a study drug, whether or not considered related to the study drug (ICH E2A Guideline. Clinical Safety Data Management: Definitions and Standards for Expedited Reporting, Oct 1994).

[0197] *Serious Adverse Event.* An SAE is any untoward medical occurrence that at any dose:

- Results in death – includes all deaths, even those that appear to be completely unrelated to study drug (*e.g.*, a car accident in which a patient is a passenger). Is life-threatening – in the view of the investigator, the patient is at immediate risk of death at the time of the event. This does not include an AE that had it occurred in a more severe form, might have caused death.
- Requires in-patient hospitalization or prolongation of existing hospitalization. Inpatient hospitalization is defined as a hospital admission (any duration) or an emergency room visit for longer than 24 hours. Prolongation of existing hospitalization is defined as a hospital stay that is longer than was originally

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anticipated for the event or is prolonged due to the development of a new AE as determined by the investigator or treating physician

- Results in persistent or significant disability/incapacity (substantial disruption of one's ability to conduct normal life functions).
- Is a congenital anomaly/birth defect
- Is an important medical event - Important medical events may not be immediately life-threatening or result in death or hospitalization, but may jeopardize the patient or may require intervention to prevent one of the other serious outcomes listed above (*e.g.*, intensive treatment in an emergency room or at home for allergic bronchospasm; blood dyscrasias or convulsions that do not result in hospitalization; or development of drug dependency or drug abuse).

[0198] *Ocular important medical event.* An ocular important medical event may include the following:

- An AE that requires either surgical or medical intervention to prevent permanent loss of vision;
- Substantial, unexplained vision loss or an AE that causes substantial vision loss

Criteria for reporting SAEs must be followed for these events.

[0199] *Severity.* The severity of AEs will be graded according to the following scale:

- Mild: Does not interfere in a significant manner with the patient 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.
- Moderate: Produces some impairment of functioning but is not hazardous to health. It is uncomfortable or an embarrassment. Treatment for symptom may be needed.
- 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. If a laboratory value is considered an AE, its severity should be based on the degree of physiological impairment the value indicates.

[0200] *Causality.* The investigator must provide causality assessment as whether or not there is a reasonable possibility that the drug caused the adverse event, based on evidence or facts, his/her clinical judgment, and the following definitions. The causality assessment must be made based on the available information and can be updated as new information becomes available.

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The following factors should be considered when assessing causality:

- Temporal relationship: time to onset vs time drug was administered;
- Nature of the reactions: immediate vs. long term;
- Clinical and pathological features of the events;
- Existing information about the drug & same class of drugs;
- Concomitant medications;
- Underlying and concurrent illnesses;
- Response to de-challenge (drug discontinuation) or dose reduction;
- Response to re-challenge (re-introduction of the drug) or dose increase, when applicable;
- Patient's medical and social history.

[0201] *Causality to the Study Drug.*

- Related: The AE follows a reasonable temporal sequence from study drug administration, and cannot be reasonably explained by the nature of the reaction, patient's clinical (*e.g.*, disease under study, concurrent diseases, concomitant medications), or other external factors; or the AE follows a reasonable temporal sequence from study drug administration, and is a known reaction to the drug under study or its class of drugs, or is predicted by known pharmacology.
- Not Related: The AE does not follow a reasonable sequence from study drug administration, or can be reasonably explained by the nature of the reaction, patient's clinical state (*e.g.*, disease under study, concurrent diseases, and concomitant medications) or other external factors.

[0202] *Causality to the Injection Procedure.* The relationship of AEs to the injection procedure is assessed by the investigator, and is a clinical decision based on all available information. The following question is addressed: Is there a reasonable possibility that the AE may have been caused by the injection procedure?

The possible answers are:

- Not Related: There is a reasonable possibility that the event may have been caused by the injection procedure
- Related: There is a reasonable possibility that the event may have been caused by the injection procedure

Causality to the Study Conduct (Protocol-Specified Procedure).

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- Related: The AE follows a reasonable temporal sequence from a protocol-specified procedure, and cannot be reasonably explained by the nature of the reaction, patient's clinical (*e.g.*, disease under study, concurrent diseases, concomitant medications), or other external factors.
- Not Related: The AE does not follow a reasonable sequence from a protocol-specified procedure, or can be reasonably explained by the nature of the reaction, patient's clinical state (*e.g.*, disease under study, concurrent diseases, and concomitant medications) or other external factors

Analysis of Drug Concentration Data-Main Study

[0203] The concentrations of free and bound aflibercept over time will be summarized by descriptive statistics for each treatment group. No formal statistical hypothesis testing will be performed.

Dense PK Sub-study

[0204] The PK parameters to be determined after the first dose for free and bound aflibercept may include, but are not limited to:

- C_{max};
- C_{max}/Dose;
- t_{max};
- t_{last};
- C_{last};
- AUC_{inf};
- AUC_{inf}/Dose;
- $t_{1/2}$;
- C_{trough};

[0205] After repeat dosing in the dense PK sub-study, PK parameters to be determined may include, but are not limited to, C_{trough}, time to reach steady-state, and accumulation ratio. The concentrations of free and bound aflibercept over time and selected PK parameters will be summarized by descriptive statistics by treatment group. This descriptive statistical assessment will include the geometric means and ratios of the geometric means

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for selected PK parameters, as deemed appropriate. No formal statistical hypothesis testing will be performed.

Results at week 16 (n=55)-values as reported at the time for patients reaching week 16.

[0206] The baseline demographics, eye characteristics, and blood pressure of "All patients" at this point in this trial are set forth in Figures 2-4.

[0207] After 16 weeks of study duration, patients receiving the 8 mg doses of aflibercept (HD dosing regimen) maintained (on average) greater vision improvements and anatomical improvements than that of patients receiving the 2 mg dose (IAI dosing regimen) (relative to baseline).

[0208] Patients (Completers) receiving the HD dosing regimen maintained a greater mean change in best corrected visual acuity (7.4) than the IAI patients (5.2) at week 16 (Figure 6).

[0209] Anatomical improvements were also remarkable at week 16. The central retinal thickness (CRT) of patients receiving the HD dosing regimen remained below that of patients (Completers) receiving the IAI dosing regimen-a mean change of -142 micrometers in HD patients vs -133 micrometers in IAI patients at week 16 (Figure 5).

[0210] Measures of retinal dryness were also strikingly better in patients receiving the HD dosing regimen. Overall, more patients (Completers) receiving the HD regimen had complete resolution of intraretinal/subretinal fluid at week 16. The proportion of HD patients with dry retinas at week 16 was 44% whereas the proportion of IAI patients with dry retinas was just 9% (Figure 7). A dry retina was regarded as a retina exhibiting no intraretinal fluid (IRF) and subretinal fluid (SRF). Conversely, a greater proportion of patients receiving the IAI dosing regimen had "not-dry" retinas at week 16 than that of HD patients (86% vs 56%, respectively) (Figure 8). Fewer HD patients exhibited IRF (IRF only or both IRF and SRF) or SRF (SRF only or both IRF and SRF) at week 16 than IAI patients (Figures 9-10, respectively). Similar trends were apparent when the retinal fluid status (Dry, IRF only, SRF only or both IRF and SRF) of IAI and HD patients at baseline, week 4, week 8, week 12 and week 16 was observed (Figures 11-12, respectively). The number of treatments in the HD and IAI treatment groups ("All patients") was comparable (Figure 13).

[0211] The HD and IAI dosing regimens were generally well tolerated. The ocular treatment-emergent adverse events (TEAEs) (Figure 14-15), intraocular pressure (IOP) (Figure 16), non-ocular TEAEs (Figures 17-18), non-ocular SAEs (Figure 19), hypertension AEs (Figure 20), mean systolic blood pressure (Figure 21), mean diastolic blood pressure

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(Figure 22), mean IOP (Figure 23), mean change of IOP (Figure 24) were comparable in each treatment group.

Results- additional patients reaching week 16 (n=106) -values as reported at the time for a larger set of patients reaching week 16

[0212] At week 16, a higher proportion of these patients in the HD aflibercept 8 mg group had no retinal or subretinal fluid (50.9%, n=27/53) in the center (1 mm) subfield on optical coherence tomography compared to patients treated with IAI EYLEA 2 mg (34.0%, n=18/53) (p=0.08). During the initial 16 weeks of the trial, treatment emergent adverse events (TEAEs) in the study eye occurred in 17.0% (9 of 53) of aflibercept 8 mg patients and 22.6% (12 of 53) of EYLEA 2 mg patients. AEs that occurred more frequently in the aflibercept 8 mg group were conjunctival hemorrhage (5.7% aflibercept 8 mg, 3.8% EYLEA 2 mg) and vitreous detachment (3.8% aflibercept 8 mg, 1.9% EYLEA 2 mg). Serious ocular AEs (SAEs) occurred in two patients overall, one in the aflibercept 8 mg group (retinal tear) and one in the EYLEA 2 mg group (visual acuity reduced). No intraocular inflammation, occlusive vasculitis, arterial thromboembolic events (adjudicated according to the Anti-Platelet Trialists' Collaboration definitions) or death in either patient group were identified through week 16.

[0213] The disposition and exposure, baseline demographics, baseline characteristics, and baseline blood pressure and medical history of patients in this analysis are set forth in Figures 26, 27, 28 and 29.

[0214] The proportion of HD patients achieving a dry retina (no IRF and no SRF in the central subfield on SD-OCT (spectral domain optical coherence tomography)) was 51% whereas the proportion of IAI patients was 34% (Figure 30) (last observation carried forward (LOCF) values). HD patients without IRF (dry or with SRF only) was 70% (IAI patients: 68%) and without SRF (dry or with IRF only) was 70% (IAI patients: 51%) (Figure 31) (LOCF). At week 16, there was a greater median (Figure 32) and mean (Figure 33) decrease in central retinal thickness from baseline in HD patients (-161.0 micrometers, -156.2 micrometers, respectively) relative to IAI patients (-96.0 micrometers, -143.5 micrometers, respectively) (LOCF). The mean change from baseline to week 12 in choroidal neovascularization and total lesion size is set forth in Figure 34 showing greater reductions in the HD group relative to the IAI group. Regarding best corrected visual acuity (BCVA), HD patients achieved a mean change, from baseline through week 16, of 8.4

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(ETDRS letters) whereas IAI patients achieved 6.5 (Figure 35) (LOCF). A smaller proportion of HD patients lost letters than IAI patients; and a greater proportion of HD patients gained letters than IAI patients at week 16 (Figure 36).

[0215] The proportion of HD patients receiving additional treatment at week 16 (19%) was smaller than that of IAI patients (27%). Also, the occurrence of ocular TEAEs through week 16 was 17% among HD patients and 22.6% among IAI patients (Figure 38(A)). The occurrence of ocular serious treatment emergent adverse events and intraocular inflammation TEAEs among HD and IAI patients, through week 16, was comparable (Figure 38(B), Figure 38(C)). Mean intraocular pressure change from baseline, occurrence of intraocular pressure events, occurrence of APTC events or deaths, hypertension adverse events among HD and IAI patients was comparable at week 16 (Figure 39, Figure 40, Figure 41, Figure 42). Mean changes in blood pressure (systolic or diastolic) through week 16 among all HD and IAI patients or patients in a dense PK sub-study was comparable (Figure 43 (A-B), Figure 44(A-B)).

Results- patients reaching week 44 (n=100) -values as reported at the time for patients reaching week 44

[0216] The study ended at week 44 with 100 patients. With an identical dosing regimen and slightly fewer rescue and/or PRN doses, a higher proportion of eyes receiving 8 mg aflibercept (HD) were dry in the center subfield relative to the eyes receiving 2 mg aflibercept (IAI). In addition, a change from baseline in central subfield thickness (CST) suggested better anatomic outcomes in the 8 mg HD group relative to the 2 mg IAI group. Changes in visual acuity from baseline favored the 8 mg dosing regimen (HD) over the 2 mg regimen (IAI) (+7.9 letters vs. +5.1 letters).

[0217] No new safety signals were seen and the safety profile for the HD group was comparable to that of IAI. There was one case of mild iritis in the HD group that resolved with topical therapy. Changes from baseline blood pressure and intraocular pressure were similar between the groups.

[0218] Out of the 106 patients that started in the study, 100 reached the 44-week point-49 in the IAI group and 51 in the HD group (Figure 45). The baseline demographics of the patients in the study were majority white and having more females than males with an average age of about 77 years as set forth in Figure 46. In addition, the baseline characteristics of the study eye in patients are set forth in Figure 47. The IAI and HD

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groups received the same mean number of injections (5.8) through week 44 (see Figure 48) with slightly more HD patients than IAI patients not receiving additional or PRN treatments.

[0219] Patients in the HD group achieved numerically superior anatomical improvements in the eye. Retinal drying (lack of fluid in the center subfield-no intraretinal fluid (IRF) and no subretinal fluid (SRF)) at weeks 16 to 44 was higher in the HD group relative to that of the IAI group (Figure 49, Figure 51). Moreover, at weeks 16 and 44, there was a greater proportion of eyes, in the HD group than in the IAI group, without fluid in the macula (no IRF and no SRF in the macula by SD-OCT) (Figure 50). The proportion of patients in each group without IRF (dry or SRF only) at weeks 16 and 44 is set forth in Figure 52. At weeks 16 and 44, 70% of patients in the HD group exhibited no subretinal fluid (SRF) (dry or with IRF only) whereas 51% of the IAI group patients exhibited no SRF (Figure 53). Through much of the 44-week trial, the HD group achieved greater mean and median reductions in central retinal thickness (CRT) from baseline (Figure 54, Figure 55).

[0220] Patients in the HD group also achieved greater gains in vision. By week 44, the mean change, from baseline, in best corrected visual acuity (BCVA) was 7.9 in the HD group and 5.1 in the IAI group (Figure 56). Fewer patients in the HD group than in the IAI group lost vision ($\geq$ 5, $\geq$10 or $\geq$15 letters lost) by week 44. Moreover, more patients in the HD group gained vision ($\geq$10 or $\geq$15 letters gained) (Figure 57) by week 44.

[0221] Ocular TEAEs and ocular serious TEAEs, intraocular inflammation TEAEs, intraocular pressure events, non-ocular TEAEs and non-ocular serious TEAEs were comparable between treatment groups (Figure 58, Figure 59 and Figure 60, Figure 61, Figure 63, Figure 64). Intraocular pressure (IOP) observed between treatment groups were also comparable (Figure 62). Moreover, the administration of the 8 mg dose in the HD group was not observed to have an effect on the occurrence of hypertension TEAEs (Figure 65). One patient died during the trial due to glioblastoma (Figure 66).

[0222] **Example 2: Characterization of Dose Accuracy**

[0223] This Example documents the procedure and execution results of deliverable volume characterization testing conducted to compare different presentations of a formulation including aflibercept (REGN3) at a concentration of 114.3 mg/ml.

[0224] The following devices will be compared:

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- **REGN3-PFS-0.5mL** (see International Design Registration, DM/212509 and International Patent Application Publication No. WO2020/247686)

and

- **1mL BD (Becton Dickinson) Luer Lok syringe** (a plastic disposable syringe that is currently used as part of the commercially available EYLEA Vial Kit presentation).

PFS=pre-filled syringe

Testing Procedures

[0225] Testing was conducted separately for each presentation. All samples were filled with 114.3 mg/mL aflibercept formulated drug substance.

[0226] REGN3-PFS-0.5mL testing:

- Sample Size: n = 60
- PFS filled with 187 microliters and primed (air volume expelled) by hand
- A volume of 85-87 microliters was targeted to be delivered from the PFS
- Delivered sample volume was determined by weighing (discussed below)
- PFS was fitted with BD Hypoint 30G ½" needles

[0227] 1mL BD Luer Lok Syringe testing:

- Sample Size: n = 310
- Syringe fitted with BD PrecisionGlide 30G ½" needles
- Sample was drawn up into the syringe samples from a beaker and manually primed
- A volume of 70 microliters was targeted to be delivered from the syringe
- Delivered sample volume was determined by weighing (discussed below)

Results of Deliverable Volume Testing

[0228] Deliverable volume was calculated by collecting the dose delivered through manual injection after the syringe was manually primed and weighing the collected dose on a balance. The collected mass was then divided by the density of the FDS (1.059 g/ml) to calculate the volume delivered.

$$V = (m_{dose}/\rho) * 1000,$$

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where V is the delivered volume (μL), m_{dose} is the dose mass (g), and ρ is the solution density (g/mL). Preconditioning of samples at 5°C was conducted for the testing in order to maintain drug product integrity.

[0229] REGN3-PFS-0.5mL:

- Mean delivered dose volume: 81.670 microliters
- Std Dev: 4.458
- Min: 64.684 microliters
- Max: 89.481 microliters

[0230] 1mL BD (Becton Dickinson) Luer Lok syringe:

- Mean delivered dose volume: 75.740 microliters
- Std Dev: 8.665
- Min: 44.287 microliters
- Max: 97.828 microliters

[0231] The testing of REGN3-PFS-0.5mL demonstrated that 59 out of 60 samples were able to administer a volume with high precision with 1 sample delivering volume of 64.684 microliters. See the histogram of delivered doses of REGN3-PFS-0.5mL in Figure 25 (A).

[0232] Three hundred six (306) samples delivered with the 1mL BD (Becton Dickinson) Luer Lok syringe were utilized for data analysis. Samples 21, 74, 117 and 125 were removed from analysis due to operator error in dose preparation prior to collection. Testing showed that 252 out of 306 samples were 70 microliters -100 microliters. See the histogram of delivered doses in Figure 25(B).

[0233] The deliverable volume data of both devices showed the difference in variability and accuracy. The 1mL BD (Becton Dickinson) Luer Lok syringe has a lower average delivered volume at 74.7 microliters than the 0.5mL PFS with 81.6 microliters. However, it has a larger spread of volumes delivered with a range of 53.5 microliters compared to 24.8 microliters for the 0.5 mL PFS. The increased variability depicted in the 1mL BD (Becton Dickinson) Luer Lok syringe may be attributed to user variability in setting the dose to 70

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microliters as well as the variability in graduation printing on the syringe. Individual delivered volumes for each device are set forth in Figure 25 (C).

[0234] Both devices were able to provide a dose within a 70 microliters -100 microliters range; however, the REGN3-PFS-0.5mL was able to deliver more consistent doses within a limited deliverable volume range with increased precision.

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We claim:

1. A method for treating or preventing an angiogenic eye disorder, in a subject in need thereof, comprising administering to an eye of the subject, a single initial dose of about 8 mg or more of a VEGF antagonist, followed by one or more secondary doses of about 8 mg or more of the VEGF antagonist, followed by one or more tertiary doses of about 8 mg or more of the VEGF antagonist; wherein each secondary dose is administered about 2 to 4 weeks after the immediately preceding dose; and wherein each tertiary dose is administered about 4, 8 or 12 weeks after the immediately preceding dose.

2. The method of claim 1 wherein, while receiving said treatment:
(i) with respect to visual acuity or best corrected visual acuity (BCVA), the subject achieves:

- no loss in visual acuity or BCVA;
- a gain in visual acuity or BCVA;
- maintenance of visual acuity or BCVA
- no loss of visual acuity or BCVA by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44, following the initial dose wherein visual acuity or BCVA is according to ETDRS or the Snellen equivalent;
- no loss of visual acuity or BCVA of about 5 or more, about 10 or more, or about 15 or more letters by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44, following the initial dose wherein visual acuity or BCVA is according to ETDRS or the Snellen equivalent;
- a gain in visual acuity or BCVA, of about 5 or more, about 6 or more, about 7 or more, about 8 or more, about 9 or more, about 10 or more or about 15 or more letters, by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44 following the initial dose, wherein visual acuity or BCVA is according to ETDRS or the Snellen equivalent; and/or
- a gain in visual acuity or BCVA of about 6 or 7 or 8 letters by about week 8 and maintaining a gain of about 6 or 7 or 8 letters until at least about week 44 wherein visual acuity or BCVA is according to ETDRS or the Snellen equivalent;

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(ii) with respect to central retinal thickness (CRT), the subject achieves:

- a decrease in central retinal thickness;
- no increase in central retinal thickness;
- maintenance in central retinal thickness;
- a decrease in central retinal thickness by at least about 123, 125, 131, 142, 147, 149, 150, 151, 156, 157, 158, 159, 161, 162, 166, 167, 168, 172, 173, 175, 177, 178 or 183 micrometers by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44 following the initial dose;
- a decrease in central retinal thickness of about 47 micrometers from about week 12 to about week 20 following the initial dose;
- a decrease in central retinal thickness of about 17 micrometers from about week 24 to about week 32 following the initial dose;
- a decrease in central retinal thickness of about 18 micrometers from about week 36 to about week 44 following the initial dose; and/or
- a decrease in central retinal thickness of about 149, about 150, about 160 or about 149-160 micrometers by about week 4 following the initial dose and achieving said decrease (±1, 2, 3, 4, 5, 10, 12, 13, 14 or 15 micrometers) by about week 44 following the initial dose;
- a decrease ranging from about 131-178 or about 123-175 micrometers from about weeks 4 to 44;
- a reduction in central retinal thickness of about 160 or 161 or 162 micrometers by about week 12 and maintaining said reduction (±1, 2, 3, 4 or 5 micrometers) by about week 44;
- a reduction in central retinal thickness of about 156 micrometers by about week 16 and maintaining said reduction (±1, 2, 3, 4 or 5 micrometers) by about week 44

(iii) with respect to retinal fluid, the subject achieves:

- a dry retina having no intraretinal fluid and no subretinal fluid; or no intraretinal fluid; or no subretinal fluid, in the center subfield or macula as measured by spectral domain optical coherence tomography;

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- a dry retina having no intraretinal fluid and no subretinal fluid; or no intraretinal fluid; or no subretinal fluid; in the center subfield as measured by spectral domain optical coherence tomography by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44, following the initial dose;
- no SRF and IRF in the macula as measured by SD-OCT by week 16 or week 44 following the initial dose;
- no sub-retinal pigment epithelium (RPE) fluid until at least about week 44 following the initial dose as measured by spectral domain optical coherence tomography; and/or
- maintenance of a dry retina once achieved until at least about week 44 following the initial dose as measured by spectral domain optical coherence tomography; and/or

and/or

(iv) the subject achieves:

- a reduction in total choroidal neovascularization (CNV) lesion size by at least about 3.2 or 3.3 micrometers by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44 following the initial dose;
- no significant increase in intraocular pressure from baseline by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44 or later following the initial dose; and/or
- no significant increase in systolic (S) and/or diastolic (D) blood pressure from baseline by about week 4, 8, 9, 12, 16, 20, 24, 28, 32, 36, 40 or 44 or later following the initial dose.

3. The method of claim 2, wherein a dry retina lacks intraretinal fluid and/or subretinal fluid.

4. The method of claim 2 or 3, wherein, at or before said initial dose, the subject has one or more of the following characteristics:

- best corrected visual acuity of about 57, 58 or 57-58 ETDRS letters;
- central retinal thickness, as measured by SD-OCT, of about 488, 516, 502 or 488-516 micrometers;
- intraocular pressure of about 14, 15 or 14-15 mmHg;

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- neovascular age-related macular degeneration lesion size of about 7, 8 or 7-8 mm^2;
- choroidal neovascularization lesion size of about 7, 8 or 7-8 mm^2;
- has occult choroidal neovascularization as measured by fluorescein angiography;
- has minimal classic choroidal neovascularization as measured by fluorescein angiography; and/or
- has predominantly classic choroidal neovascularization as measured by fluorescein angiography.

5. A method for improving best corrected visual acuity, decreasing central retinal thickness and/or achieving a dry retina, in an eye of a subject in need thereof suffering from an angiogenic eye disorder, comprising administering to the eye of the subject, a single initial dose of about 8 mg or more of a VEGF antagonist, followed by one or more secondary doses of about 8 mg or more of the VEGF antagonist, followed by one or more tertiary doses of about 8 mg or more of the VEGF antagonist; wherein each secondary dose is administered about 2 to 4 weeks after the immediately preceding dose; and wherein each tertiary dose is administered about 4, 8 or 12 weeks after the immediately preceding dose.

6. A method for promoting retinal drying, in an eye of a subject with an angiogenic eye disorder, comprising administering to the eye of the subject, a single initial dose of about 8 mg or more of a VEGF antagonist, followed by one or more secondary doses of about 8 mg or more of the VEGF antagonist, followed by one or more tertiary doses of about 8 mg or more of the VEGF antagonist; wherein each secondary dose is administered about 2 to 4 weeks after the immediately preceding dose; and wherein each tertiary dose is administered about 4, 8 or 12 weeks after the immediately preceding dose.

7. The method of any one of claims 1-6 wherein 4, 8 or 12 weeks is 12 weeks.

8. The method of any one of claims 1-7, wherein about 2 to 4 weeks is 2, 3, 4 or 5 weeks.

9. The method of any one of claims 1-8 wherein 2 to 4 weeks is 4 weeks.

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10. The method of any one of claims 1-9 wherein:

- the single initial dose is followed by only 2 secondary doses,
- each secondary dose is administered about 4 weeks after the immediately preceding dose,
- the first tertiary dose is administered about 8 weeks after the immediately preceding dose,
- the first tertiary dose is administered about 12 weeks after the immediately preceding dose,
- each tertiary dose is administered about 12 weeks after the immediately preceding dose.

11. The method of any one of claims 1-10 further comprising administering one or more additional *pro re nata* doses.

12. The method of any one of claims 1-11 wherein the angiogenic eye disorder is:

- neovascular age-related macular degeneration,
- macular edema (ME),
- macular edema following retinal vein occlusion (ME-RVO),
- retinal vein occlusion (RVO),
- central retinal vein occlusion (CRVO),
- branch retinal vein occlusion (BRVO),
- diabetic macular edema (DME),
- choroidal neovascularization (CNV),
- iris neovascularization,
- neovascular glaucoma,
- post-surgical fibrosis in glaucoma,
- proliferative vitreoretinopathy (PVR),
- optic disc neovascularization,
- corneal neovascularization,
- retinal neovascularization,
- vitreal neovascularization,

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- pannus,
- pterygium,
- vascular retinopathy,
- diabetic retinopathy (DR),
- non-proliferative diabetic retinopathy,
- diabetic retinopathy characterized by a Diabetic Retinopathy Severity Scale (DRSS) level of about 47 or 53,
- proliferative diabetic retinopathy,
- proliferative diabetic retinopathy in a subject that does not suffer from DME, and/or
- diabetic retinopathy in a patient who has diabetic macular edema (DME).

13. The method of any one of claims 1-12 wherein
- the method comprises administering the secondary doses to a subject who has received the initial dose,
- the method comprises administering the remaining secondary doses to a subject who has already received one or more secondary doses,
- the method comprises administering one or more tertiary doses to a subject who has already received the secondary doses, or
- the method comprises administering one or more tertiary doses to a subject who has already received one or more tertiary doses.

14. The method of claim 6, wherein retinal drying is characterized by no intraretinal fluid (IRF) and no subretinal fluid (SRF) in the eye of the subject, after the subject has received three monthly doses of the VEGF antagonist.

15. The method of any one of claims 1-14, wherein the VEGF antagonist is a VEGF receptor fusion protein.

16. The method of any one of claims 1-15, wherein the VEGF antagonist is:
(i) a VEGF receptor fusion protein comprising two polypeptides that comprise (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO: 2; (2) a VEGFR2 component

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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) a VEGF receptor fusion protein comprising two polypeptides that comprise an immunoglobin-like (Ig) domain 2 of VEGFR1, an Ig domain 3 of a VEGFR2, and a multimerizing component;
(iii) a VEGF receptor fusion protein comprising two polypeptides that comprise an immunoglobin-like (Ig) domain 2 of VEGFR1, an Ig domain 3 of VEGFR2, an Ig domain 4 of VEGFR2 and a multimerizing component;
(iv) a VEGF receptor fusion protein comprising two VEGFR1R2-FcΔC1(a) polypeptides encoded by the nucleic acid sequence of SEQ ID NO: 1; or
(v) selected from the group consisting of: aflibercept, conbercept, an anti-VEGF antibody or antigen-binding fragment thereof or biopolymer conjugate thereof, bevacizumab, ranibizumab, pegaptanib, brolucizumab, faricimab, abicipar pegol, an anti-VEGF DARPin and a bispecific anti-VEGF/ANG2 antibody.

17. The method of any one of claims 1-16, wherein the VEGF antagonist is aflibercept or conbercept.

18. The method of any one of claims 1-17 wherein VEGF antagonist is in a pharmaceutical formulation comprising:
at least about 100 mg/ml of a VEGF receptor fusion protein,
L-arginine, and
a histidine-based buffer.

19. The method of any one of claims 1-18, wherein the VEGF antagonist is in a pharmaceutical formulation selected from the group consisting of A-KKKK.

20. The method of any one of claims 1-19, wherein the VEGF antagonist is in a pre-filled syringe.

21. The method of claim 20, wherein the pre-filled syringe is glass or plastic, and/or sterile.

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22. The method of any one of claims 1-21 wherein said about 8 mg or more is a dose of about 7.2 mg, 8 mg, 8.01 mg, 8.8 mg, 7.2-8.8 mg, 9.3 mg, 9.33 mg, 9.7 mg, 9.8 mg, 9.9 mg, or 9.7-9.9 mg, or more.

23. The method of any one of claims 1-22 wherein said dose is ± about 10%, ± about 0.5, or ± about 0.51 mg.

24. The method of any one of claims 1-23 wherein said VEGF antagonist is delivered in a volume of about 70, 81, 82, 81.7, 85, 86, 87, 85-87 microliters.

25. The method of any one of claims 1-24 wherein VEGF antagonist is delivered in a volume that is ± about 4, 4.45, 4.5, or 5 microliters.

26. The method of any one of claims 1-25 wherein said VEGF antagonist is delivered by intravitreal dose with a dose delivery device which is a syringe.

27. The method of any one of claims 1-26 wherein the VEGF antagonist is administered by intravitreal injection of a volume that is device-determined.

28. The method of any one of claims 1-27 wherein the VEGF antagonist is administered by intravitreal injection, of a formulation comprising the VEGF antagonist, with a pre-filled syringe wherein said method comprises the steps:
(a) priming the syringe by advancing the plunger rod by a predetermined distance into the body until advancement of the plunger rod is resisted by a stop;
(b) rotating the plunger rod about a longitudinal axis; and
(c) actuating the plunger rod to dispense a predetermined volume of the formulation.

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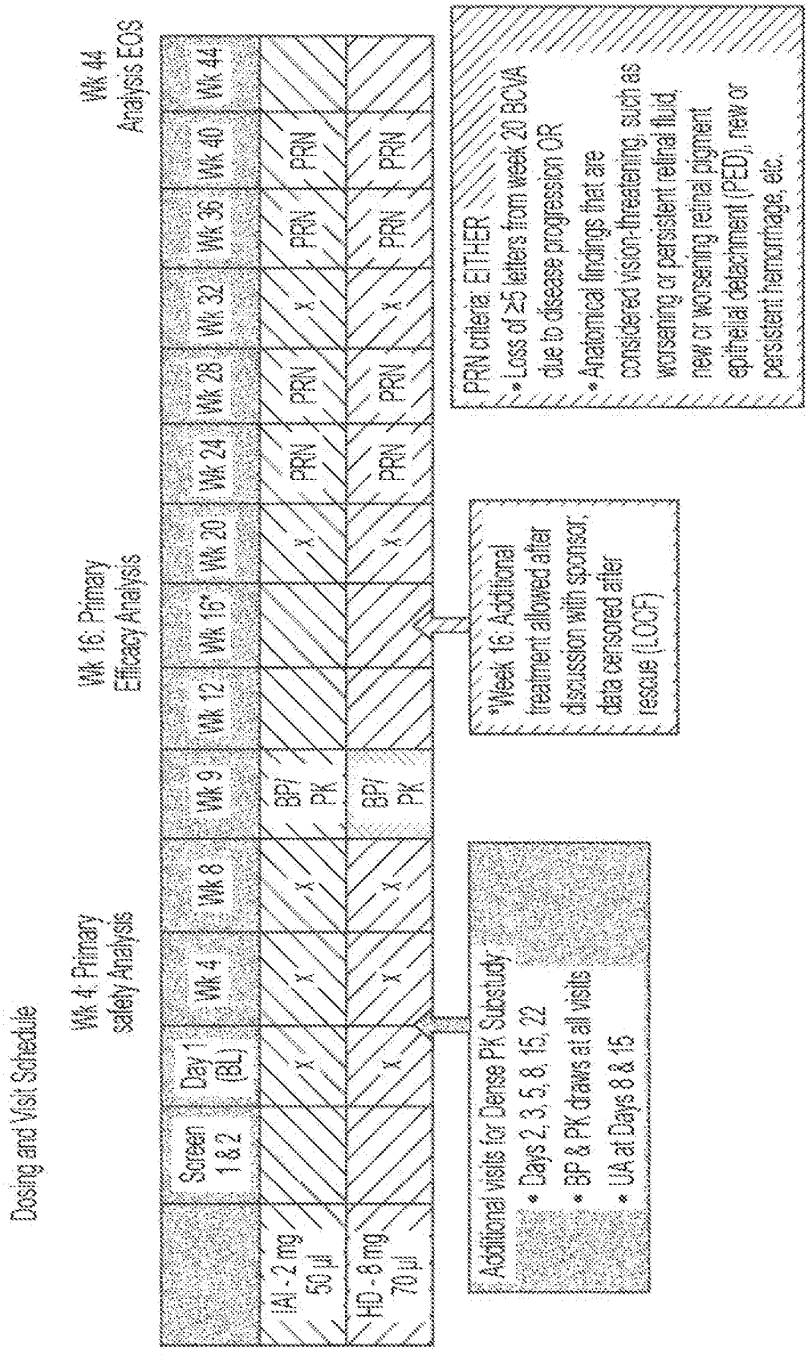

Dosing and Visit Schedule
Wk 4: Primary safety Analysis
Wk 16: Primary Efficacy Analysis
Wk 44 Analysis EOS
Screen 1 & 2
Day 1 (BL)
Wk 4
Wk 8
Wk 9
Wk 12
Wk 16*
Wk 20
Wk 24
Wk 28
Wk 32
Wk 36
Wk 40
Wk 44
IAI - 2 mg 50 µl
HD - 6 mg 70 µl
BP/ PK
PRN
Additional visits for Dense PK Substudy
• Days 2, 3, 5, 8, 15, 22
• BP & PK draws at all visits
• UA at Days 8 & 15
*Week 16: Additional treatment allowed after discussion with sponsor, data censored after rescue (LOCF)
PRN criteria: EITHER
• Loss of ≥5 letters from week 20 BCVA due to disease progression OR
• Anatomical findings that are considered vision-threatening, such as worsening or persistent retinal fluid, new or worsening retinal pigment epithelial detachment (PED), new or persistent hemorrhage, etc.

FIG. 1

Baseline Demographics (All patients*)

	IAI (n=27)	HD (n=28)	ALL (n=55)
Sex			
Male	9 (33.3%)	12 (42.9%)	21 (38.2%)
Female	18 (66.7%)	16 (57.1%)	34 (61.8%)
Ethnicity			
All	27 (100%)	28 (100%)	55 (100%)
HISPANIC OR LATINO	4 (14.8%)	1 (3.6%)	5 (9.1%)
NOT HISPANIC OR LATINO	23 (85.2%)	27 (96.4%)	50 (90.9%)
Race			
All	27 (100%)	28 (100%)	55 (100%)
WHITE	27 (100%)	28 (100%)	55 (100%)
Age (years)			
Mean (SD)	76.1 (8.90)	77.7 (7.86)	76.9 (8.35)

Percentages are calculated based on N

FIG. 2

Baseline Characteristics (All patients*)

	IAI (n=27)	HD (n=28)	ALL (n=55)
Mean (SD) BL BCVA in Study Eye (ETDRS letters)	57.1 (9.24)	59.3 (13.25)	58.2 (11.41)
Mean (SD) BL CRT in Study Eye (µm)	474.0 (222.18)	497.3 (127.66)	
Mean (SD) Baseline IOP in Study Eye (mmHg)	14.5 (3.43)	14.7 (3.32)	14.6 (3.35)

FIG. 3

Baseline Blood Pressure (All patients*)

	IAI (n=27)	HD (n=28)
Systolic Pressure (Mean (SD) mmHg)	126.27 (10.1)	122.8 (11.0)
Diastolic Pressure (Mean (SD) mmHg)	71.73 (9.7)	69.89 (9.5)

FIG. 4

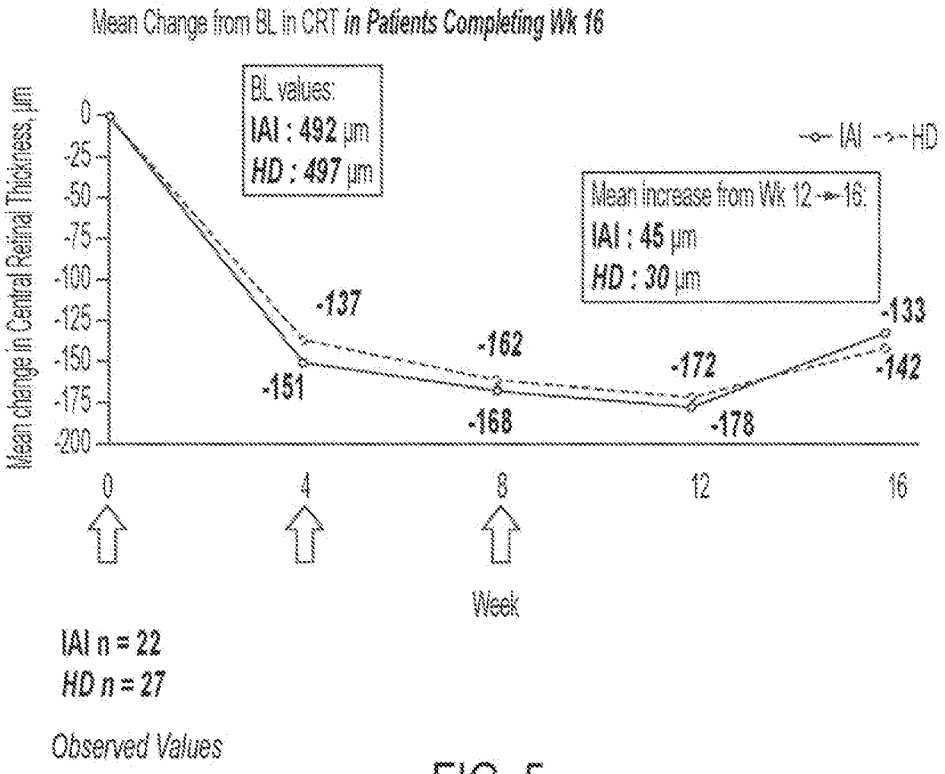

Mean Change from BL in CRT in Patients Completing Wk 16
BL values:
IAI : 492 µm
HD : 497 µm
IAI
HD
Mean increase from Wk 12 to 16:
IAI : 45 µm
HD : 30 µm
Mean change in Central Retinal Thickness, µm
0
-25
-50
-75
-100
-125
-150
-175
-200
-137
-151
-162
-168
-172
-178
-133
-142
0
4
8
12
16
Week
IAI n = 22
HD n = 27
Observed Values

FIG. 5

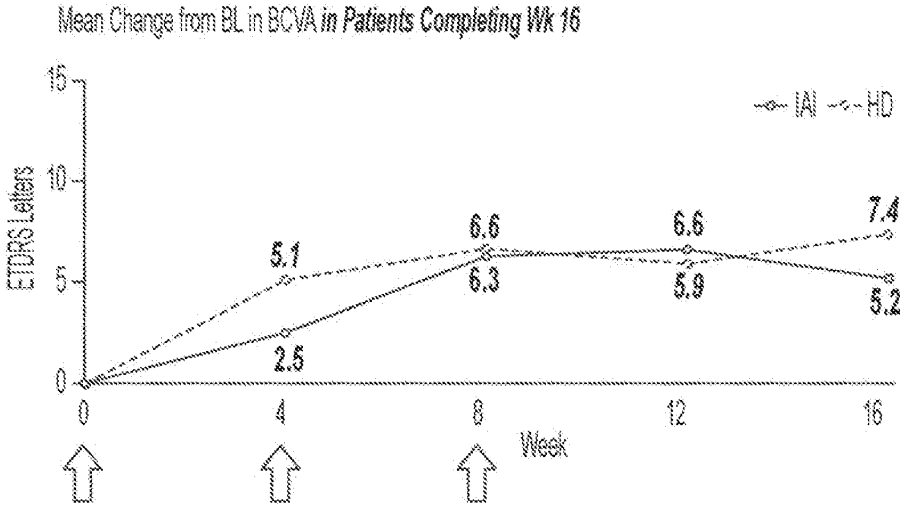

Mean Change from BL in BCVA in Patients Completing Wk 16
IAI
HD
ETDRS Letters
15
10
5
0
5.1
6.6
6.6
7.4
2.5
6.3
5.9
5.2
0
4
8
12
16
Week
IAI n = 22
HD n = 27
Observed Values

FIG. 6

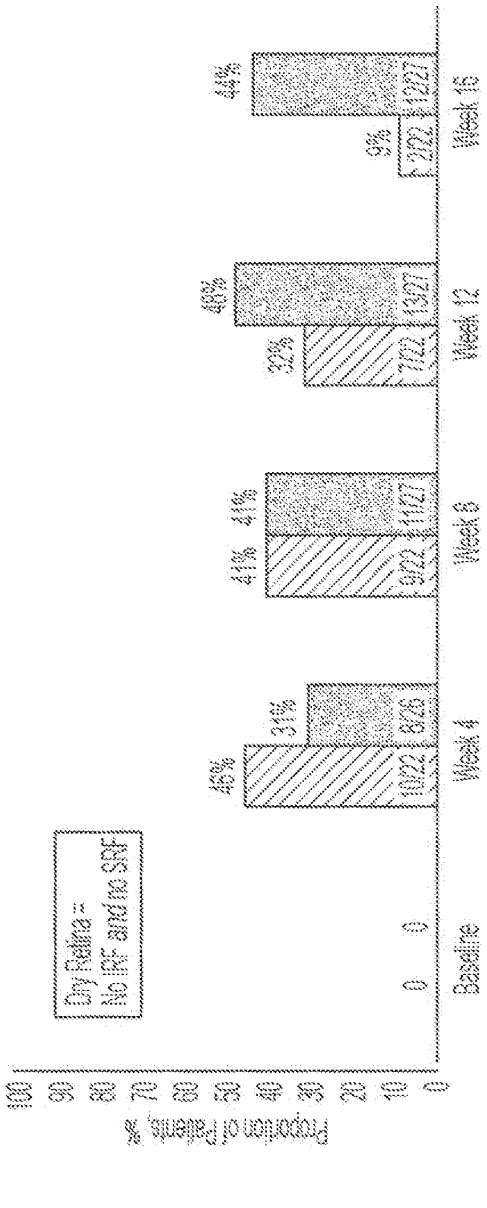

Proportion of Pts with Dry Retina in Patients Completing Wk 16
Proportion of Patients, %
100
90
80
70
60
50
40
30
20
10
0
Dry Retina =
No IRF and no SRF
0 0
46%
31%
41% 41%
32%
46%
9%
44%
1022 876
922 1027
722 1327
202 1227
Baseline
Week 4
Week 8
Week 12
Week 16
Observed Values

FIG. 7

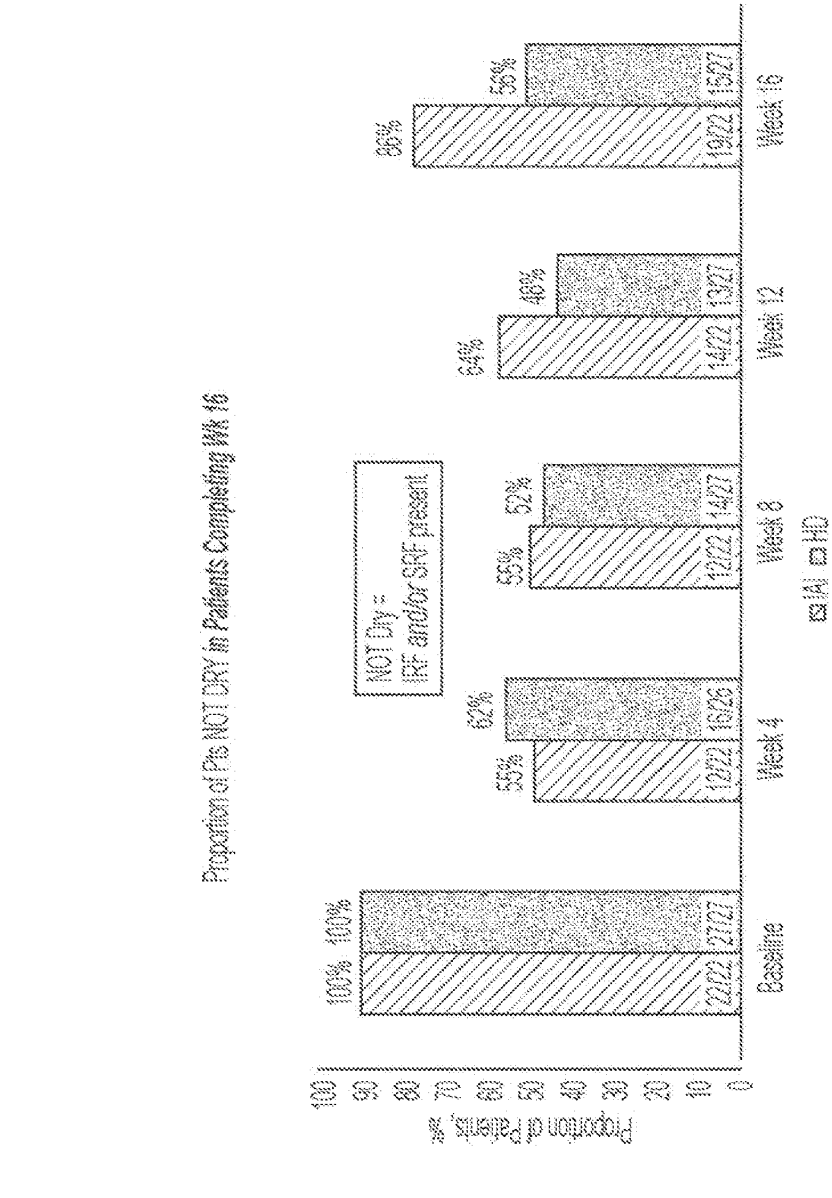

Proportion of Pts NOT DRV in Patients Completing Wk 16
Proportion of Patients, %
100
90
80
70
60
50
40
30
20
10
0
NOT Dry =
IRF and/or SRF present
100%
100%
55%
62%
55%
52%
64%
46%
86%
56%
12/22
27/27
12/22
16/26
12/22
14/27
14/22
12/27
19/22
15/27
Baseline
Week 4
Week 8
Week 12
Week 16
IAI
HD
Observed Values

FIG. 8

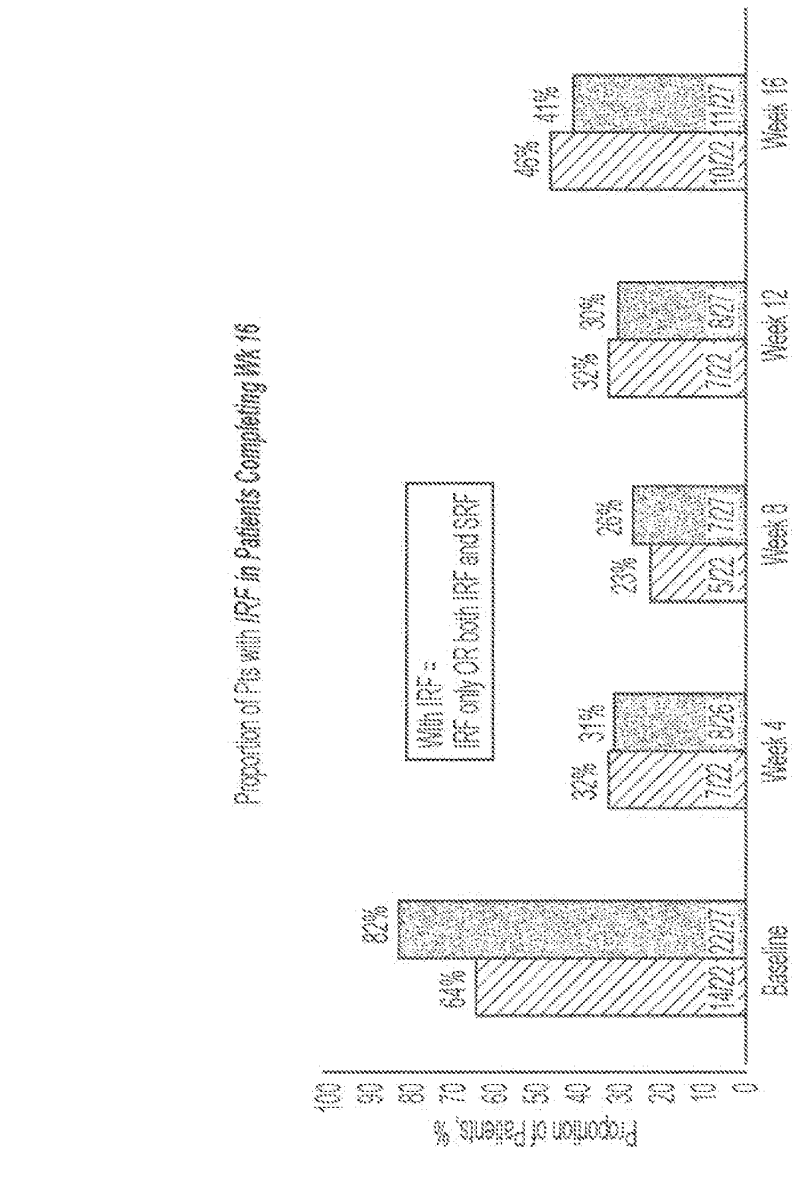

Proportion of Pts with IRF in Patients Completing Wk 16
Proportion of Patients, %
100
90
80
70
60
50
40
30
20
10
0
82%
64%
32%
31%
23%
26%
32%
30%
46%
41%
1422/2277
1722/926
522/1727
1722/927
1022/1127
Baseline
Week 4
Week 8
Week 12
Week 16
With IRF =
IRF only OR both IRF and SRF
IAI HD
Observed Values

FIG. 9

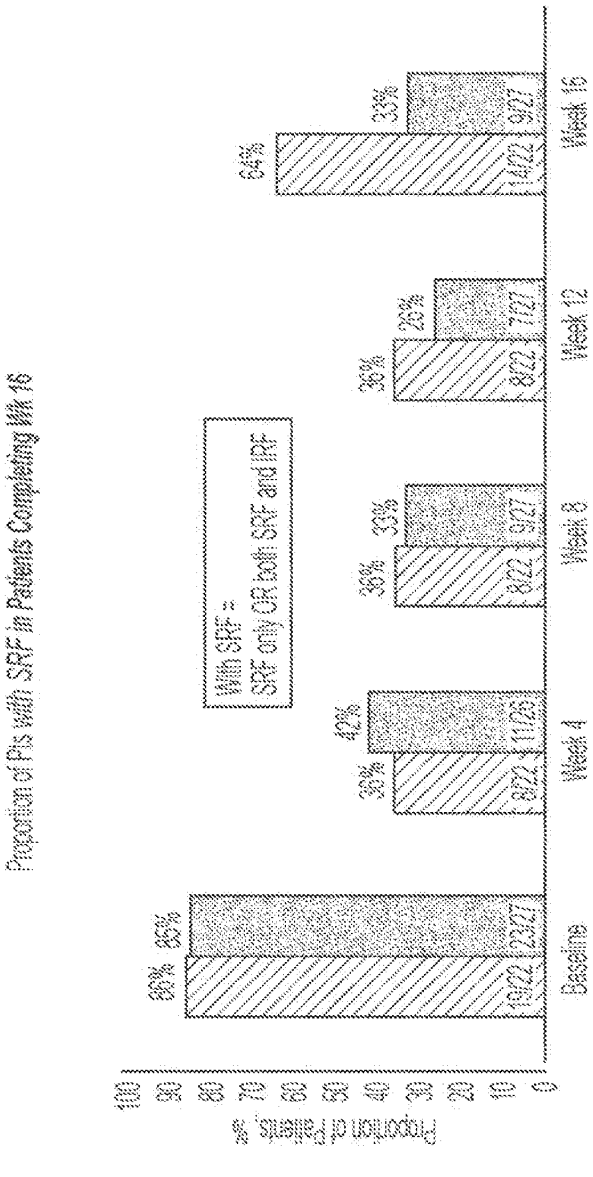

Proportion of Pts with SRF in Patients Completing Wk 16
Proportion of Patients, %
100
90
80
70
60
50
40
30
20
10
0
With SRF =
SRF only OR both SRF and IRF
68%
85%
10/22
23/27
Baseline
36%
42%
8/22
11/26
Week 4
35%
33%
8/22
9/27
Week 8
36%
26%
8/22
7/27
Week 12
64%
33%
14/22
9/27
Week 16
All
HD
Observed Values

FIG. 10

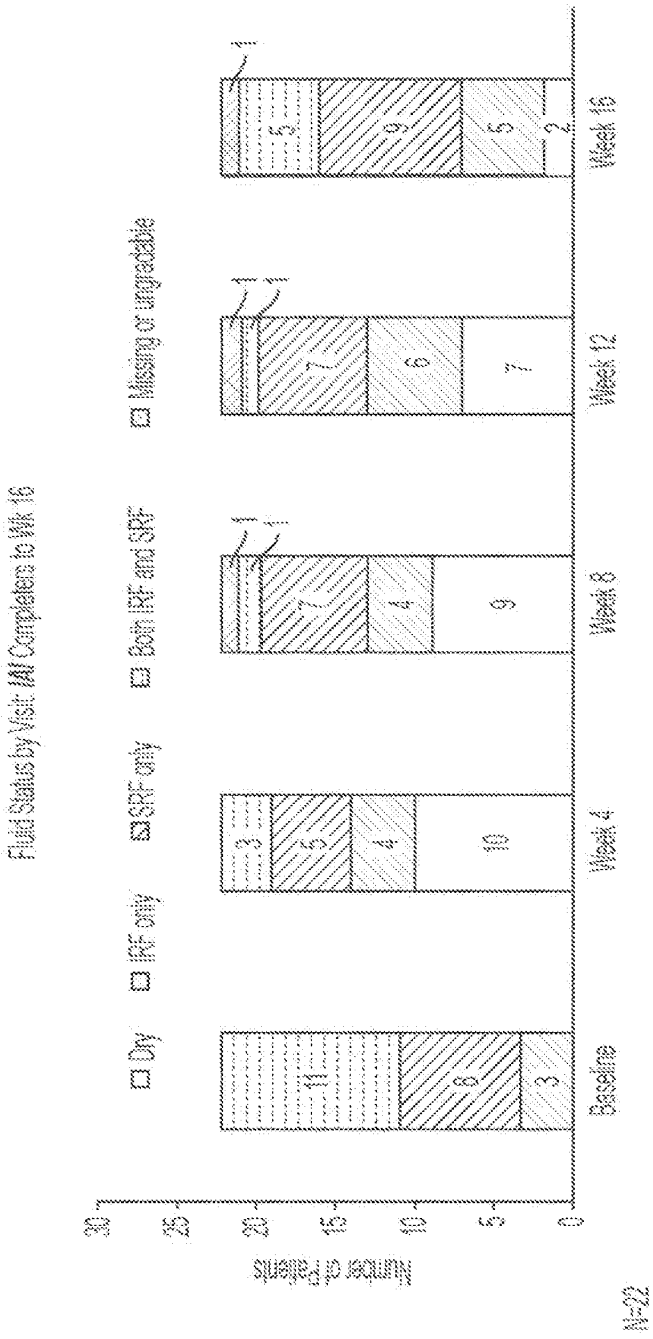

Fluid Status by Visit: IWI Completers to Wk 16
Dry
IRF only
SRF only
Both IRF and SRF
Missing or ungradable
Number of Patients
30
25
20
15
10
5
0
Baseline
Week 4
Week 8
Week 12
Week 16
N=22
3
11
6
1
3
3
4
10
1
1
7
4
9
1
1
7
6
7
1
1
3
9
5
2

FIG. 11

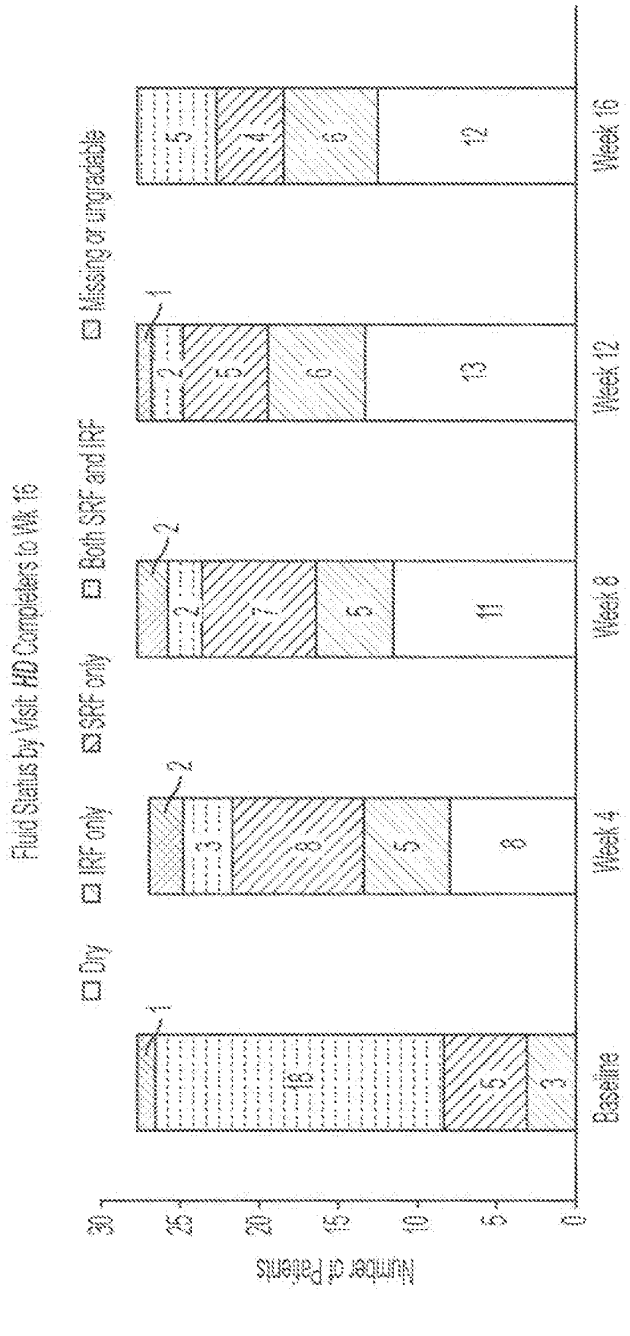

Fluid Status by Visit: HD Completers to Wk 16
Dry
IRF only
SRF only
Both SRF and IRF
Missing or ungradable
Number of Patients
30
25
20
15
10
5
0
Baseline
Week 4
Week 8
Week 12
Week 16
N=27 at BL, Wks 8, 12, 16;
n=26 at Wk 4

FIG. 12

Treatment Exposure (All patients*)

	IAI (n=27)	HD (n=28)
Mean (SD)	4.1 (1.07)	3.9 (0.92)
Median	4.0	4.0
Q1:Q3	4.0 : 5.0	3.0 : 4.5

FIG. 13

Summary of Ocular TEAEs in Study Eye

	AI (N=27)	HD (N=27)
Number of Patients with at Least One Such AE, n(%)	8 (29.6%)	4 (14.3%)
CHALAZION	0	1 (3.6%)
CONJUNCTIVAL DISORDER	0	1 (3.6%)
CONJUNCTIVAL HEMORRHAGE	1 (3.7%)	1 (3.6%)
CONJUNCTIVAL HYPEREMIA	0	1 (3.6%)
DRY EYE	0	1 (3.6%)
EYE PAIN	0	1 (3.6%)
MACULOPATHY	0	1 (3.6%)
NEOVASCULAR AMD	0	1 (3.6%)
PUNCTATE KERATITIS	1 (3.7%)	1 (3.6%)
RETINAL CYST	1 (3.7%)	0
RETINAL HEMORRHAGE	1 (3.7%)	0
RETINAL EDEMA	0	1 (3.6%)
SUBRETINAL FLUID	0	1 (3.6%)
TRICHIASIS	0	1 (3.6%)
VISION BLURRED	0	1 (3.6%)
VISUAL ACUITY REDUCED*	2 (7.4%)	0
VISUAL IMPAIRMENT	1 (3.7%)	0
VITREOUS DETACHMENT	2 (7.4%)	2 (7.1%)
CORNEAL ABRASION	0	1 (3.6%)

*1 of these events is an SAE

FIG. 14

Summary of TEAE IOI of Study Eye

	IAI (N=27)	HD (N=27)
Number of Patients with at Least One Such AE, n (%)	0	0

FIG. 15

Intraocular Pressure

	IAI (n=27)	HD (n=28)
# pts with ≥10 mmHg increase in pre-injection IOP from BL	0	1 (3.6%)
# of patients with ≥10 mmHg increase in post-injection IOP from pre-injection IOP at any visit	7 (25.9%)	4 (14.3%)
# pts with post-injection IOP > 25 mmHg at any visit	3 (11.1%)	5 (17.9%)
# pts with pre-injection IOP ≥ 25 mmHg at any visit	0	0
# pts with any visit IOP ≥ 35 mmHg at Any Time	0	0

Safety Analysis Set

Percentages are calculated based on N

FIG. 16

Summary of Non-Ocular TEAEs (1 of 2)

	IAI (N=27)	HD (N=28)
Number of Patients with at Least One Such AE, n(%)	*8 (29.6%)*	*10 (35.7%)*
DIARRHEA	1 (3.7%)	1 (3.6%)
GERD	1 (3.7%)	0
SMALL INTESTINAL OBSTRUCTION	1 (3.7%)	0
PERIPHERAL SWELLING	1 (3.7%)	0
EAR INFECTION FUNGAL	1 (3.7%)	0
SEASONAL ALLERGY	0	1 (3.6%)
SINUSITIS	1 (3.7%)	1 (3.6%)
BACK INJURY	0	1 (3.6%)
FALL	0	1 (3.6%)
BACTERIAL TEST POSITIVE	0	1 (3.6%)
HEPATIC ENZYME INCREASED	1 (3.7%)	0

FIG. 17

Summary of Non-Ocular TEAEs (2 of 2)

	IAI (N=27)	HD (N=28)
Number of Patients with at Least One Such AE, n(%)	*8 (29.6%)*	*10 (35.7%)*
URINE KETONE BODIES PRESENT	1 (3.7%)	0
WHITE BLOOD CELLS URINE	0	1 (3.6%)
TRANSIENT ISCHAEMIC ATTACK	0	1 (3.6%)
COUGH	0	1 (3.6%)
DERMATITIS CONTACT	0	1 (3.6%)
HYPERTENSION	1 (3.7%)	1 (3.6%)

FIG. 18

Non-Ocular Serious Adverse Events

	IAI (N=27)	HD (N=28)
Number of Patients with at Least One Non-Ocular SAE, n(%)	1 (3.7%)	1 (3.6)
Small Intestinal Obstruction	1	
TIA		1

FIG. 19

Hypertension Adverse Events

	IAI (N=27)	HD (N=28)
Number of Patients with at Least One Such AE, n(%)	1 (3.7%)	1 (3.6%)
Hypertension	1 (3.7%)	1 (3.6%)

FIG. 20

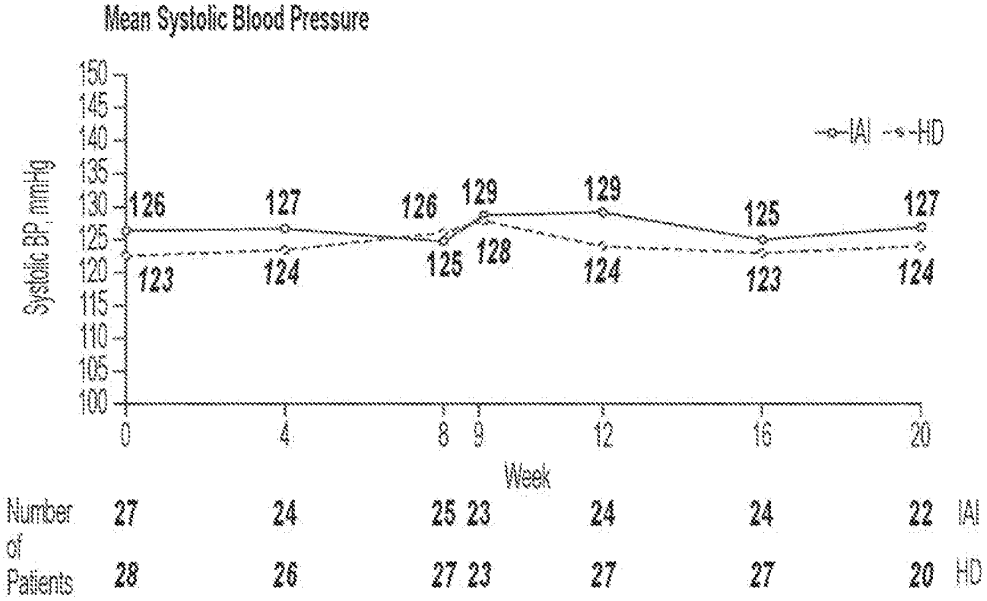

Mean Systolic Blood Pressure
Systolic BP, mmHg
150
145
140
135
130
125
120
115
110
105
100
IAI
HD
126
127
126
129
129
125
127
123
124
125
128
124
123
124
0
4
8
9
12
16
20
Week
Number of Patients
27
24
25
23
24
24
22
IAI
28
26
27
23
27
27
20
HD

FIG. 21

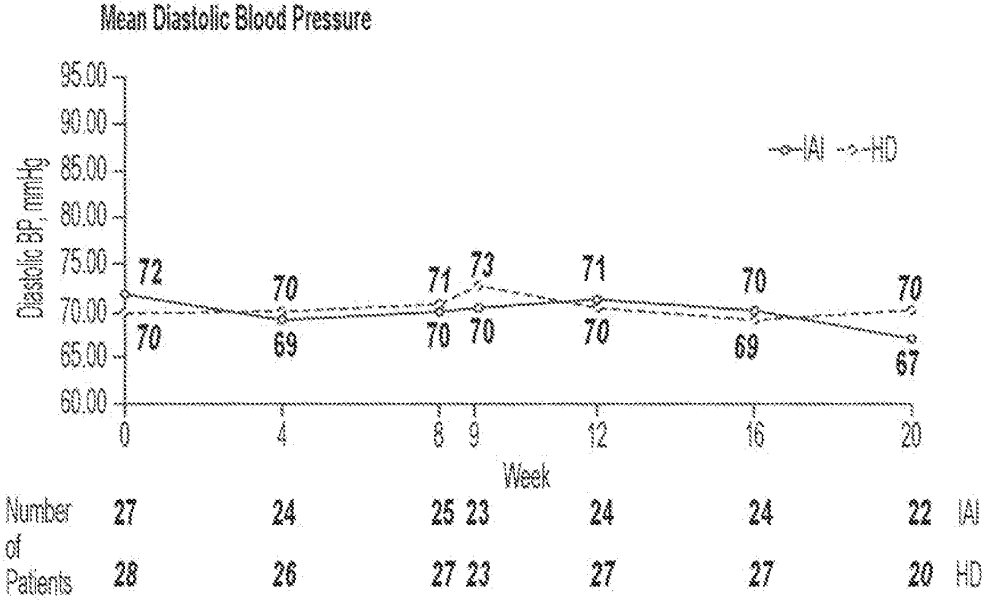

Mean Diastolic Blood Pressure
Diastolic BP, mmHg
95.00
90.00
85.00
80.00
75.00
70.00
65.00
60.00
IAI
HD
72
70
71
73
71
70
70
70
69
70
70
70
69
67
0
4
8
9
12
16
20
Week
Number of Patients
27
24
25
23
24
24
22
IAI
28
26
27
23
27
27
20
HD

FIG. 22

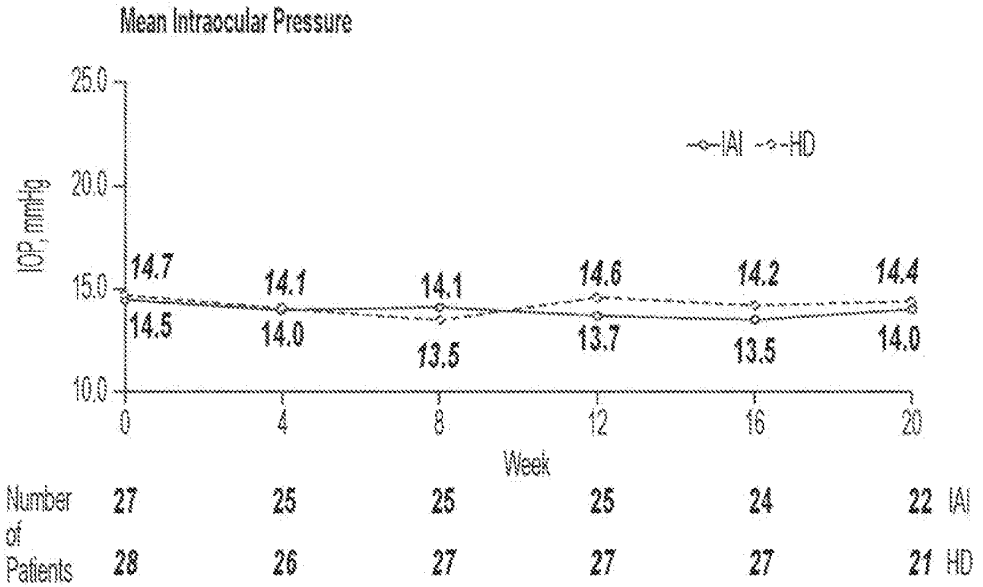

Mean Intraocular Pressure
IAI
HD
IOP, mmHg
25.0
20.0
15.0
10.0
14.7
14.1
14.1
14.6
14.2
14.4
14.5
14.0
13.5
13.7
13.5
14.0
0
4
8
12
16
20
Week
Number of Patients
27 25 25 25 24 22 IAI
28 26 27 27 27 21 HD

FIG. 23

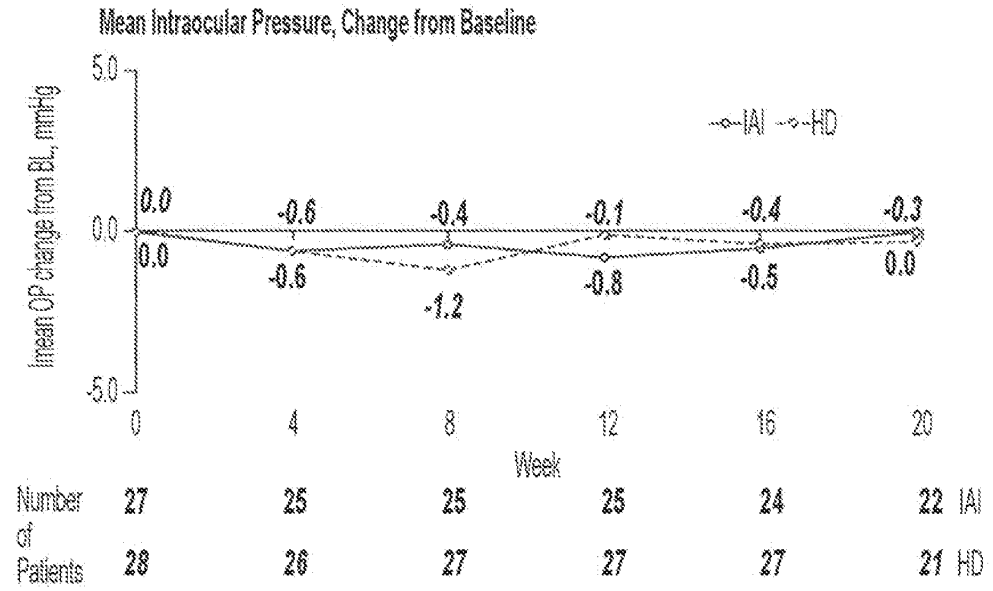

Mean Intraocular Pressure, Change from Baseline
IAI
HD
Imean OP change from BL, mmHg
5.0
0.0
-5.0
0.0
-0.6
-0.4
-0.1
-0.4
-0.3
0.0
-0.6
-1.2
-0.8
-0.5
0.0
0
4
8
12
16
20
Week
Number of Patients
27 25 25 25 24 22 IAI
28 26 27 27 27 21 HD

FIG. 24

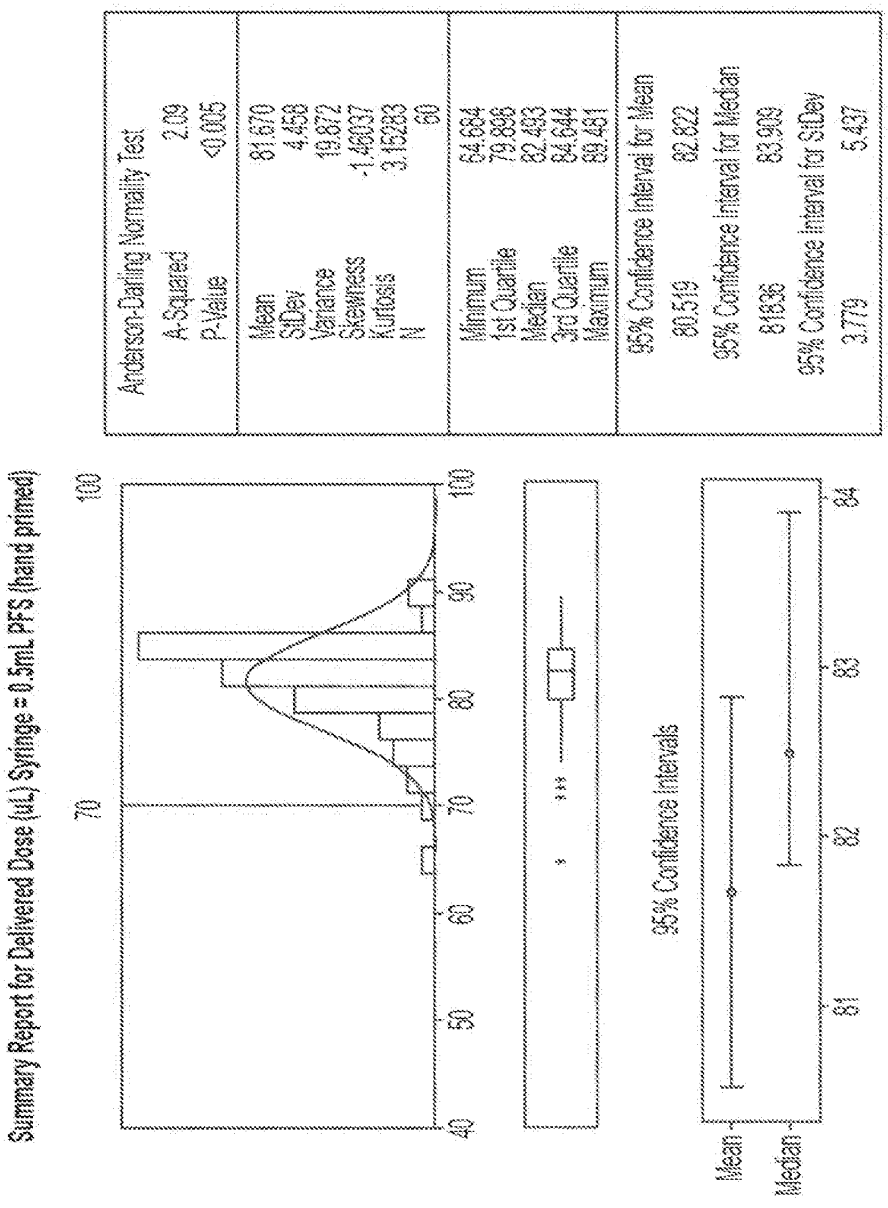

Summary Report for Delivered Dose (uL) Syringe = 0.5mL PFS (hand primed)
70
100
40
50
60
70
80
90
100
95% Confidence Intervals
Mean
Median
81
82
83
84
Anderson-Darling Normality Test
A-Squared 2.09
P-Value <0.005
Mean 81.670
StDev 4.458
Variance 19.872
Skewness -1.46037
Kurtosis 3.15283
N 60
Minimum 64.664
1st Quartile 79.896
Median 82.493
3rd Quartile 84.644
Maximum 89.481
95% Confidence Interval for Mean
80.519 82.822
95% Confidence Interval for Median
81.636 83.909
95% Confidence Interval for StDev
3.779 5.437

FIG. 25A

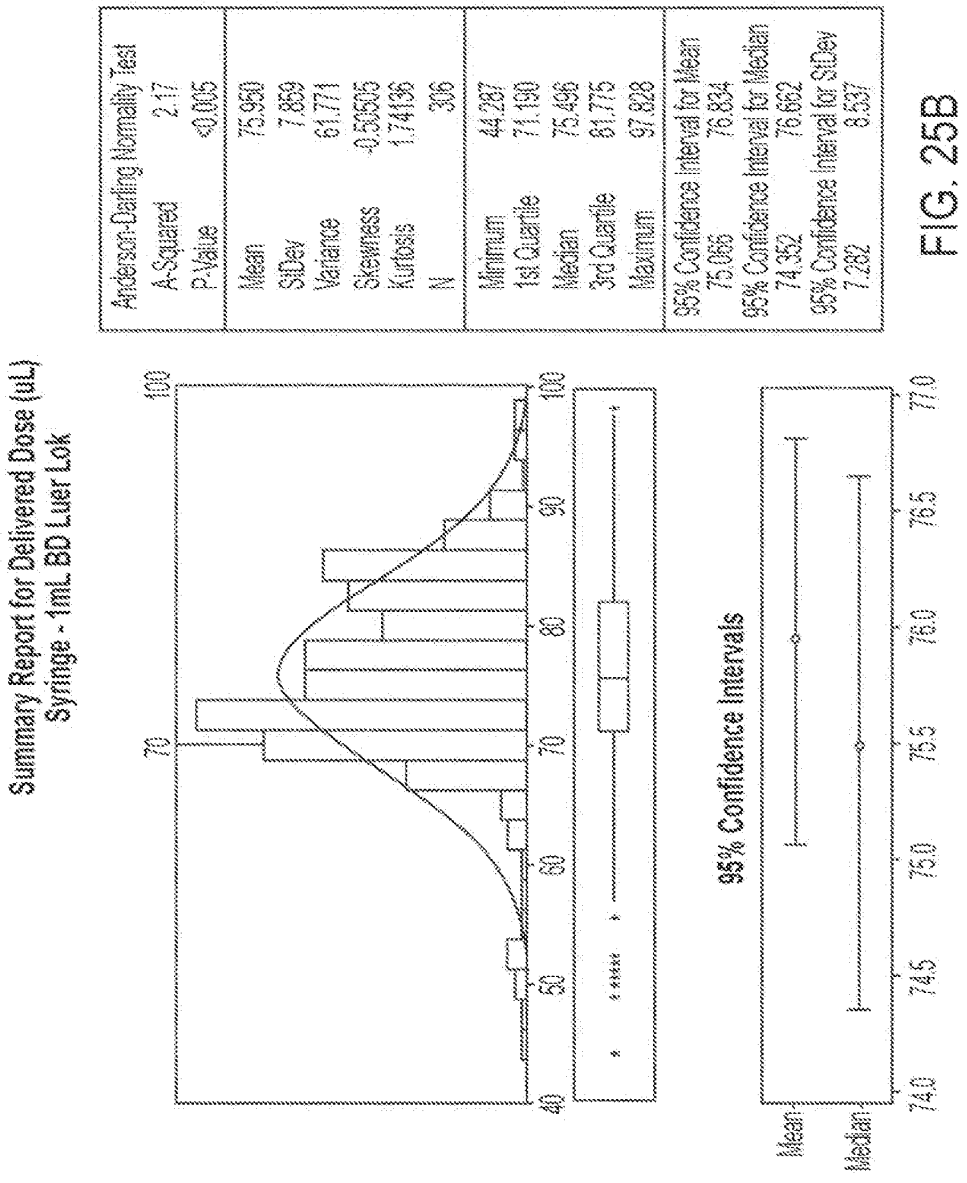

Summary Report for Delivered Dose (uL)
Syringe - 1mL BD Luer Lok
Anderson-Darling Normality Test
A-Squared 2.17
P-Value <0.005
Mean 75.950
StDev 7.859
Variance 61.771
Skewness -0.30505
Kurtosis 1.74196
N 306
Minimum 44.287
1st Quartile 71.190
Median 75.496
3rd Quartile 81.775
Maximum 97.828
95% Confidence Interval for Mean
75.065 76.834
95% Confidence Interval for Median
74.352 76.662
95% Confidence Interval for StDev
7.282 8.537
95% Confidence Intervals
Mean
Median
74.0
74.5
75.0
75.5
76.0
76.5
77.0
40
50
60
70
80
90
100

FIG. 25B

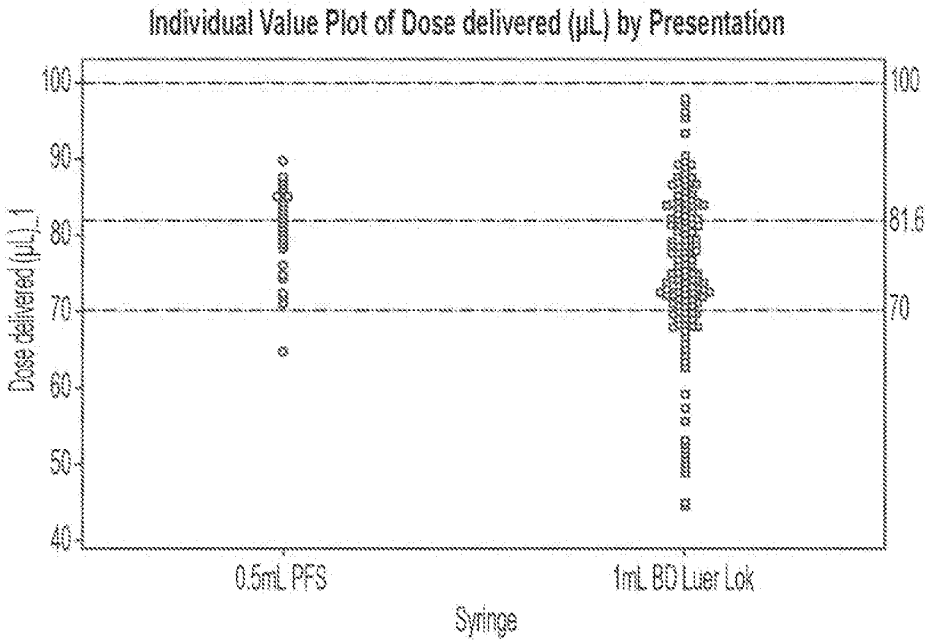

Individual Value Plot of Dose delivered (µL) by Presentation
Dose delivered (µL) (
100
90
80
70
60
50
40
100
81.6
70
0.5mL PFS
1mL BD Luer Lok
Syringe

FIG. 25C

Disposition and Exposure Through Week 16

	IAI	HD	Total
Total Number Randomized	53	53	106
Main Study	38	38	76
Dense PK Substudy	15	15	30
Number of Patients Completing Week 16	51	53	104
Number of Patients Who Discontinued	2	0	2
Adverse Event	0	0	0
Withdrawal of consent by subject	2	0	2
Due to COVID-19	*1*	0	*1*

Exposure	IAI (n=53)	HD (n=53)
Mean (SD) number of injections (of 3 planned)	2.9 (0.47)	3.0 (0.14)

FIG. 26

Baseline Demographics

	IAI (n=53)	HD (n=53)	ALL (n=106)
Sex			
Male	17 (32.1%)	23 (43.4%)	40 (37.7%)
Female	36 (67.9%)	30 (56.6%)	66 (62.3%)
Ethnicity			
All	53 (100%)	53 (100%)	106 (100%)
Hispanic or Latino	4 (7.5%)	2 (3.8%)	6 (5.7%)
Not Hispanic or Latino	49 (92.5%)	51 (96.2%)	100 (94.3%)
Race			
White	51 (96.2%)	52 (98.1%)	103 (97.2%)
Age (years)			
Mean (SD)	77.7 (8.27)	77.0 (7.69)	77.4 (7.95)

FIG. 27

Baseline Characteristics

	IAI (n=53)	HD (n=53)	All (n=106)
Mean (SD) BL BCVA in Study Eye, ETDRS letters	58.2 (10.5)	57.9 (13.6)	58.0* (11.9)
Mean (SD) BL CRT in Study Eye, µm	498.1 (204.9)	516.2 (175.64)	502.1 (190.6)
Mean (SD) Baseline IOP in Study Eye, mmHg	14.9 (3.4)	14.8 (3.4)	14.9 (3.4)
Mean (SD) Lesion Size, mm	7.9 (6.21)	7.7 (6.84)	7.8 (6.50)
Mean (SD) CNV Size, mm	7.9 (6.20)	7.5 (6.86)	7.7 (6.51)
Baseline FA classification [%]			
Occult CNV	22 (41.5%)	26 (49.1%)	48 (45.3%)
Minimally Classic	26 (49.1%)	19 (35.8%)	45 (42.5%)
Predominantly Classic	4 (7.5%)	8 (15.1%)	12 (11.3%)
Missing	1 (1.9%)	0	1 (0.9%)

*58 ETDRS letters ~20/60-20/70 Snellen visual acuity

FIG. 28

Baseline Blood Pressure and Medical History of Hypertension (All patients)

	IAI (n=53)	HD (n=53)
Systolic Pressure (Mean (SD) mmHg)	125.4 (10.6)	124.6 (10.6)
Diastolic Pressure (Mean (SD) mmHg)	70.7 (9.0)	71.7 (8.5)

	IAI (n=53)	HD (n=53)
Medical History of Vascular Hypertensive Disorders, n (%)	31 (58.5%)	26 (49.1%)

FIG. 29

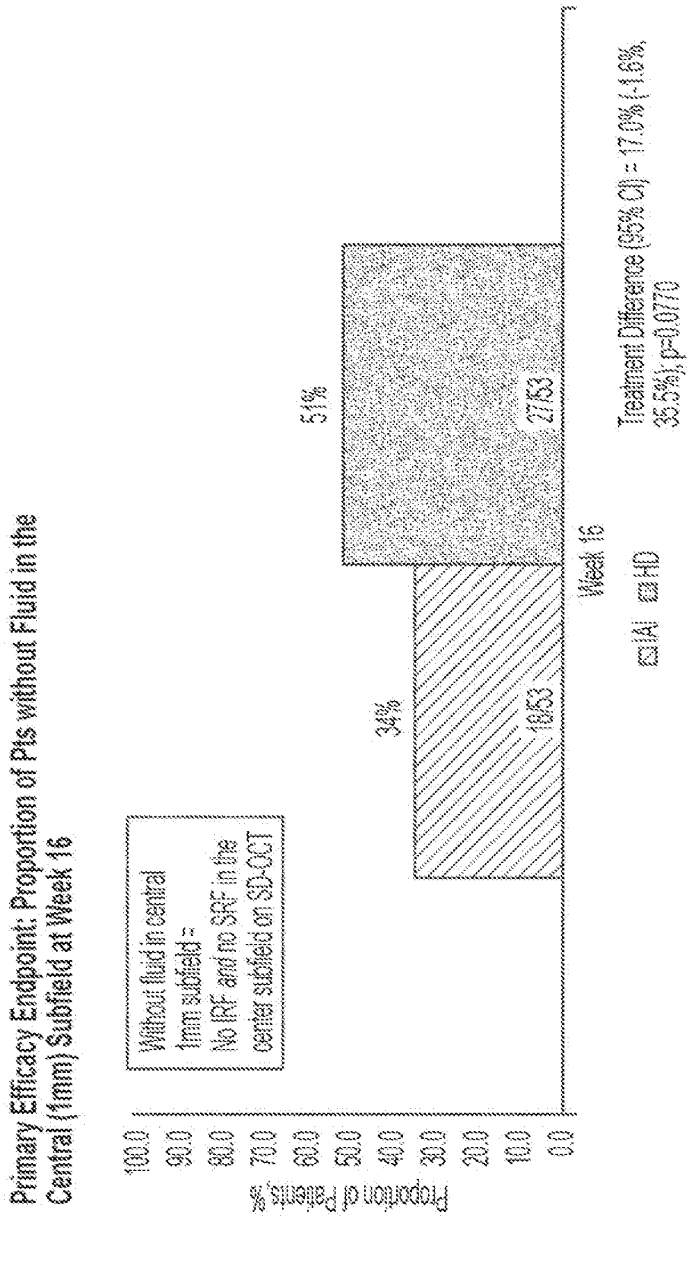

Primary Efficacy Endpoint: Proportion of Pts without Fluid in the Central (1mm) Subfield at Week 16
Without fluid in central 1mm subfield = No IRF and no SRF in the center subfield on SD-OCT
Proportion of Patients, %
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
34%
51%
16/53
27/53
Week 16
IAI
HD
Treatment Difference (95% CI) = 17.0% (-1.6%, 35.5%); p=0.0770
LOCF

FIG. 30

Proportion of Pts without Intraretinal Fluid & without Subretinal Fluid in the Center (1mm) Subfield at Week 16
Proportion of Patients, %
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Without IRF (Intraretinal Fluid) (Dry or with SRF only)
68%
70%
36/53
37/53
Without SRF (Subretinal Fluid) (Dry or with IRF only)
51%
70%
27/53
37/53
Treatment effect for SRF= 18.9% (95% CI= 0.6, 37.1); p=0.0471
IAI
HD
LOCF

FIG. 31

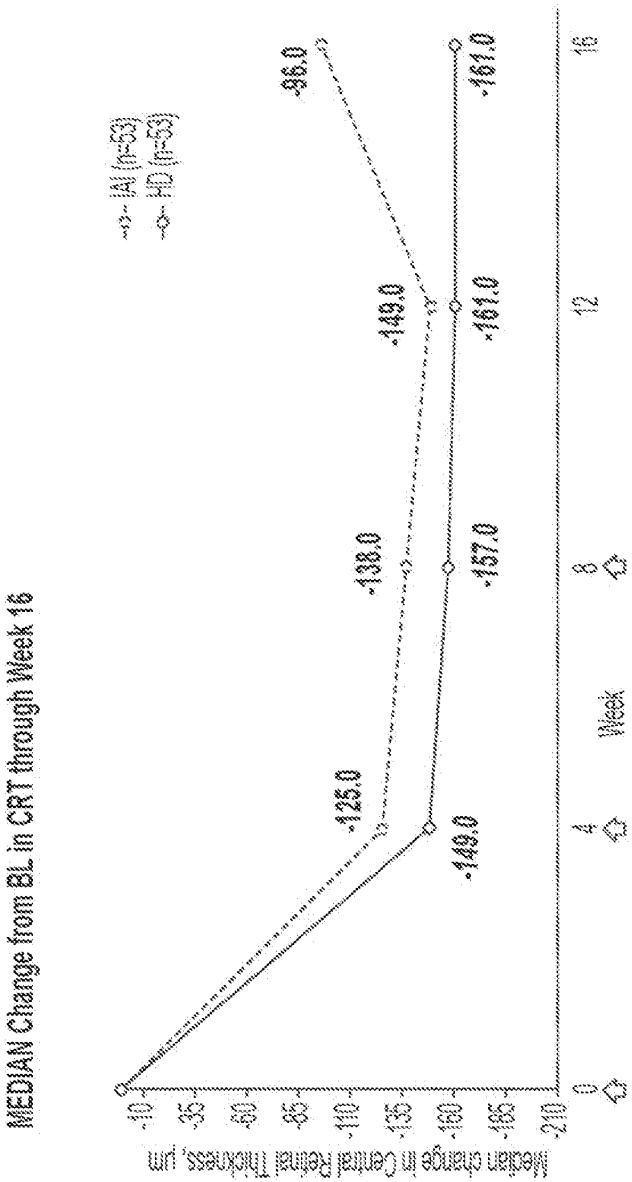


FIG. 32

FAS, LOCF

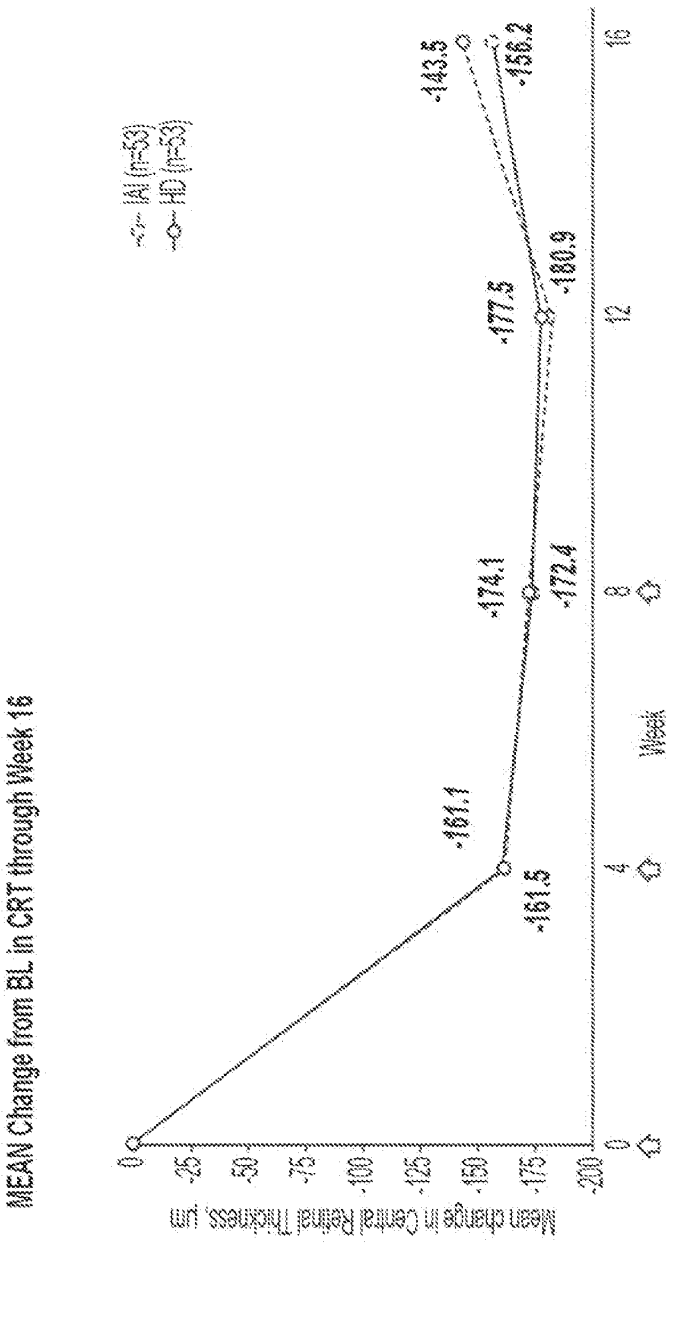


FIG. 33

FAS, LOCF

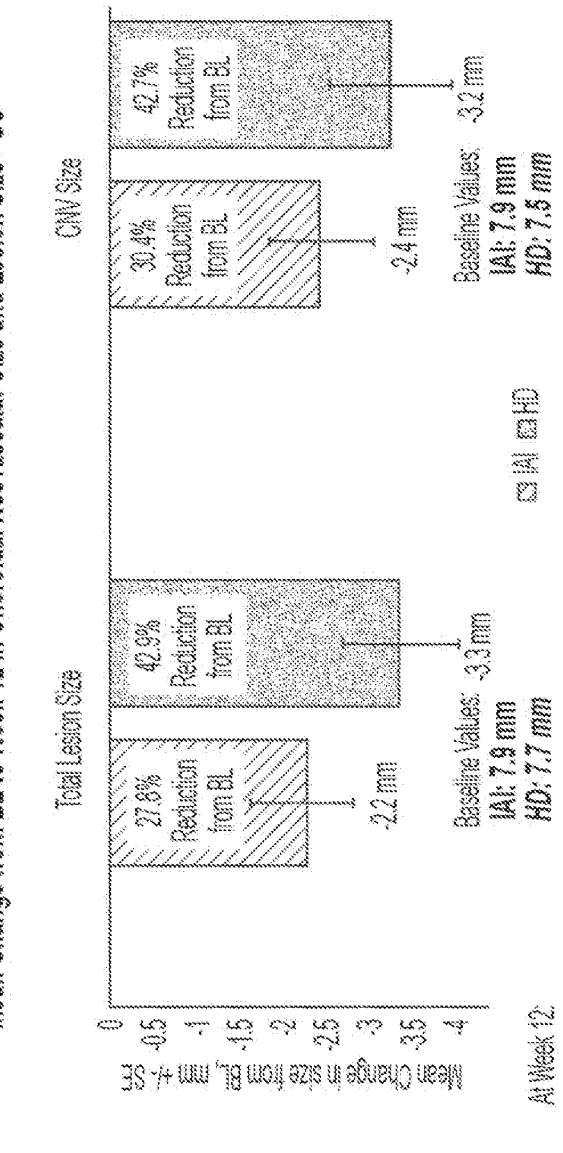

Mean Change from BL to Week 12 in Choroidal Neovascular Size and Lesion Size - OC
Total Lesion Size
CNV Size
Mean Change in size from BL, mm +/- SE
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
27.8% Reduction from BL
42.9% Reduction from BL
30.4% Reduction from BL
42.7% Reduction from BL
-2.2 mm
-3.3 mm
-2.4 mm
-3.2 mm
Baseline Values:
IAI: 7.9 mm
HD: 7.7 mm
Baseline Values:
IAI: 7.9 mm
HD: 7.5 mm
IAI
HD
At Week 12:
IAI n=44
HD n=46

FIG. 34

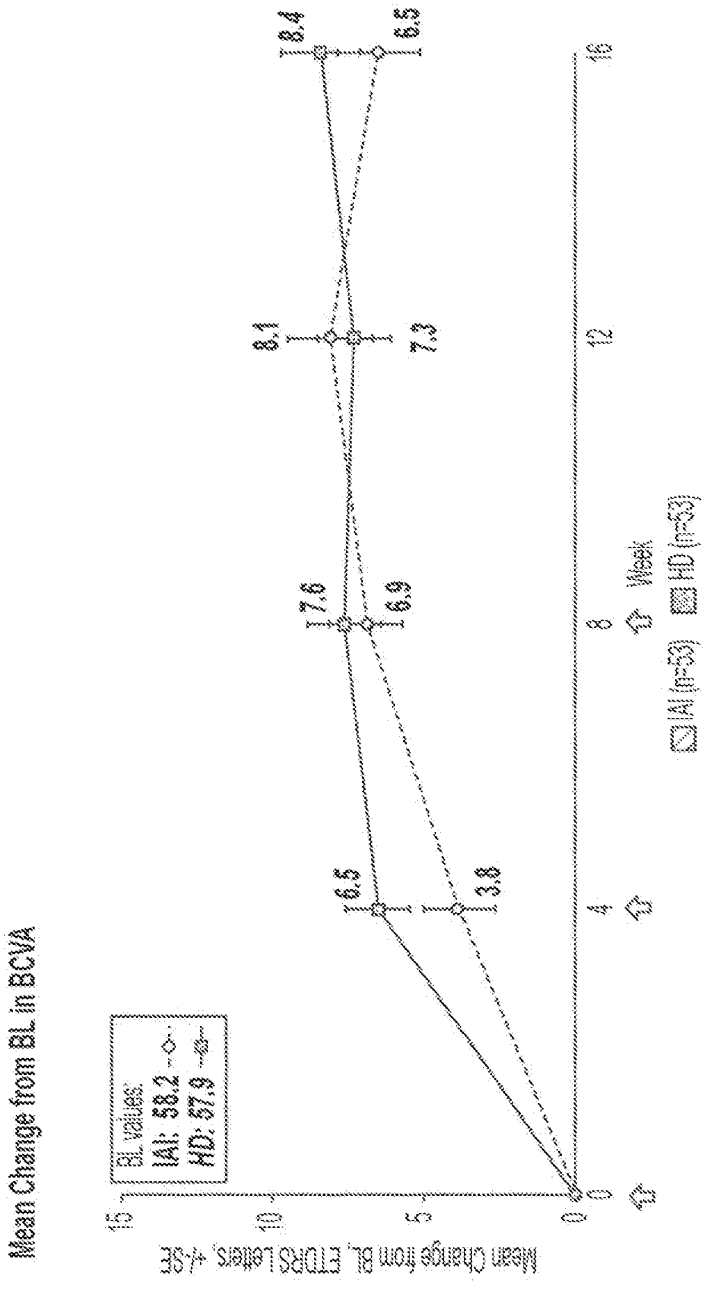

Mean Change from BL in BCVA
LOCF
Mean Change from BL, ETDRS Letters, +/-SE
15
10
5
0
BL values:
IAI: 58.2
HD: 57.9
0
4
8
12
16
Week
IAI (n=53)
HD (n=33)
6.5
3.8
7.6
6.9
8.1
7.3
8.4
6.5

FIG. 35

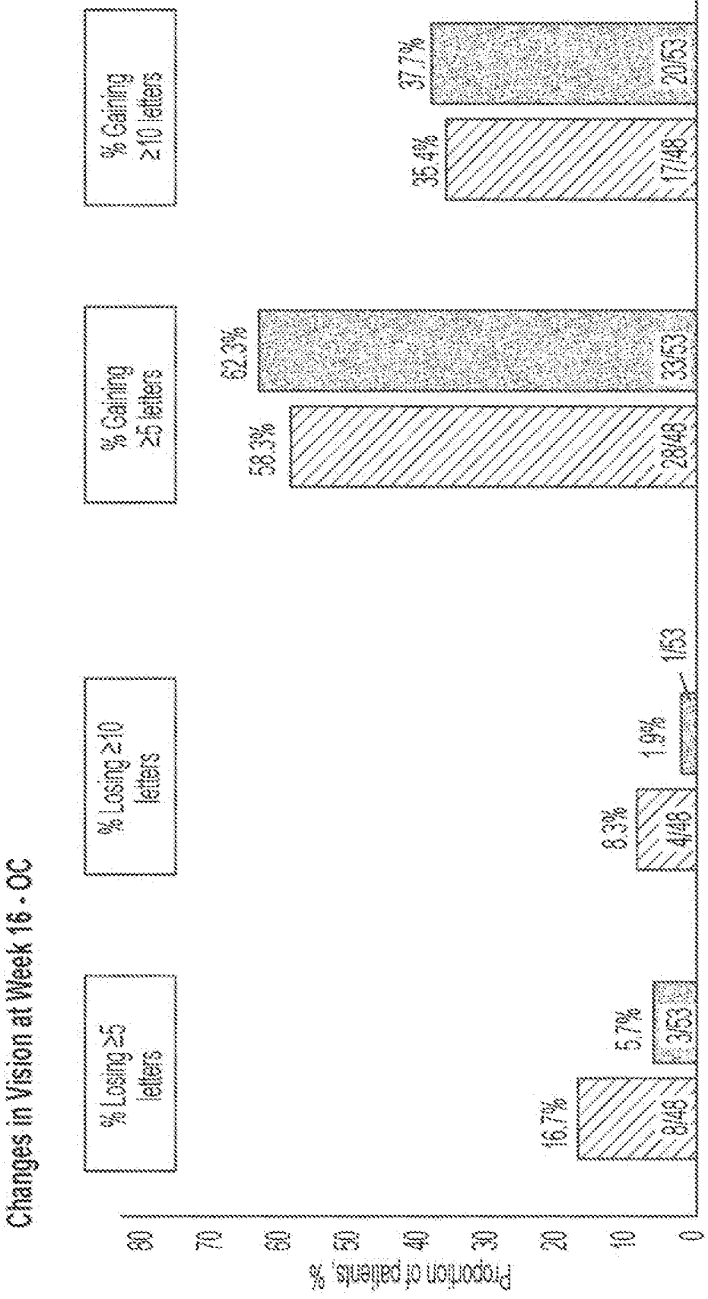

Changes in Vision at Week 16 - OC
% Losing ≥5 letters
% Losing ≥10 letters
% Gaining ≥5 letters
% Gaining ≥10 letters
Proportion of patients, %
0
10
20
30
40
50
60
70
80
16.7%
8/48
5.7%
3/53
8.3%
4/48
1.9%
1/53
58.3%
28/48
62.3%
33/53
35.4%
17/48
37.7%
20/53
IAI
HD

FIG. 36

Proportion of patients Receiving Additional Treatment at Week 16

	IAI (N=53)	HD (N=53)
Number of patients who had a Week 16 visit	48	53
Proportion of patients who had a Week 16 visit and who received Additional Treatment	27%	19%

FIG. 37

Ocular TEAEs in Study Eye Occurring in ≥2% of Patients Through Week 16

	IAI (N=53)	HD (N=53)
Number of Patients with at Least One TEAE in Study Eye, n (%)	12 (22.6%)	9 (17.0%)
CONJUNCTIVAL HAEMORRHAGE	2 (3.8%)	3 (5.7%)
NEOVASCULAR AGE-RELATED MACULAR DEGENERATION	2 (3.8%)	0
RETINAL HAEMORRHAGE	2 (3.8%)	0
VISUAL ACUITY REDUCED	2 (3.8%)	0
VISUAL IMPAIRMENT	2 (3.8%)	0
VITREOUS DETACHMENT	1 (1.9%)	2 (3.8%)

FIG. 38A

Ocular Serious Treatment Emergent Adverse Events in Study Eye Through Week 16

	All (N=53)	HD (N=53)
Number of Patients with at Least One Ocular SAE in the Study Eye*, n (%)	1 (1.9%)	1 (1.9%)
VISUAL ACUITY REDUCED	1 (1.9%)	0
RETINAL TEAR	0	1 (1.9%)

FIG. 38B

Intraocular Inflammation TEAEs in the Study Eye Through Week 16

	IAI (N=53)	HD (N=53)
Number of Patients with at Least One Such AE, n	0	0

FIG. 38C

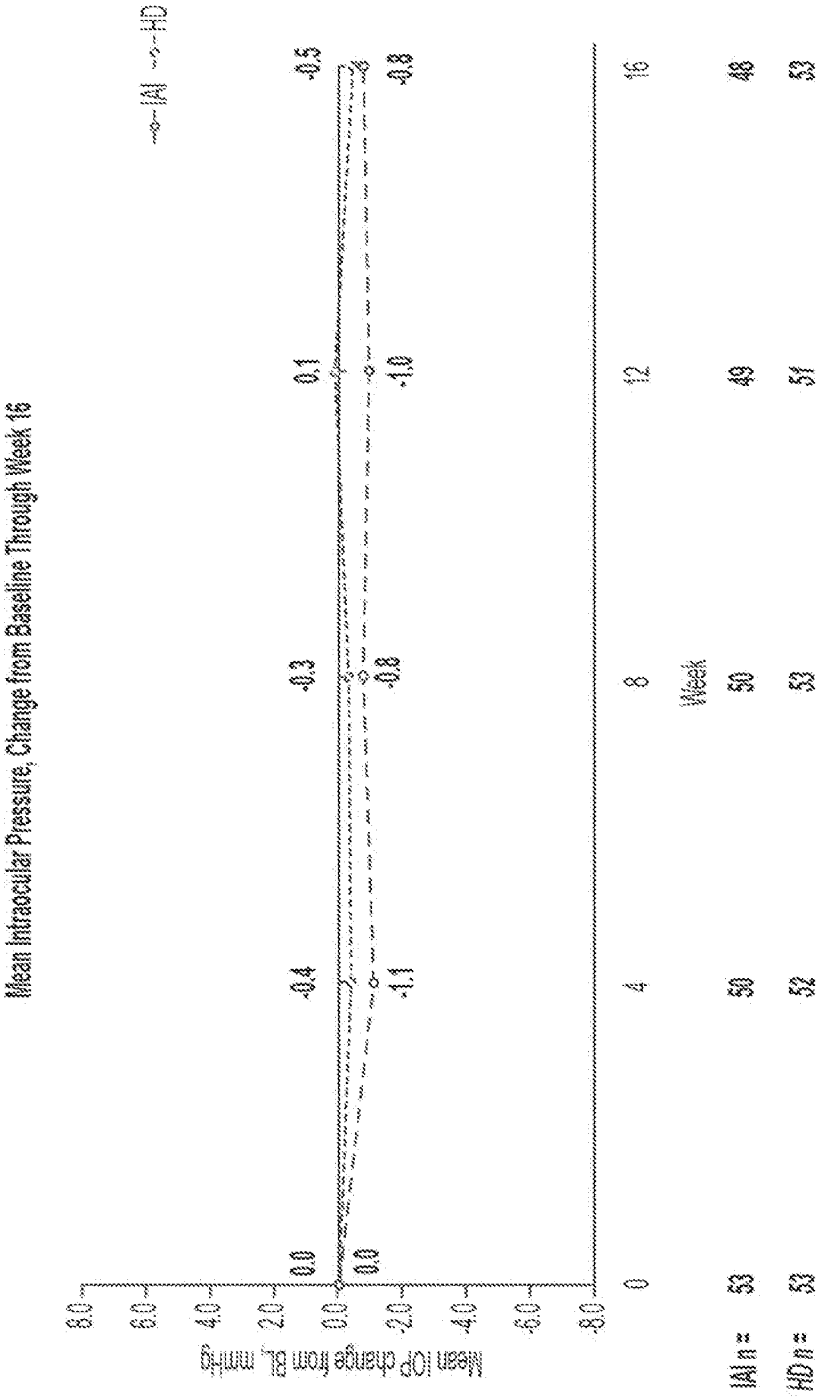

Mean Intraocular Pressure, Change from Baseline Through Week 16
IAI
HD
Mean IOP change from BL, mmHg
8.0
6.0
4.0
2.0
0.0
-2.0
-4.0
-6.0
-8.0
0.0
0.0
-0.4
-0.3
-0.8
-1.1
0.1
-1.0
-0.5
-0.8
0
4
8
12
16
Week
IAI n =
53
50
50
49
48
HD n =
53
52
53
51
53

FIG. 39

Summary of Intraocular Pressure Events Through Week 16

	IAI (N=53)	HD (N=53)
# pts with ≥10 mmHg increase in pre-injection IOP from BL	0	0
# pts with pre-injection IOP ≥25 mmHg at any visit	0	1 (1.9%)
# pts with any visit IOP ≥35 mmHg at Any Time	0	0

Safety Analysis Set

Percentages are calculated based on N

FIG. 40

APTC Events or Deaths Through Week 16

	IAI (N=53)	HD (N=53)
Number of Patients with at Least One Such AE, n	0	0

FIG. 41

Hypertension Adverse Events Through Week 16

	IAI (N=53)	HD (N=53)
Number of Patients with at Least One Such AE, n (%)	1 (1.9%)	1 (1.9%)
Worsening Hypertension	1 (1.9%)	1 (1.9%)

FIG. 42

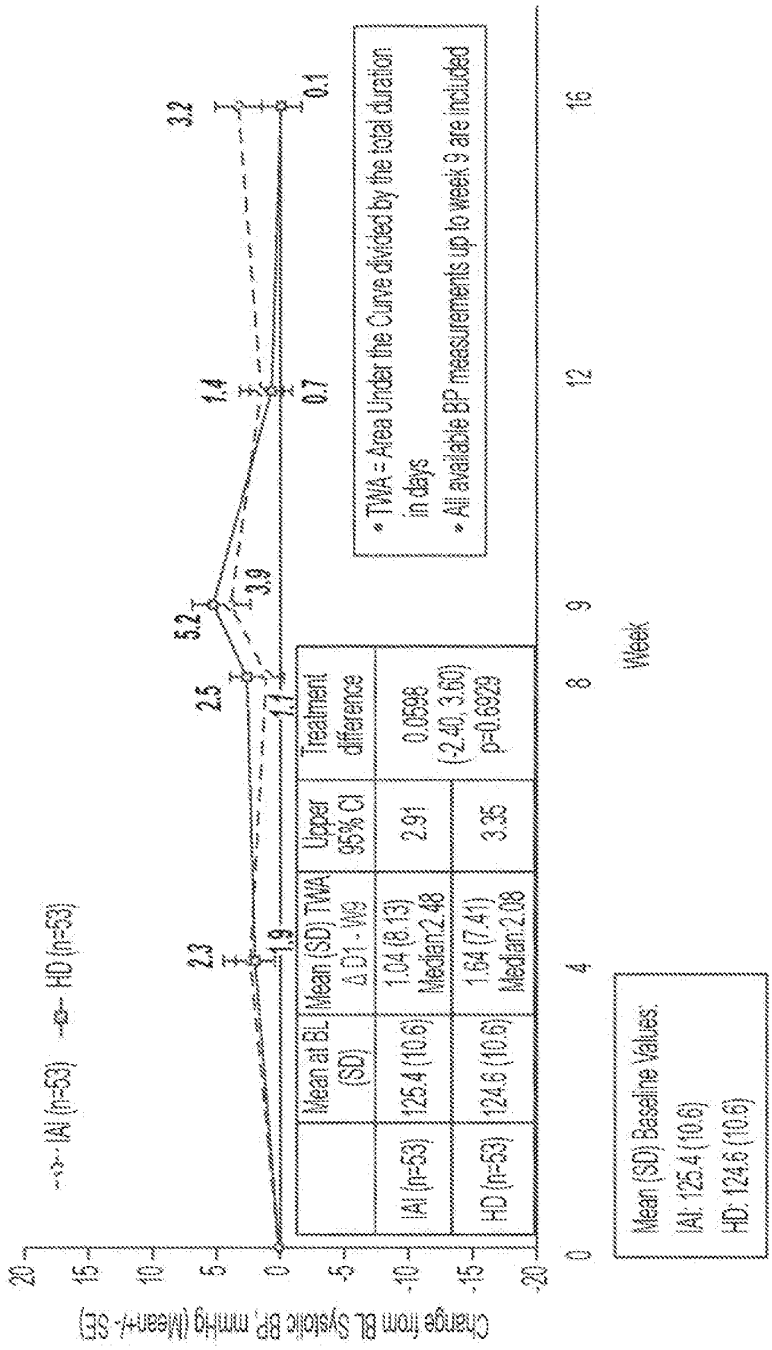
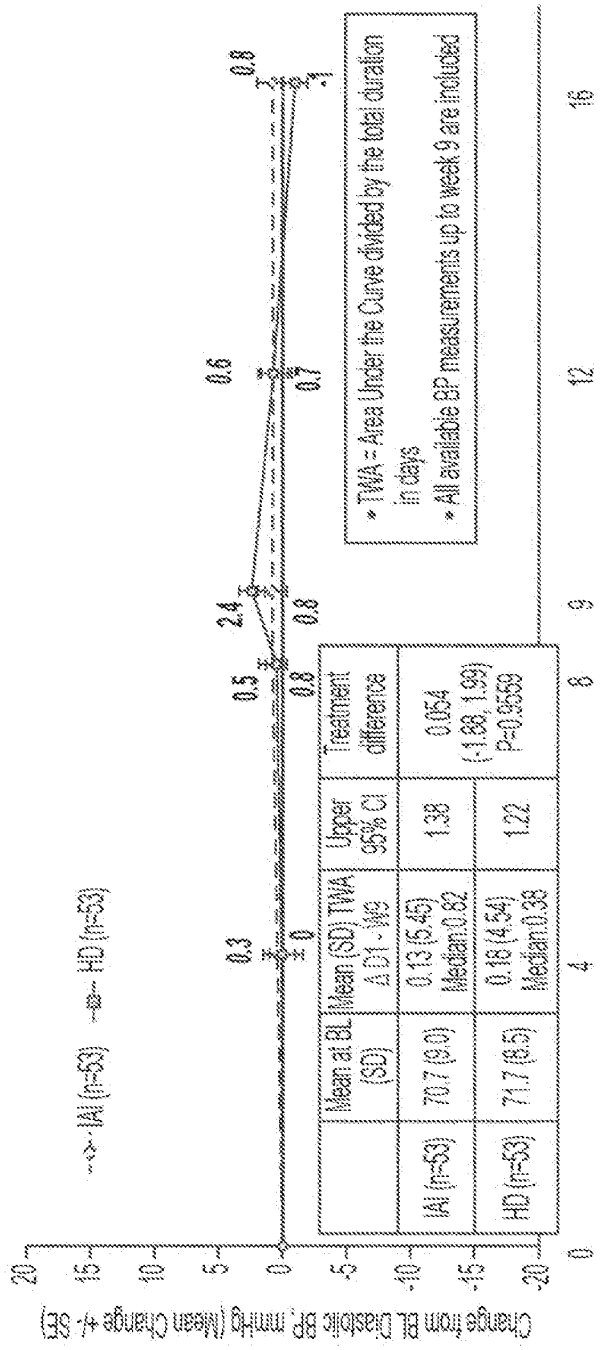

Mean Change from Baseline in Systolic Blood Pressure through Week 16, All Patients
IAI (n=53)
HD (n=53)
Change from BL Systolic BP, mmHg (Mean+/- SE)
Week
2.3
1.9
2.5
1.7
5.2
3.9
1.4
0.7
3.2
0.1
Mean (SD) Baseline Values:
IAI: 125.4 (10.6)
HD: 124.6 (10.6)
* TWA = Area Under the Curve divided by the total duration in days
* All available BP measurements up to week 9 are included
Mean at BL (SD)
Mean (SD) TWA Δ D1 - W9
Upper 95% CI
Treatment difference
IAI (n=53)
125.4 (10.6)
1.04 (8.13) Median:2.48
2.91
0.0598 (-2.40, 3.60) p=0.6929
HD (n=53)
124.6 (10.6)
1.64 (7.41) Median:2.08
3.35

FIG. 43A



	Mean at BL (SD)	Mean (SD) TWA Δ D1 - W9	Upper 95% CI	Treatment difference
IAI (n=53)	70.7 (9.0)	0.13 (5.45) Median 0.82	1.38	0.054 (-1.88, 1.99) P=0.9559
HD (n=53)	71.7 (8.5)	0.16 (4.54) Median 0.38	1.22	

FIG. 43B

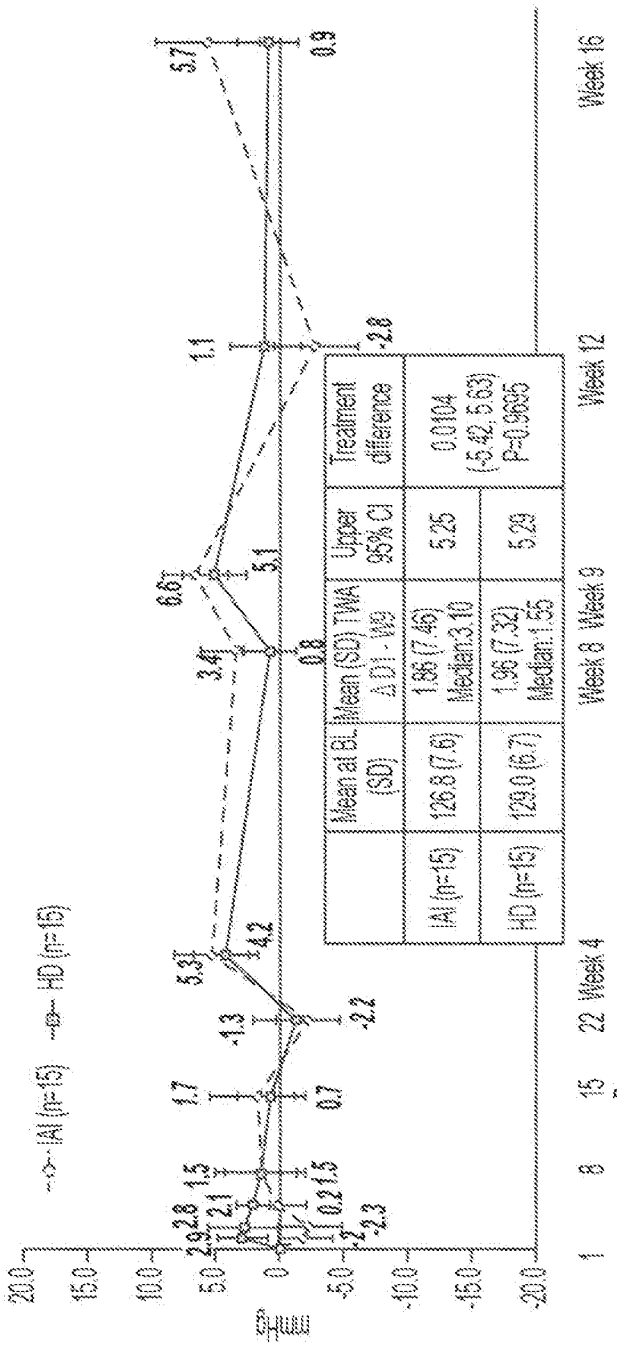

Mean Change from BL in Systolic BP through Wk 16: Dense PK Substudy Patients
IAI (n=15)
HD (n=15)
20.0
15.0
10.0
5.0
0
-5.0
-10.0
-15.0
-20.0
mmHg
1
8
15
22
Week 4
Week 8
Week 9
Week 12
Week 16
Day
2.9
2.8
1.5
2.1
1.1
0.2
1.5
-2.3
1.7
0.7
-1.3
-2.2
5.3
4.2
3.4
0.8
6.6
5.1
1.1
2.8
5.7
0.9
Mean at BL (SD)
Mean (SD) TWA Δ D1 - W9
Upper 95% CI
Treatment difference
IAI (n=15)
126.8 (7.6)
1.86 (7.46) Median:3.10
5.25
0.0104 (-5.42, 5.63) P=0.9635
HD (n=15)
129.0 (6.7)
1.96 (7.32) Median:1.55
5.29
Mean (SD) Baseline Values:
IAI: 126.8 (7.6)
HD: 129.0 (6.7)

FIG. 44A

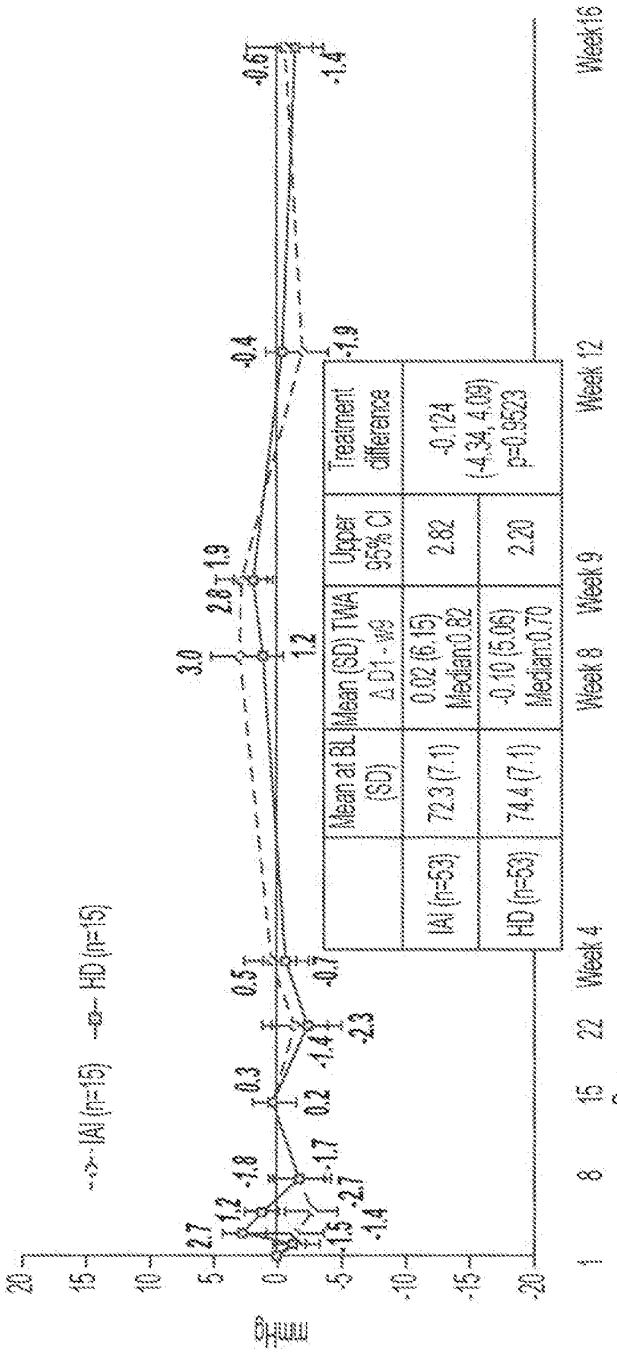

Mean Change from BL in Diastolic BP through Wk 16; Dense PK Substudy Patients
IAI (n=15)
HD (n=15)
mmHg
Day
1
8
15
22
Week 4
Week 8
Week 9
Week 12
Week 16
20
15
10
5
0
-5
-10
-15
-20
2.7
1.2
-1.8
1.5
-1.7
2.7
-1.4
0.3
0.2
0.5
-1.4
-0.7
-2.3
3.0
2.0
1.9
1.2
-0.4
-1.9
-0.6
-1.4
Mean (SD) Baseline Values:
IAI: 72.3 (7.1)
HD: 74.4 (7.1)
Mean at BL (SD)
Mean (SD) TWA Δ D1 - W8
Upper 95% CI
Treatment difference
IAI (n=53)
72.3 (7.1)
0.02 (6.15) Median 0.82
2.82
-0.124 (-4.34, 4.09) p=0.9523
HD (n=53)
74.4 (7.1)
-0.10 (5.06) Median 0.70
2.20

FIG. 44B

Patient Disposition

	Aflibercept 2 mg	Aflibercept 8 mg	Total
Randomized patients, n	53	53	106
Patients completing Week 16, n (%)	51 (96)	53 (100)	104 (98)
Patients completing Week 44, n (%)	49 (92)	51 (96)	100 (94)

FIG. 45

Baseline Demographics

	Aflibercept 2 mg (n=53)	Aflibercept 8 mg (n=53)	Total (N=106)
Sex, n(%)			
Male	17 (32.1)	23 (43.4)	40 (37.7)
Female	36 (67.9)	30 (56.6)	66 (62.3)
Race, n(%)			
White	51 (96.2)	52 (98.1)	103 (97.2)
Ethnicity, n (%)			
Hispanic or Latino	4 (7.5)	2 (3.8)	6 (5.7)
Not Hispanic or Latino	49 (92.5)	51 (96.2)	100 (94.3)
Age, mean (SD), years	77.7 (8.3)	77.0 (7.7)	77.4 (8.0)

SD, standard deviation.

FIG. 46

Baseline Characteristics - Study Eye

	Aflibercept 2 mg (n=53)	Aflibercept 8 mg (n=53)	Total (N=106)
BCVA, mean (SD), ETDRS letters	58.2 (10.5)	57.9 (13.6)	58.0[a] (11.9)
CRT, mean (SD), µm	488.1 (204.9)	516.2 (175.6)	502.1 (190.6)
IOP, mean (SD), mm Hg	14.9 (3.4)	14.8 (3.4)	14.9 (3.4)
Lesion size, mean (SD), mm^2	7.9 (6.2)	7.7 (6.8)	7.8 (6.5)
CNV size, mean (SD), mm^2	7.9 (6.2)	7.5 (6.9)	7.7 (6.5)
FA classification, n (%)			
Occult	22 (41.5)	26 (49.1)	48 (45.3)
Minimally classic	26 (49.1)	19 (35.8)	45 (42.5)
Predominantly classic	4 (7.5)	8 (15.1)	12 (11.3)
Missing	1 (1.9)	0	1 (0.9)

[a]58 ETDRS letters ≈ 20/60-20/70 Snellen visual acuity.
CRT, central retinal thickness; IOP, intraocular pressure.

FIG. 47

Treatment Exposure and PRN Treatment through Week 44

Exposure	Aflibercept 2 mg (n=53)	Aflibercept 8 mg (n=53)
Mean number of aflibercept injections through Week 44	5.8	5.8
Proportion of patients who did NOT receive additional or PRN treatment	24 (45%)	28 (53%)
Total number of PRN injections given	38	33

FIG. 48

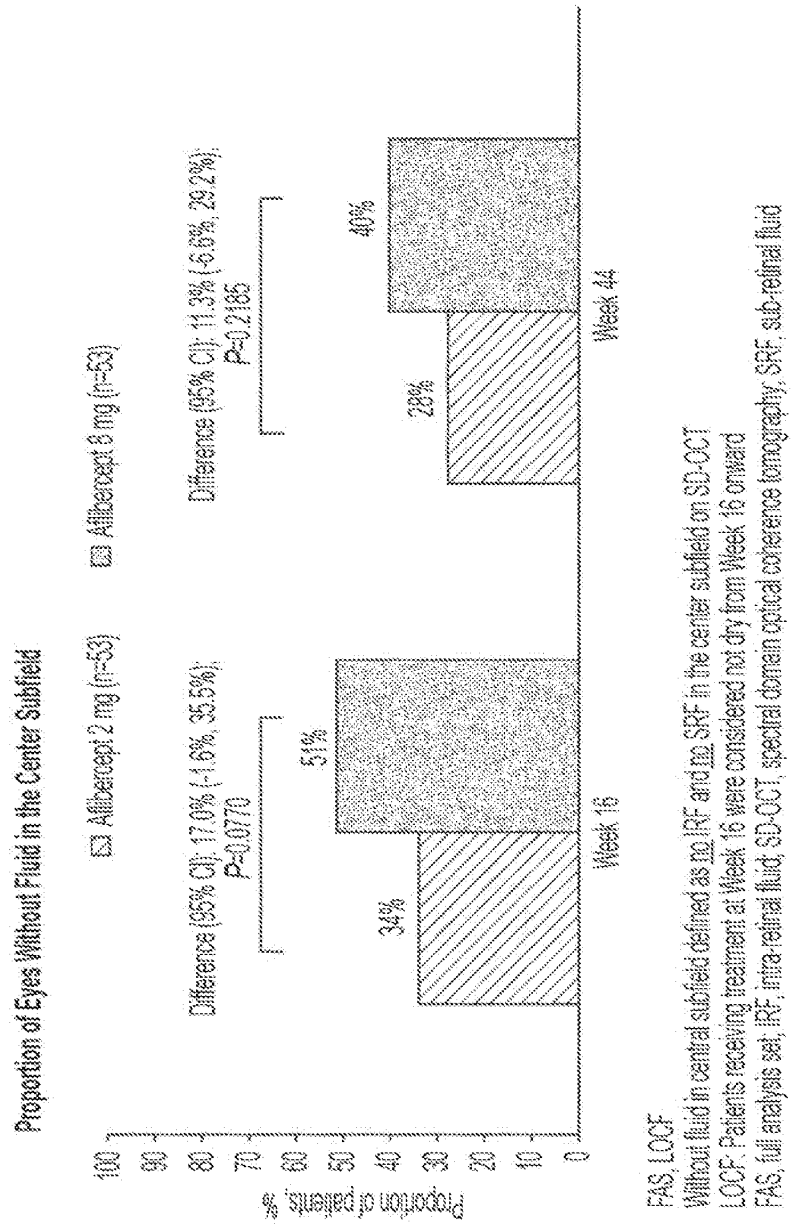

Proportion of Eyes Without Fluid in the Center Subfield
Aflibercept 2 mg (n=53)
Aflibercept 8 mg (n=53)
Difference (95% CI): 17.0% (-1.6%, 35.5%)
P=0.0770
Difference (95% CI): 11.3% (-6.6%, 29.2%)
P=0.2185
34%
51%
28%
40%
Week 16
Week 44
Proportion of patients, %
100
90
80
70
60
50
40
30
20
10
0
FAS, LOCF
Without fluid in central subfield defined as no IRF and no SRF in the center subfield on SD-OCT
LOCF: Patients receiving treatment at Week 16 were considered not dry from Week 16 onward
FAS, full analysis set, IRF, intra-retinal fluid, SD-OCT, spectral domain optical coherence tomography, SRF, sub-retinal fluid

FIG. 49

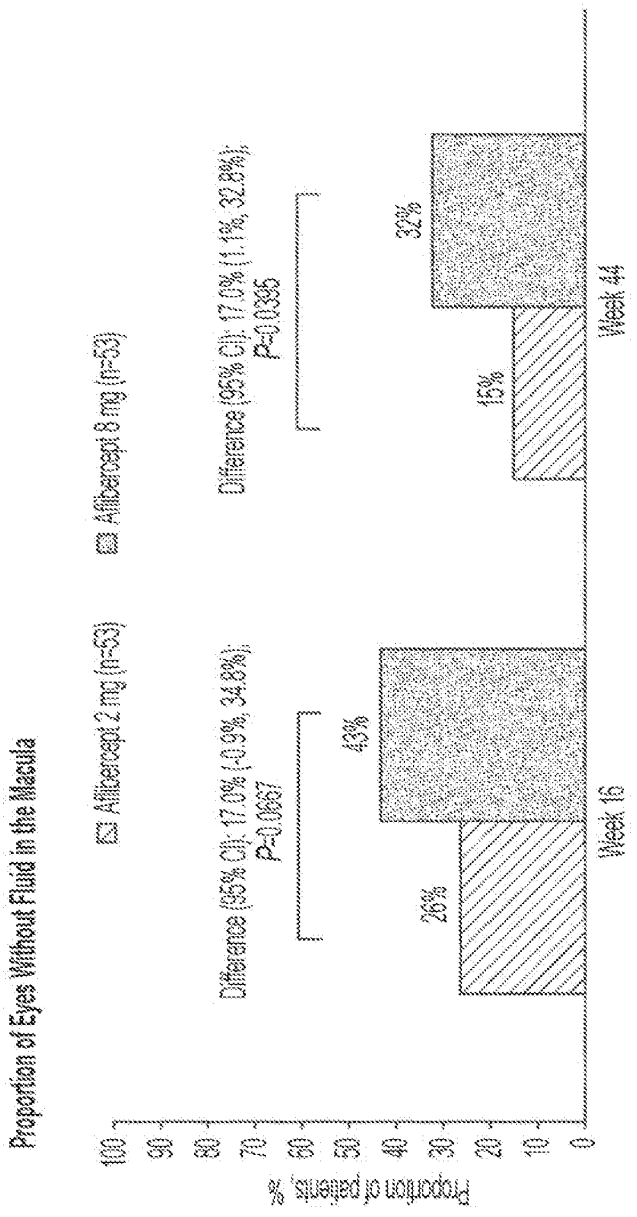

Proportion of Eyes Without Fluid in the Macula
Aflibercept 2 mg (n=53)
Aflibercept 8 mg (n=53)
Difference (95% CI): 17.0% (-0.9%, 34.8%);
P=0.0667
Difference (95% CI): 17.0% (1.1%, 32.8%);
P=0.0335
26%
43%
15%
32%
Week 16
Week 44
Proportion of patients, %
100
90
80
70
60
50
40
30
20
10
0
FAS, LOCF
Without fluid in macula defined as no IRF and no SRF in the macula on SD-OCT
LOCF: Patients receiving treatment at Week 16 were considered not dry from Week 16 onward

FIG. 50

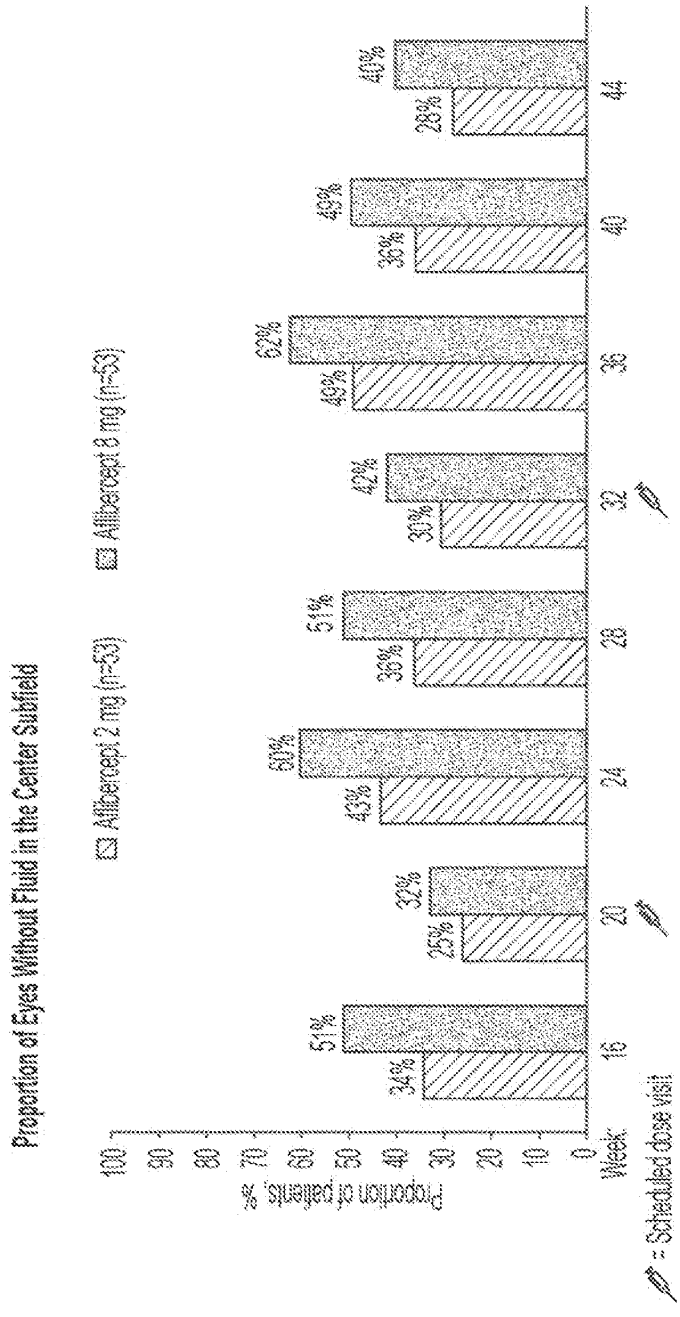

Proportion of Eyes Without Fluid in the Center Subfield
Aflibercept 2 mg (n=53)
Aflibercept 8 mg (n=53)
Percentage of patients, %
100
90
80
70
60
50
40
30
20
10
0
Week
16
20
24
28
32
36
40
44
34%
51%
25%
32%
43%
50%
36%
51%
30%
42%
49%
62%
36%
49%
28%
40%
= Scheduled dose visit
FAS, LOCF;
Without fluid in central subfield defined as no IRF and no SRF in the center subfield on SD-OCT
LOCF: Patients receiving treatment at Week 16 were considered not dry from Week 16 onward

FIG. 51

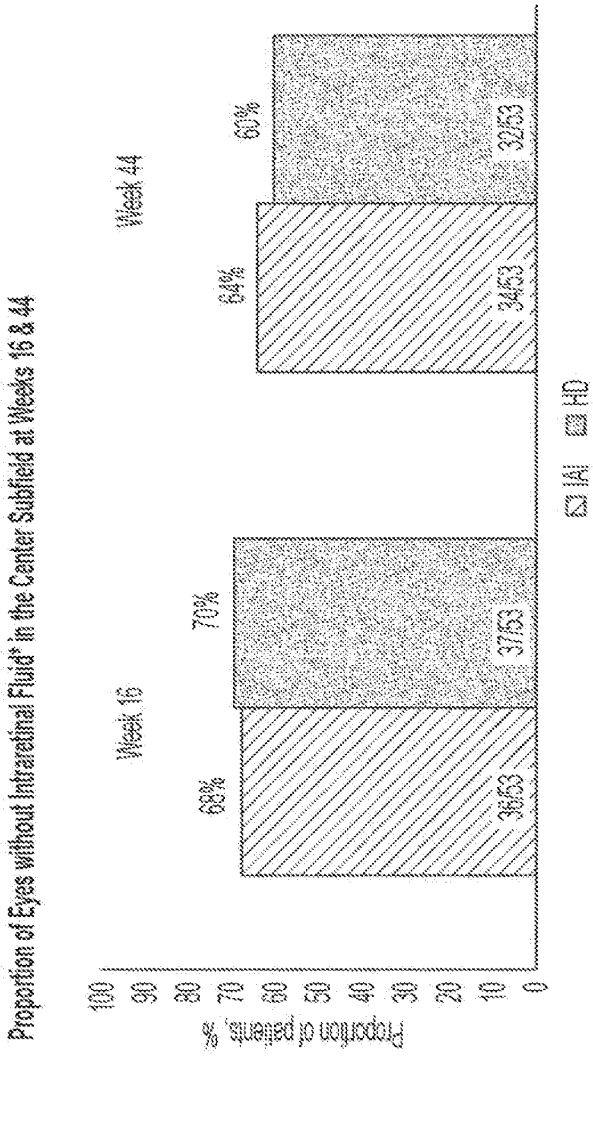

Proportion of Eyes without Intraretinal Fluid* in the Center Subfield at Weeks 16 & 44
Week 16
Week 44
68%
70%
64%
60%
36/53
37/53
34/53
32/53
Proportion of patients, %
100
90
80
70
60
50
40
30
20
10
0
IAI
HD
FAS: 2 mg N=53; 8 mg N=53; LOCF
*Without Intraretinal Fluid = Dry
or with Subretinal Fluid only

FIG. 52

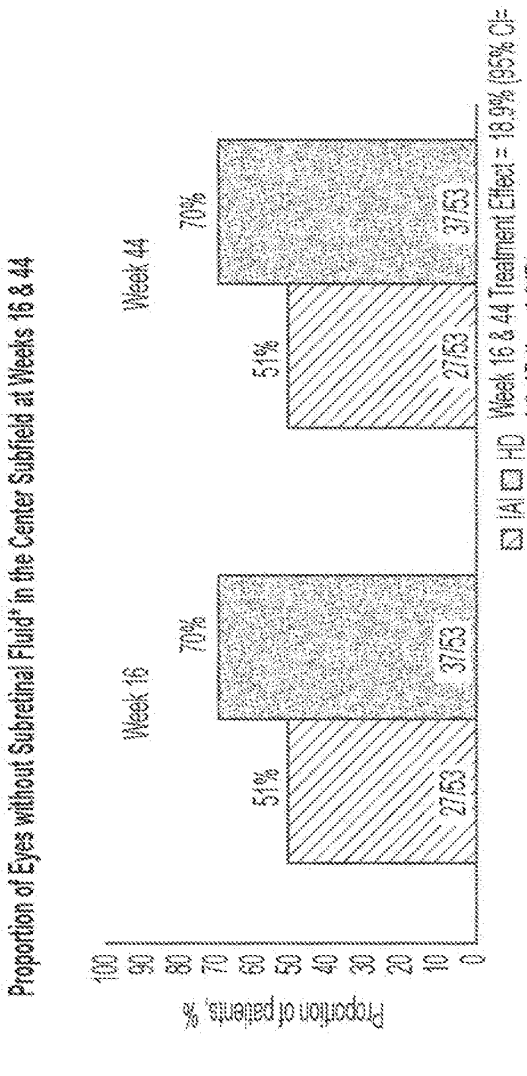

Proportion of Eyes without Subretinal Fluid* in the Center Subfield at Weeks 16 & 44
Proportion of patients, %
100
90
80
70
60
50
40
30
20
10
0
Week 16
Week 44
51%
70%
51%
70%
27/53
37/53
27/53
37/53
IAI
HD
IAI HD Week 16 & 44 Treatment Effect = 18.9% (95% CI= 0.6, 37.1); p=0.0471
FAS: 2 mg N=53; 8 mg N=53; LOCF
Week 16 & 44 Treatment Effect = 18.9% (95% CI= 0.6, 37.1); p=0.0471
*Without Subretinal Fluid = Dry or with Intraretinal Fluid only

FIG. 53

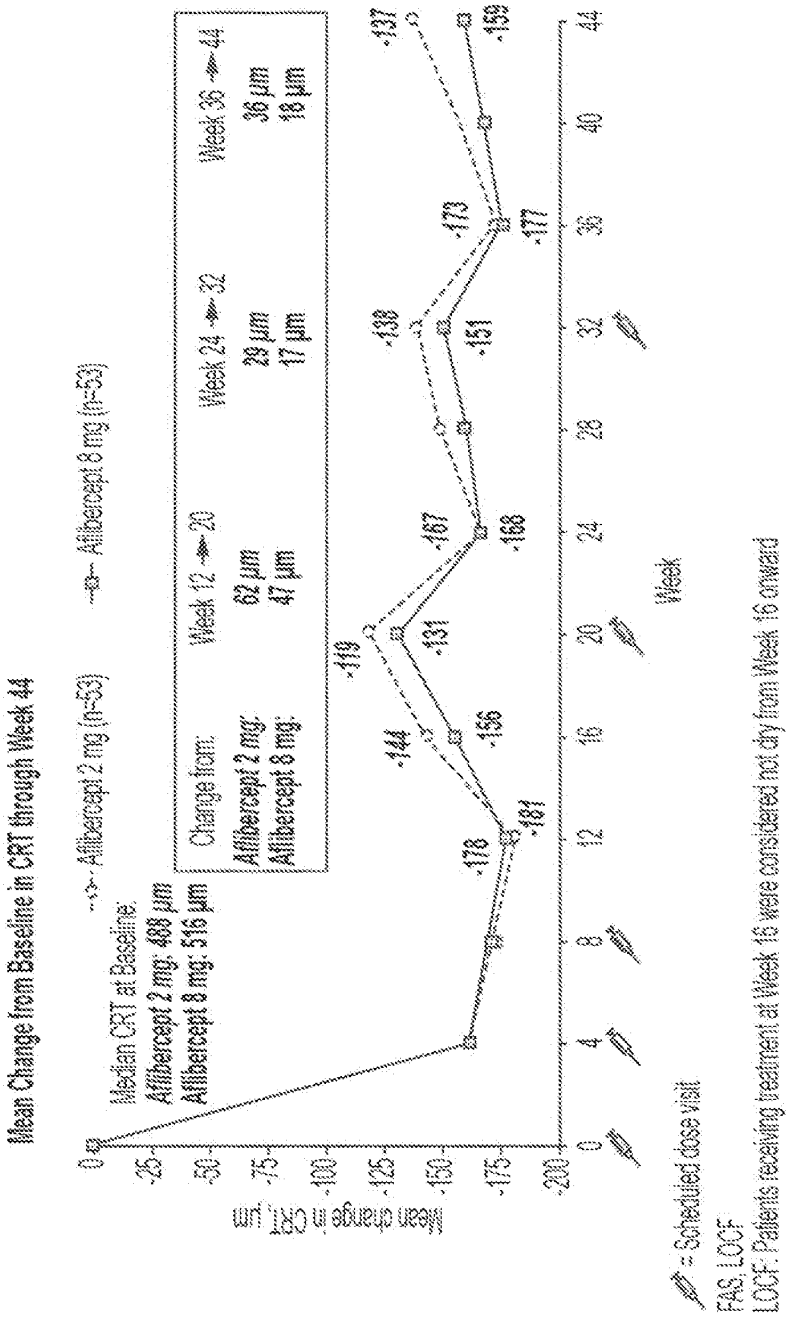

Mean Change from Baseline in CRT through Week 44
Aflibercept 2 mg (n=53)
Aflibercept 8 mg (n=53)
Median CRT at Baseline:
Aflibercept 2 mg: 408 µm
Aflibercept 8 mg: 516 µm
Change from:
Week 12 → 20
Week 24 → 32
Week 36 → 44
Aflibercept 2 mg:
62 µm
29 µm
36 µm
Aflibercept 8 mg:
47 µm
17 µm
16 µm
Mean change in CRT, µm
Week
= Scheduled dose visit
FAS, LOCF
LOCF: Patients receiving treatment at Week 16 were considered not dry from Week 16 onward

FIG. 54

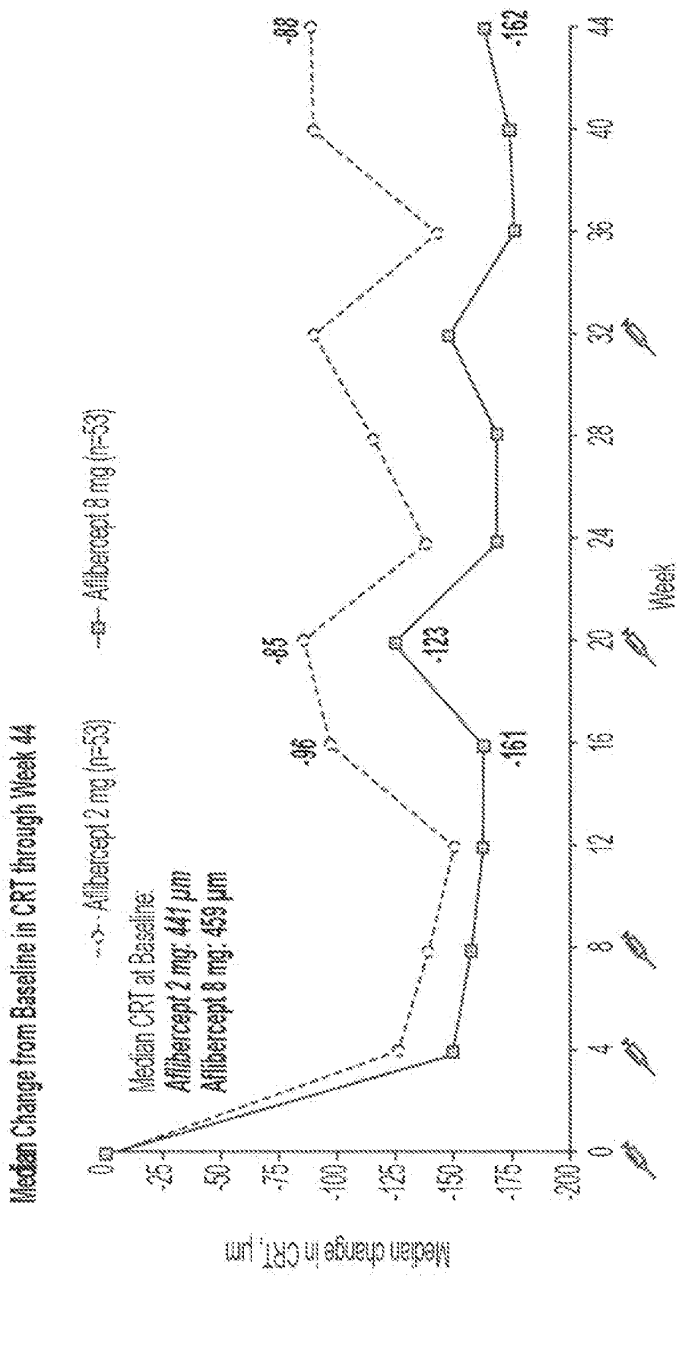

Median Change from Baseline in CRT through Week 44
Aflibercept 2 mg (n=53)
Aflibercept 8 mg (n=53)
Median CRT at Baseline
Aflibercept 2 mg: 441 µm
Aflibercept 8 mg: 459 µm
Median change in CRT, µm
0
-25
-50
-75
-100
-125
-150
-175
-200
-96
-85
-123
-161
-88
-162
0
4
8
12
16
20
24
28
32
36
40
44
Week
= Scheduled dose visit
FAS, LOCF
LOCF: Patients receiving treatment at Week 16 were considered not dry from Week 16 onward

FIG. 55

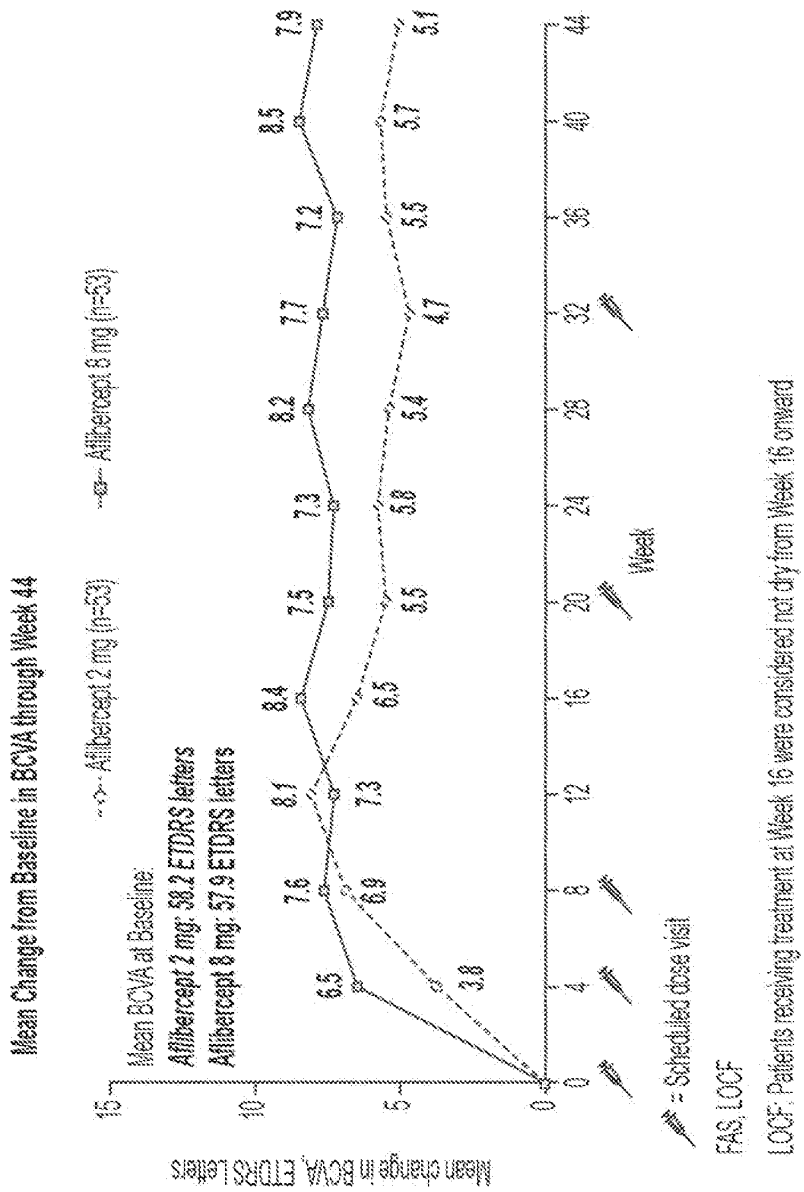

Mean Change from Baseline in BCVA through Week 44
Aflibercept 2 mg (n=53)
Aflibercept 8 mg (n=53)
Mean BCVA at Baseline:
Aflibercept 2 mg: 58.2 ETDRS letters
Aflibercept 8 mg: 57.9 ETDRS letters
Mean change in BCVA, ETDRS Letters
15
10
5
0
6.5
7.6
8.1
8.4
7.5
7.3
8.2
7.7
7.2
8.5
7.9
3.8
6.9
7.3
6.5
5.5
5.0
5.4
4.7
5.6
5.7
5.1
0
4
8
12
16
20
24
28
32
36
40
44
Week
= Scheduled dose visit
FAS, LOCF
LOCF: Patients receiving treatment at Week 16 were considered not dry from Week 16 onward

FIG. 56

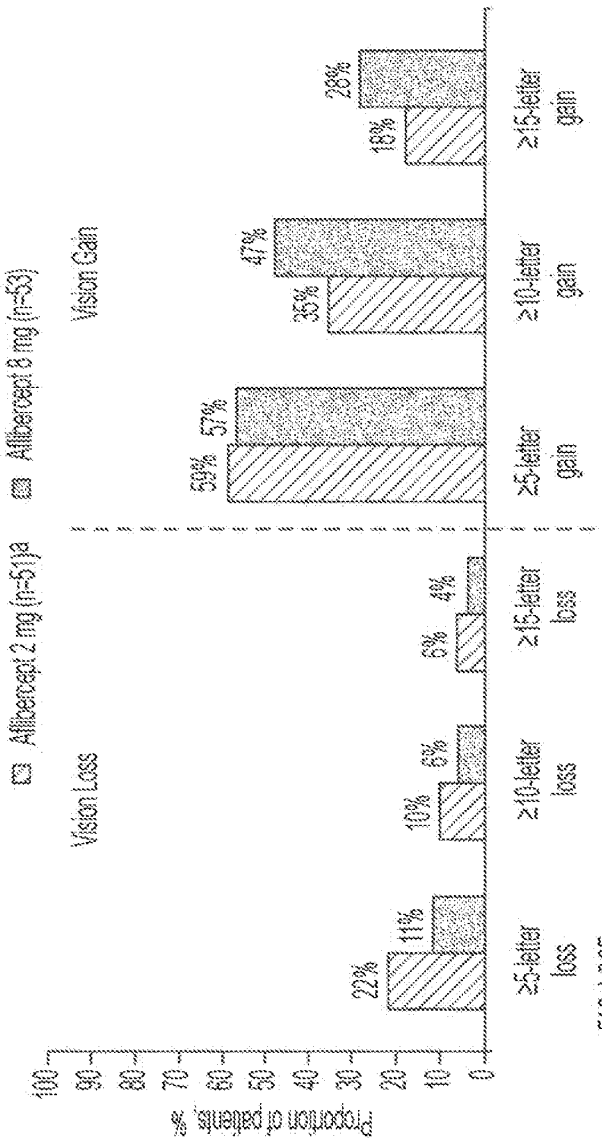

Proportion of Patients with Vision Loss or Gain at Week 44
Aflibercept 2 mg (n=51)a
Aflibercept 8 mg (n=53)
Vision Loss
Vision Gain
Proportion of patients, %
100
90
80
70
60
50
40
30
20
10
0
22%
11%
10%
6%
6%
4%
59%
57%
47%
35%
28%
18%
≥5-letter loss
≥10-letter loss
≥15-letter loss
≥5-letter gain
≥10-letter gain
≥15-letter gain
FAS, LOCF
aTwo patients in the aflibercept 2 mg group did not have post-baseline values

FIG. 57

Ocular TEAEs in Study Eye Occurring in ≥2% of Pts

	IAI (N=53)	HD (N=53)
Number of Patients with at Least One Ocular TEAE in Study Eye, n(%)	20 (37.7%)	20 (37.7%)
CONJUNCTIVAL HAEMORRHAGE	2 (3.8%)	3 (5.7%)
DRY EYE	2 (3.8%)	2 (3.8%)
MACULAR DEGENERATION	1 (1.9%)	1 (1.9%)
NEOVASCULAR AGE-RELATED MACULAR DEGENERATION	4 (7.5%)	2 (3.8%)
PUNCTATE KERATITIS	2 (3.8%)	1 (1.9%)
RETINAL HAEMORRHAGE	2 (3.8%)	1 (1.9%)
RETINAL TEAR	0	2 (3.8%)
SUBRETINAL FLUID	1 (1.9%)	1 (1.9%)
VISUAL ACUITY REDUCED	2 (3.8%)	1 (1.9%)
VISUAL IMPAIRMENT	2 (3.8%)	1 (1.9%)
VITREOUS DETACHMENT	2 (3.8%)	4 (7.5%)

FIG. 58

Ocular Serious TEAEs in Study Eye

	IAI (N=53)	HD (N=53)
Number of Patients with at Least One Ocular SAE in the Study Eye*, n (%)	1 (1.9%)	2 (3.8)
VISUAL ACUITY REDUCED	1 (1.9%)	0
RETINAL TEAR	0	1 (1.9%)
VISUAL IMPAIRMENT	0	1 (1.9%)

*No Ocular SAEs occurred in the Fellow Eye

FIG. 59

Intraocular Inflammation TEAEs in Study Eye

	IAI (N=53)	HD (N=53)
Number of Patients with at Least One Such AE, n (%)	0	1 (1.9%)
IRITIS	0	1 (1.9%)

Iritis was characterized as mild cells in the anterior chamber which resolved within 1 month with topical therapy

FIG. 60

Summary of Intraocular Pressure Events

	IAI (n=53)	HD (n=53)
# pts with ≥ 10 mmHg increase in pre-injection IOP from BL	0/51	2/53 (3.8%)
# pts with pre-injection IOP ≥25 mmHg at any visit	0/53	2/53 (3.8%)
# pts with any visit IOP ≥35 mmHg at Any Time	0/53	0/53

Safety Analysis Set
Percentages are calculated based on N

FIG. 61

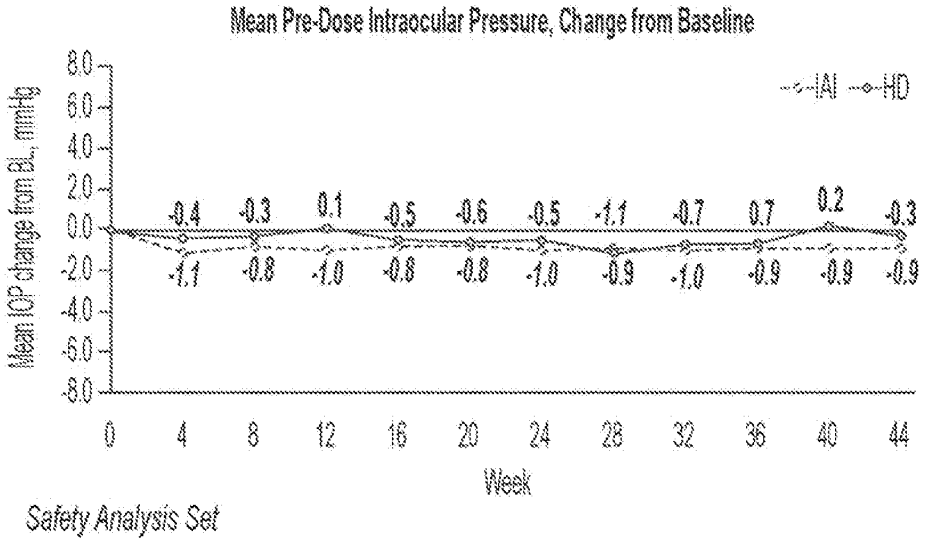


FIG. 62

Non-Ocular TEAEs Occurring in ≥2% of Patients

	IAI (N=53)	HD (N=53)
Number of Patients with at Least One Non-Ocular TEAE, n(%)	24 (45.3%)	28 (52.8%)
DIARRHEA	1 (1.9%)	2 (3.8%)
GASTRITIS	1 (1.9%)	1 (1.9%)
GASTROOESOPHAGEAL REFLUX DISEASE	2 (3.8%)	0
NAUSEA	0	2 (3.8%)
CHEST PAIN	0	2 (3.8%)
COVID-19	2 (3.8%)	2 (3.8%)
SINUSITIS	2 (3.8%)	1 (1.9%)
URINARY TRACT INFECTION	1 (1.9%)	2 (3.8%)
FALL	2 (3.8%)	3 (5.7%)
ARTHRALGIA	2 (3.8%)	0
CARPAL TUNNEL SYNDROME	1 (1.9%)	1 (1.9%)
DIZZINESS	0	3 (5.7%)
HYPERTENSION	1 (1.9%)	1 (1.9%)

FIG. 63

Non-Ocular Serious TEAEs

	IAI (N=53)	HD (N=53)
Number of Patients with at Least One Non-Ocular SAE, n(%)	4 (7.5%)	5 (9.4%)
ACUTE LEFT VENTRICULAR FAILURE	0	1 (1.9%)
COLITIS	1 (1.9%)	0
SMALL INTESTINAL OBSTRUCTION	1 (1.9%)	0
CHEST PAIN	0	1 (1.9%)
NON-CARDIAC CHEST PAIN	0	1 (1.9%)
COVID-19	1 (1.9%)	0
FEMUR FRACTURE	1 (1.9%)	0
LUMBAR SPINAL STENOSIS	0	1 (1.9%)
HEPATIC ENCEPHALOPATHY	0	1 (1.9%)
TRANSIENT ISCHEMIC ATTACK	0	1 (1.9%)
ACUTE RESPIRATORY FAILURE	0	1 (1.9%)

FIG. 64

Hypertension TEAEs

	IAI (N=53)	HD (N=53)
Number of Patients with at Least One Such AE, n (%)	1 (1.9%)	1 (1.9%)
Worsening Hypertension	1 (1.9%)	1 (1.9%)

FIG. 65

Adjudicated APTC Events and Deaths

	IAI (N=53)	HD (N=53)
Adjudicated APTC Events, n	0	0
Deaths, n	0	1*

*Death due to glioblastoma

FIG. 66

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/029462

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/17 A61P27/02
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 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)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, FSTA, 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	Regeneron Pharmaceuticals: "Safety, Tolerability, and Efficacy of Aflibercept in Patients With Neovascular Age-Related Macular Degeneration. NCT04126317", , 15 October 2019 (2019-10-15), pages 1-8, XP055952673, Retrieved from the Internet: URL:https://clinicaltrials.gov/ct2/show/record/NCT04126317 [retrieved on 2022-08-18] the whole document ------	1-28
X	WO 2012/097019 A1 (REGENERON PHARMA [US]; YANCOPOULOS GEORGE D [US]) 19 July 2012 (2012-07-19) paragraph [0030] ------ -/--	1-28

[x] Further documents are listed in the continuation of Box C. [x] 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
18 August 2022	26/08/2022

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
Dolce, Luca

Form PCT/ISA/210 (second sheet) (April 2005)

PGR2026-00039
Biocon Ex.1002
Page 2129 of 4945

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/029462

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2019/217927 A1 (REGENERON PHARMA [US]) 14 November 2019 (2019-11-14) paragraph [0027]	1-28
X	US 2018/298092 A1 (GEKKIEVA MARGARITA [CH] ET AL) 18 October 2018 (2018-10-18) paragraph [0087]	1,5,6
A	WO 2021/048779 A2 (LUPIN LTD [IN]) 18 March 2021 (2021-03-18) the whole document	1-28
A	WO 2017/120601 A1 (CLEARSIDE BIOMEDICAL INC [US]) 13 July 2017 (2017-07-13) the whole document	1-28
A	WO 2019/108770 A1 (REGENERON PHARMA [US]) 6 June 2019 (2019-06-06) the whole document	1-28

1

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

PGR2026-00039
Biocon Ex.1002
Page 2130 of 4945

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/029462

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. [X] forming part of the international application as filed:

      [X] 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/US2022/029462

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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Page 2132 of 4945

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2022/029462

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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Page 2133 of 4945

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2022/029462

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PTO/SB/06 (09-11)
Approved for use through 1/31/2014, OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 17/384,070	Filing Date 07/23/2021	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $480 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

AMENDMENT	08/01/2023	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 38	Minus	** 20	= 18		x $100 =	1800
	Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0		x $480 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	1800

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/TINA J BARDEN/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
17/384,070	07/23/2021	Kenneth S. Graham	125984-266215

CONFIRMATION NO. 1061

147803
Schwabe W&W, PC / Regeneron Pharmaceuticals, Inc.
PacWest Center
1211 SW Fifth Avenue, Suite 1900
Portland, OR 97204

POA ACCEPTANCE LETTER

OC000000137686245

Date Mailed: 04/07/2023

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 03/22/2023.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/sleutchit/

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82B (07-13)
Approved for use through 03/31/2021. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date
17/384,070	July 23, 2021

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

☑ I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 147803

OR

☐ I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

☑ The address associated with the above-mentioned Customer Number

OR

☐ The address associated with Customer Number:

OR

☐ Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

Regeneron Pharmaceuticals, Inc.

☐ Inventor or Joint Inventor (title not required below)

☐ Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

☑ Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

☐ Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature	/Frank R. Cottingham/	Date (Optional)	
Name	Frank R. Cottingham		
Title	Vice President Intellectual Property, Regeneron Pharmaceuticals, Inc.		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

☑ Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Acknowledgement Receipt

EFS ID:	47728100
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
Filer Authorized By:	
Attorney Docket Number:	125984-266215
Receipt Date:	22-MAR-2023
Filing Date:	23-JUL-2021
Time Stamp:	18:06:14
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	10430US02_POA.pdf	1123774 bf312ce998e73c565d305d02b44db156688c5c30	no	1

Warnings:

Information:	
Total Files Size (in bytes):	1123774

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.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/384,070	07/23/2021	Kenneth S. Graham	125984-266215	1061

147803 7590 03/22/2023

Schwabe W&W, PC / Regeneron Pharmaceuticals, Inc.
PacWest Center
1211 SW Fifth Avenue, Suite 1900
Portland, OR 97204

EXAMINER
LOCKARD, JON MCCLELLAND

ART UNIT	PAPER NUMBER
1647	

NOTIFICATION DATE	DELIVERY MODE
03/22/2023	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

LTermes@schwabe.com
ipdocketing@schwabe.com

Office Action Summary	Application No. 17/384,070	Applicant(s) Graham et al.	
	Examiner JON M LOCKARD	Art Unit 1647	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 23 July 2021.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 30-32,35 and 39-52 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 30-32,35 and 39-52 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☑ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 23 July 2021 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

Notice of Pre-AIA or AIA Status

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

DETAILED ACTION

Status of Application, Amendments, and/or Claims

2. The Preliminary Amendment filed on 23 July 2021 has been entered in full. Claims 1-29, 33-34 and 36-38 have been cancelled, claims 30-32 and 35 have been amended, and claims 39-52 have been added. Therefore, claims 30-32, 35 and 39-52 are pending and the subject of this Office Action.

Information Disclosure Statement

3. The information disclosure statements (IDS) filed 03 December 2021, 06 December 2021, 17 December 2021, 04 January 2022, 11 April 2022, 16 May 2022, and 16 November 2022 have been considered by the examiner.

Specification

4. The disclosure is objected to because of the following informalities:

5. An updated status of the parent nonprovisional applications should be included in the first sentence of the specification.

6. The title of the invention is not descriptive. A new title is required that is clearly indicative of the invention to which the claims are directed.

Claim Objections

7. Claim 35 is objected to because of the following informalities: In line 4 of the claim, there should be a comma separating "(CRVO)" and "branch retinal vein". Appropriate correction is required.

Claim Rejections - 35 USC § 112

8. The following is a quotation of 35 U.S.C. 112(b):

> (b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

9. Claims 30-32, 35 and 39-52 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor (or for applications subject to pre-AIA 35 U.S.C. 112, the applicant), regards as the invention.

10. Claim 30 is rejected as being indefinite for reciting the limitation "at least about" in line 3 of the claim. Since the specification does not provide any indication as to what range is covered by the term "at least about", the metes and bounds of the claim cannot be determined.

11. Claims 31-32, 35 and 39-52 are rejected for depending from an indefinite claim.

Claim Rejections - 35 USC § 112, 1st Paragraph (Written Description)

12. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such

> full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

13. Claims 30-32, 35 and 39-52 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

14. The claims are drawn quite broadly to a method for treating an angiogenic eye disorder in a human patient in need thereof comprising administering an intravitreal injection of at least about 8 mg of a VEGF receptor fusion protein comprising two polypeptides that comprise an immunoglobulin-like (Ig) domain 2 of VEGFR1, an Ig domain 3 of VEGFR2 receptor, and a multimerizing domain.

15. The guidelines for the Examination of Patent Applications Under the 35 U.S.C. 112, ¶1 "Written Description" Requirements make clear that the written description requirement for a claimed genus may be satisfied by disclosure of relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying

characteristics, sufficient to show the applicant was in possession of the genus (Federal Register, Vol. 66, No. 4, pages 1099-1111, Friday January 5, 2001, see especially page 1106 3rd column).

16. In the instant case, the claim recites a high concentration of a VEGF receptor fusion protein without any recitation of the elements of the composition that permit such a high concentration of the protein. The Specification discloses that L-arginine monohydrochloride increases the stability of high concentrations of the VEGF trap Aflibercept, a fusion protein that comprises Ig domain 2 of VEFGFR1, Ig domain 3 of VEGFR2, and an Fc (See Example 4 at pp. 55-56). Therefore, while the specification provides adequate written description for stable, high concentration formulations of a VEGF receptor fusion protein along with 5% sucrose, polysorbate, a histidine based buffer, and L-arginine, it does not provide adequate written description for the breadth of the formulation encompassed by the claims.

17. *Vas-Cath Inc. v. Mahurkar*, 19USPQ2d 1111, clearly states that "applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession *of the invention*. The invention is, for purposes of the 'written description' inquiry, *whatever is now claimed*." (See page 1117.) The specification does not "clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed." (See *Vas-Cath* at page 1116).

18. One cannot describe what one has not conceived. See *Fiddes v. Baird*, 30 USPQ2d 1481 at 1483. In *Fiddes*, claims directed to mammalian FGF's were found to be unpatentable due to lack of written description for that broad class. The specification provided only the bovine sequence.

19. Therefore, only high concentration formulations of a VEGF receptor fusion protein along with 5% sucrose, polysorbate, a histidine-based buffer, and L-arginine, but not the full breadth of the claims, meet the written description provision of 35 U.S.C. §112, first paragraph. Applicant is reminded that *Vas-Cath* makes clear that the written description provision of 35 U.S.C. §112 is severable from its enablement provision (see page 1115).

Double Patenting

20. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

21. A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope

of a joint research agreement. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

22. The USPTO internet Web site contains terminal disclaimer forms which may be used. Please visit http://www.uspto.gov/forms/. The filing date of the application will determine what form should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

23. Claims 30-32, 35 and 39-52 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-36 of U.S. Patent No. 11,103,552. Although the conflicting claims are not identical, as they recite different dosing schedules, they are not patentably distinct from each other because claims 1-26 of the '338 patent are drawn to a method for treating an angiogenic eye disorder, including age-related macular degeneration (wet), macular edema, central retinal vein occlusion (CRVO), branch retinal vein occlusion (BRVO), diabetic retinal edema, choroidal neovascularization, proliferative vitreoretinopathy, vascular retinopathy, diabetic retinopathy, and non-proliferative or proliferative diabetic retinopathy, comprising the intravitreal injection of a VEGF receptor fusion protein comprising two polypeptides that comprise an immunoglobulin-like (Ig) domain 2 of VEGFR1, Ig domain 3 of a VEGFR2 and a multimerizing component. The claims of the '552 patent also recite wherein the dosage of the VEGF receptor fusion protein is about 8 mg, and also recite wherein the VEGF receptor fusion protein is aflibercept.

Therefore, the claims are overlapping in scope.

Summary

24. No claim is allowed.

Advisory Information

Any inquiry concerning this communication or earlier communications from the examiner should be directed to **Jon M. Lockard** whose telephone number is **(571) 272-2717**. The examiner can normally be reached on Monday through Friday, 8:00 AM to 4:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, **Joanne Hama**, can be reached on **(571) 272-2911**. The fax number for the organization where this application or proceeding is assigned is **571-273-8300**.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see **http://pair-direct.uspto.gov**. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at **866-217-9197** (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/JON M LOCKARD/
Examiner, Art Unit 1647
March 16, 2023

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/384,070	Graham et al.
	Examiner	Art Unit
	JON M LOCKARD	1647

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner
NONE		03/16/2023	JML

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
PE2E Search (USPAT, US-PGPUB, EPO, DERWENT): See attached search history.	03/16/2023	JML
STN (MEDLINE, SCISEARCH, EMBASE, BIOSIS): See attached search history.	03/16/2023	JML
PALM: Inventor search.	03/16/2023	JML

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

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Receipt date: 12/03/2021

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~~ 1647
	Confirmation Number	1061
	Examiner Name	~~Not Yet Assigned~~ Jon Lockard
	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
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	101	6,171,586		01-09-2001	Lam et al.	
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	036	7,087,411		08-08-2006	Daly et al.	
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U.S. PATENT APPLICATION PUBLICATIONS						
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	Attorney Docket Number	125984-266215 / 10430US02

NON-PATENT LITERATURE DOCUMENTS			
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EXAMINER SIGNATURE			
Examiner Signature	/JON M LOCKARD/	Date Considered	03/15/2023

*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.

[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.

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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

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.L/

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Page 2167 of 4945

Receipt date: 05/16/2022

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	First Named Inventor	Kenneth S. Graham
	Art Unit	~~Not Yet Assigned~~ 1647
	Confirmation Number	1061
	Examiner Name	~~Not Yet Assigned~~ Jon Lockard
	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
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Examiner Signature	/JON M LOCKARD/	Date Considered	03/15/2023

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☒ None

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Signature	/Lance A. Termes/	Date (YYYY-MM-DD)	2022-05-16
Name/Print	Lance A. Termes	Registration Number	43184

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PE2E SEARCH - Search History (Prior Art)

/J.L./

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
L1	7076	vegf ADJ trap	(US-PGPUB; USPAT; EPO; DERWENT)	OR	ON	ON	2023/03/16 01:06 PM
L2	8296	aflibercept	(US-PGPUB; USPAT; EPO; DERWENT)	OR	ON	ON	2023/03/16 01:06 PM
L3	737	eylea	(US-PGPUB; USPAT; EPO; DERWENT)	OR	ON	ON	2023/03/16 01:06 PM
L4	269	vegf ADJ trap-eye	(US-PGPUB; USPAT; EPO; DERWENT)	OR	ON	ON	2023/03/16 01:06 PM
L5	1290	(L1 L2 L3 L4) SAME formulation	(US-PGPUB; USPAT; EPO; DERWENT)	OR	ON	ON	2023/03/16 01:06 PM
L6	32	L5 SAME "high concentration"	(US-PGPUB; USPAT; EPO; DERWENT)	OR	ON	ON	2023/03/16 01:07 PM
L7	49	L5 SAME (8mg OR "8 mg")	(US-PGPUB; USPAT; EPO; DERWENT)	OR	ON	ON	2023/03/16 01:07 PM
L8	1	L5 AND "high concentration".clm.	(US-PGPUB; USPAT; EPO; DERWENT)	OR	ON	ON	2023/03/16 01:09 PM
L9	6	L5 AND (8mg OR "8 mg").clm.	(US-PGPUB; USPAT; EPO; DERWENT)	OR	ON	ON	2023/03/16 01:09 PM
L10	80	(graham-k$-s$ washawa-s$).in.	(US-PGPUB; USPAT; EPO; DERWENT)	OR	ON	ON	2023/03/16 01:10 PM
L11	16	L5 AND L10	(US-PGPUB; USPAT; EPO; DERWENT)	OR	ON	ON	2023/03/16 01:11 PM
L12	3	L6 SAME l-arginine	(US-PGPUB; USPAT; EPO; DERWENT)	OR	ON	ON	2023/03/16 02:08 PM
L13	0	L7 SAME l-arginine	(US-PGPUB; USPAT; EPO; DERWENT)	OR	ON	ON	2023/03/16 02:08 PM
L14	5	L5 SAME l-arginine	(US-PGPUB; USPAT; EPO; DERWENT)	OR	ON	ON	2023/03/16 02:09 PM

PE2E SEARCH - Search History (Interference)

There are no Interference searches to show.

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/J.L/	406	CHANG et al., "Ziv-Aflibercept as a Possible Alternative to Aflibercept," Medical Retina, Retina Today, Pages 67-68, (2014).	

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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

EXAMINER SIGNATURE			
Examiner Signature	/JON M LOCKARD/	Date Considered	03/15/2023

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Biocon Ex.1002
Page 2173 of 4945

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (not for submission under 37 CFR 1.99)		
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CERTIFICATION STATEMENT

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☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ None

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Signature	/Lance A. Termes/	Date (YYYY-MM-DD)	2021-12-17
Name/Print	Lance A. Termes	Registration Number	43184

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Biocon Ex.1002
Page 2174 of 4945

Bibliographic Data

Application No: 17/384,070

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☐ No ☐ Met After Allowance

Verified and Acknowledged: /JON M LOCKARD/ (Examiner's Signature) | (Initials)

Title: High Concentration VEGF Receptor Fusion Protein Containing Formulations

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
07/23/2021	424	1647	125984-266215
RULE			

APPLICANTS

Regeneron Pharmaceuticals, Inc., Tarrytown, NY,

INVENTORS

Kenneth S. Graham, Pleasant Valley, NY, UNITED STATES

Saurabh WADHWA, Nanuet, NY, UNITED STATES

CONTINUING DATA

This application is a CON of 16409631 05/10/2019 PAT 11103552

16409631 has PRO of 62813882 03/05/2019

16409631 has PRO of 62769876 11/20/2018

16409631 has PRO of 62752127 10/29/2018

16409631 has PRO of 62669506 05/10/2018

FOREIGN APPLICATIONS

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08/09/2021

STATE OR COUNTRY

UNITED STATES

ADDRESS

Schwabe W&W, PC / Regeneron Pharmaceuticals, Inc.

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1211 SW Fifth Avenue, Suite 1900

Portland, OR 97204

UNITED STATES

FILING FEE RECEIVED

$1,820

Receipt date: 11/16/2022

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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
	446	11351256	B2	6-7-2022	Tang et al.	
	457	11459374	B2	10-4-2022	Tustian et al.	

U.S. PATENT APPLICATION PUBLICATIONS						
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	443	2022-0143137		5-12-2022	Witt et al.	
	461	2022-0151920		5-19-2022	Shepard et al.	
	447	2022-0175883		6-9-2022	Brockmeyer et al.	
	448	2022-0226477		7-21-2022	Sloey et al.	
	458	2022-0227836		7-21-2022	Wang et al.	
	453	2022-0265740		8-25-2022	Blumenkranz et al.	
	452	2022-0273764		9-1-2022	Yancopoulos e tal.	
	456	2022-0306732		9-29-2022	Bakhle et al.	
	451	2022-0331430		10-20-2022	Rosenkranz et al.	

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	459	2022/089440	WO	A1	5-5-2022	Eluminex Biosciences (Suzhou) Limited		
	460	2022/094340	WO	A1	5-5-2022	Gemini Therapeutics Sub, Inc.		
	445	2022/101826	WO	A1	5-19-2022	Astrazeneca UK Limited		
	449	2022/175601	WO	A1	8-25-2022	Rebio Technologies OY		
	444	2022/195505	WO	A1	9-22-2022	Kashiv Biosciences, LLC		
	462	2022/201084	WO	A1	9-29-2022	Novartis AG		
	463	2022/217110	WO	A1	10-13-2022	Avirmax, Inc.		
	454	3503883	EP	B1	7-3-2019	Nat Institute of Biological Sciences Beijing		

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	455	3716992	EP	B1	8-10-2022	Regeneron Pharmaceuticals, Inc.		
	450	4070786	EP	A1	10-12-2022	Sichuan Honghe Biotechnology Co., Ltd.		

NON-PATENT LITERATURE DOCUMENTS			
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	442	NAOTO et al., "Arginine and lysine reduce the high viscosity of serum albumin solutions for pharmaceutical injection," Journal of bioscience and bioengineering, Volume 117 (No. 5): 539-543, (2013). [http://hdl.handle.net/2241/00121920], [doi: 10.1016/j.jbiosc.2013.10.016].	

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☒ None

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Signature	/Lance A. Termes/	Date (YYYY-MM-DD)	2022-11-16
Name/Print	Lance A. Termes	Registration Number	43184

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.L/

Receipt date: 01/04/2022

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	Art Unit	~~Not Yet Assigned~~ 1647
	Confirmation Number	1061
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	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 Developmentl 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).	

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PGR2026-00039
Biocon Ex.1002
Page 2179 of 4945

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (not for submission under 37 CFR 1.99)		
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	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].	

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Examiner Signature	/JON M LOCKARD/	Date Considered	03/15/2023

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Signature	/Lance A. Termes/	Date (YYYY-MM-DD)	2022-01-04
Name/Print	Lance A. Termes	Registration Number	43184

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.L/

Inventor Information for 17/384070

/J.L./

Inventor Name	City	State/Country
GRAHAM, KENNETH S.	PLEASANT VALLEY	NEW YORK
WADHWA, SAURABH	NANUET	NEW YORK

Appln Info | Contents | Petition Info | Atty/Agent Info | Continuity Data | Foreign Data | **Inventors** | Applicants | Address | Fees | Post Info | Pre Gra

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Page 2182 of 4945

/J.L./

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     (FILE 'HOME' ENTERED AT 14:11:25 ON 16 MAR 2023)

     FILE 'MEDLINE, SCISEARCH, EMBASE, BIOSIS' ENTERED AT 14:11:37 ON 16 MAR
     2023
L1          1917 S VEGF (W) TRAP
L2         18711 S AFLIBERCEPT
L3          1702 S EYLEA
L4           308 S VEGF (W) TRAP-EYE
L5           144 S (L1 OR L2 OR L3 OR L4) (P) FORMULATION
L6             2 S L5 AND "HIGH CONCENTRATION"
L7             2 S L5 AND (8MG OR "8 MG")
L8             4 S L6 OR L7
L9             4 DUP REM L8 (0 DUPLICATES REMOVED)
                 E GRAHAM K S/AU
L10          270 S E3-E5 OR E136 OR E142
                 E WADHWA S/AU
L11          510 S E3 OR E42
L12            1 S L5 AND (L10 OR L11)
L13           33 S (L1 OR L2 OR L3 OR L4) (P) ("HIGH CONCENTRATION" OR 8MG OR
L14           22 DUP REM L13 (11 DUPLICATES REMOVED)
```

Receipt date: 12/06/2021

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	Filing Date	Jul-23-2021
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	Art Unit	~~Not Yet Assigned~~ 1647
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	Examiner Name	~~Not Yet Assigned~~ Jon Lockard
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PGR2026-00039
Biocon Ex.1002
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	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	

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Examiner Signature	/JON M LOCKARD/	Date Considered	03/15/2023

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Signature	/Lance A. Termes/	Date (YYYY-MM-DD)	2021-12-06
Name/Print	Lance A. Termes	Registration Number	43184

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	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].	
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	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].	
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	Attorney Docket Number	125984-266215 / 10430US02

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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

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☐ See attached certification statement.

☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

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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
	446	11351256	B2	6-7-2022	Tang et al.	
	457	11459374	B2	10-4-2022	Tustian et al.	

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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
	443	2022-0143137		5-12-2022	Witt et al.	
	461	2022-0151920		5-19-2022	Shepard et al.	
	447	2022-0175883		6-9-2022	Brockmeyer et al.	
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	451	2022-0331430		10-20-2022	Rosenkranz et al.	

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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]
	459	2022/089440	WO	A1	5-5-2022	Eluminex Biosciences (Suzhou) Limited		
	460	2022/094340	WO	A1	5-5-2022	Gemini Therapeutics Sub, Inc.		
	445	2022/101826	WO	A1	5-19-2022	Astrazeneca UK Limited		
	449	2022/175601	WO	A1	8-25-2022	Rebio Technologies OY		
	444	2022/195505	WO	A1	9-22-2022	Kashiv Biosciences, LLC		
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	463	2022/217110	WO	A1	10-13-2022	Avirmax, Inc.		
	454	3503883	EP	B1	7-3-2019	Nat Institute of Biological Sciences Beijing		

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	455	3716992	EP	B1	8-10-2022	Regeneron Pharmaceuticals, Inc.		
	450	4070786	EP	A1	10-12-2022	Sichuan Honghe Biotechnology Co., Ltd.		

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]
	442	NAOTO et al., "Arginine and lysine reduce the high viscosity of serum albumin solutions for pharmaceutical injection," Journal of bioscience and bioengineering, Volume 117 (No. 5): 539-543, (2013). [http://hdl.handle.net/2241/00121920], [doi: 10.1016/j.jbiosc.2013.10.016].	

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(54) Title: INHIBITORS OF ANGIOGENIC FACTORS

(57) Abstract: A recombinant human Fc fusion protein comprises an Ig-like domain of a first human VEGF receptor, another different Ig-like domain of a second human VEGF receptor, at least yet another Ig-like domain of a third human VEGF receptor, and the Fc portion of the human IgG1. The fusion protein is useful in treating or controlling an ocular disease, condition, or disorder that has etiology in aberrant angiogenesis.

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INHIBITORS OF ANGIOGENIC FACTORS

BACKGROUND OF THE INVENTION

[0001] The present invention relates to inhibitors of angiogenic factors. In particular, the present invention relates to inhibitors of members of the family of vascular endothelial growth factors ("VEGF family members"). More particular, the present invention relates to inhibitors of activity of VEGF family members, uses, and processes for production of such inhibitors.

[0002] The major cellular components of the mammalian vascular system are the endothelium, smooth muscle cells, and pericytes. Endothelial cells form the lining of the inner surface of all blood vessels in the mammal and constitute a non-thrombogenic interface between blood and tissue. Therefore, the proliferation of endothelial cells is an important component for the development of new capillaries and blood vessels which, in turn, is a necessary process for the growth and/or regeneration of mammalian tissues.

[0003] A family of secreted polypeptides has been shown to play an extremely important role in promoting endothelial cell proliferation and angiogenesis. A pathological feature of uncontrolled angiogenesis caused by VEGF over-expression is increased vascular permeability, which results in fluid leakage into, and swelling of, the surrounding tissues. In mammals, this family consists of five related growth factors having highly conserved receptor-binding structure: vascular endothelial growth factors A-D ("VEGF-A," "VEGF-B," "VEGF-C," and "VEGF-D") and placental growth factor ("PGF"). In this disclosure, this family of growth factors is also referred to as the VEGF family. These growth factors act through a family of cognate receptor tyrosine kinases, which exist only on the surface of vascular endothelial cells, to stimulate formation of blood vessels: VEGF receptor-1 ("VEGFR-1," also known as "flt-1"), VEGF receptor-2 ("VEGFR-2," also known as "KDR" in humans and "flk-1" in mice), VEGF receptor-3 ("VEGFR-3," also known as "flt-4").

[0004] VEGF-A (also sometimes simply referred to as VEGF) has emerged as the most important member of this family of growth factors. Human VEGF-A is expressed in a variety of

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tissues as multiple homodimeric forms (121, 145, 165, 183, 189 and 206 amino acids per monomer), wherein each form arises as a result of alternative splicing of a single RNA transcript.

[0005] Since VEGFs promote vascular endothelial cell proliferation and angiogenesis, they may be useful for the therapeutic treatment of numerous conditions in which a growth-promoting activity on the vascular endothelial cells is beneficially important; for example, in treatment of ulcers, vascular injuries, and myocardial infarction.

[0006] In contrast, however, while vascular endothelial proliferation is desirable under certain circumstances, vascular endothelial proliferation and angiogenesis are also undesirable components of a variety of diseases and disorders including tumor growth and metastasis, rheumatoid arthritis, psoriasis, atherosclerosis, diabetic retinopathy, retrolental fibroplasia, neovascular glaucoma, neovascular age-related macular degeneration, hemangiomas, immune rejection of transplanted corneal tissue and other tissues, and chronic inflammation. In individuals suffering from any of these disorders, one would want to inhibit, or at least substantially reduce, the endothelial proliferating activity of VEGFs.

[0007] Each of flt-1, KDR, and flt-4 tyrosine kinase receptors has seven extracellular immunoglobulin-like ("Ig-like") domains that are available for ligand binding, a transmembrane domain that serves to anchor the receptor on the surface of cells in which it is expressed and an intracellular catalytic tyrosine kinase domain. Flt-1 binds VEGF-A, VEGF-B, and PlGF. KDR binds VEGF-A, VEGF-C, and VEGF-D. Flt-4 binds VEGF-C and VEGF-D.

[0008] In view of the role of the growth factors of the VEGF family in vascular endothelial proliferation and angiogenesis, and the role that these processes play in many different diseases and disorders, it is desirable to have a pharmacological means for reducing or inhibiting one or more of the biological activities of these growth factors in patients whose pathological conditions are rooted in aberrant angiogenesis. It is also desirable to have a pharmacological means for improved treatment or control of pathological conditions that are rooted in aberrant angiogenesis.

SUMMARY OF THE INVENTION

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[0009] As used herein, the term "control" also includes reduction, alleviation, amelioration, or prevention.

[0010] In general, the present invention provides fusion or chimeric polypeptides or proteins, methods of producing and compositions comprising the same, and methods for treating or controlling at least a pathological condition in a subject, which condition has etiology in aberrant angiogenesis.

[0011] In one aspect, the present invention provides fusion or chimeric polypeptides that are capable of binding substantially to one or more VEGF family members; thereby, reducing or inhibiting their binding to VEGF receptors.

[0012] In another aspect, a fusion or chimeric polypeptide of the present invention comprises an Ig-like domain of a first VEGF receptor, an Ig-like domain of a second VEGF receptor, and at least an Ig-like domain of a third VEGF receptor.

[0013] In still another aspect, a fusion or chimeric polypeptide of the present invention comprises: (a) Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1 (or flt-1); (b) Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2 (or KDR); and (c) Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3 (or flt-4).

[0014] In still another aspect, a fusion or chimeric polypeptide of the present invention comprises: (a) Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1; (b) Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2; and (c) Ig-like domains 1, 2, and 3, or substantially Ig-like domains 1, 2, and 3, of human VEGFR-3.

[0015] In still another aspect, the human VEGFR-3 is the wild-type human VEGFR-3 having the amino acid sequence listed in SEQ ID NO: 13; and the Ig-like domain 1 of human VEGFR-3 that is included in any fusion protein of the present invention has the amino acid sequence listed in SEQ ID NO: 14. A fusion protein of the present invention comprises Ig-like domain 1, or substantially Ig-like domain 1, of human VEGFR-3; wherein the amino acid

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sequence at positions 80-82 of SEQ ID: 14 is NDT, NDS, NXT, or NXS; and X is any amino acid. In one embodiment, X is a naturally occurring amino acid.

[0016] In yet another aspect, the fusion or chimeric polypeptide of the present invention comprises: (a) Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1; (b) Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2; and (c) Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3; wherein the amino acid sequence of human VEGFR-3 is listed in SEQ ID NO: 13; and wherein the amino acid sequence at positions 104-106 is NDT, NDS, NXT, or NXS; wherein X is any amino acid. In one embodiment, X is a naturally occurring amino acid.

[0017] In yet another aspect, the fusion or chimeric polypeptide of the present invention comprises: (a) Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1 (or flt-1); (b) Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2 (or KDR); and (c) Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3 (or flt-4); linked to a multimerizing component.

[0018] In one embodiment, said multimerizing component comprises a portion of an Fc domain of human IgG1.

[0019] In another embodiment, said multimerizing component comprises substantially the last two heavy-chain domains at the amino terminus of IgG1; in other words, Fc CH2 and CH3 domains of human IgG1.

[0020] In still another aspect, the present invention provides an isolated nucleic acid molecule encoding said fusion or chimeric polypeptide.

[0021] In still another aspect, the present invention provides a vector that comprises said nucleic acid molecule, including an expression vector comprising said nucleic molecule operatively linked to an expression control sequence.

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[0022] In still another aspect, the present invention provides a host-vector system for the production of said fusion or chimeric polypeptide that comprises the expression vector in a suitable host cell.

[0023] In another aspect, the present invention provides a method of producing a fusion or chimeric polypeptide, which method comprises: (a) growing cells of the host-vector system under conditions permitting production of the fusion or chimeric protein; and (b) recovering the fusion or chimeric polypeptide so produced.

[0024] In still another aspect, the present invention provides a method for treating or controlling at least a disease, condition, or disorder, in a subject, which has etiology in aberrant angiogenesis.

[0025] In yet another aspect, the present invention provides a method for treating or controlling at least an ocular disease, condition, or disorder, in a subject, which has etiology in aberrant angiogenesis.

[0026] Other features and advantages of the present invention will become apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] Figure 1 shows schematically a fusion polypeptide of the present invention, comprising Ig-like domain 2 of VEGFR-1, Ig-like domain 3 of VEGFR-2, Ig-like domains 1 and 2 of VEGFR-3, and an Fc domain.

[0028] Figure 2 shows the SDS-PAGE and SEC-HPLC results for the transient expression of EB-101 in HEK293 cells.

[0029] Figure 3 shows the SDS-PAGE and SEC-HPLC results for the transient expression of EB-101 in CHO cells.

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[0030] Figure 4 shows the comparison of the binding affinity of EB-101 with aflibercept for human recombinant human VEGF-A_{165} using SPR Biacore.

[0031] Figure 5 shows the comparison of the binding affinity of EB-101 with aflibercept for human VEGF-$B_{167/189}$ using SPR Biacore.

[0032] Figure 6 shows the comparison of the binding affinity of EB-101 with aflibercept for human VEGF-C using SPR Biacore.

[0033] Figure 7 shows the comparison of the binding affinity of EB-101 with aflibercept for human VEGF-D using SPR Biacore.

[0034] Figure 8 shows the comparison of the binding affinity of EB-101 with aflibercept for human PlGF using SPR Biacore.

[0035] Figure 9 shows the comparison of the effect of EB-101 with aflibercept and bevacizumab on inhibition of VEGF-A_{165} mediated VEGFR-2 signaling in VEGFR-2-NFAT-RE luciferase reporter cells.

[0036] Figure 10 shows the comparison of the effect of EB-101 with aflibercept on inhibition of VEGF-C mediated VEGFR-2 signaling in VEGFR-2-NFAT-RE luciferase reporter cells.

[0037] Figure 11 shows the comparison of EB-101 with aflibercept and a recombinant human VEGFR-3 containing 7 extracellular domains (rhVEGFR-3-7ECDs) on their inhibitory effect on VEGF-C mediated VEGFR-2 signaling in VEGFR-2-NFAT-RE luciferase reporter cells.

[0038] Figure 12 shows the quantitative analyses of the effect of EB-101 with aflibercept and a recombinant human VEGFR-3 containing 7 extracellular domains (rhVEGFR-3) on inhibition of VEGFR-2 signaling in VEGFR-2-NFAT-RE luciferase reporter cells stimulated by VEGF-A_{165} and VEGF-C.

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[0039] Figure 13 shows the comparison of the effect of EB-101 with aflibercept and bevacizumab on VEGF-A_{165}-mediated proliferation of human lymphatic endothelial cells (HLECs).

[0040] Figure 14 shows the comparison of the effect of EB-101 with aflibercept and a recombinant human VEGFR3 containing 7 extracellular domains (rhVEGFR3 ECDs) on VEGF-C mediated HLEC proliferation.

[0041] Figure 15 shows the quantitative analyses of EB-101, aflibercept and a recombinant human VEGFR3 containing 7 extracellular domains (rhVEGFR3 ECDs), on VEGF-A_{165} plus VEGF-C mediated HLEC proliferation.

[0042] Figure 16 shows schematically a fusion polypeptide of the present invention, comprising Ig-like domain 2 of VEGFR-1, Ig-like domain 3 of VEGFR-2, Ig-like domains 1, 2, and 3 of VEGFR-3, and an Fc domain.

[0043] Figure 17 shows the comparison of the effect of EB-101BIb with aflibercept, EB-101, EB-101BIa on VEGF-A mediated VEGFR-2 signaling in VEGFR-2-NFAT-RE luciferase reporter cells.

[0044] Figure 18 shows the comparison of the effect of EB-101BIb with aflibercept, EB-101 and EB-101BIa on VEGFR-2 signaling in VEGFR-2-NFAT-RE luciferase reporter cells stimulated by recombinant human VEGF-C and -D.

[0045] Figure 19 shows the comparison of the inhibitory effect of EB-101BIb with aflibercept, EB-101 and a combination of aflibercept with a recombinant human VEGFR-3 on VEGF-A, -C, and -D mediated VEGFR-2 signaling in VEGFR-2-NFAT-RE luciferase reporter cells.

[0046] Figure 20 shows the comparison of the effect of EB-101BIb with aflibercept and EB-101BIa on VEGF-A_{165}-mediated proliferation of human lymphatic endothelial cells (HLECs).

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[0047] Figure 21 shows the comparison of the effect of EB-101BIb with aflibercept, EB-101BIa and a recombinant human VEGFR-3 containing 7 extracellular domains on VEGF-C and VEGF-D mediated HLEC proliferation.

[0048] Figure 22 shows the comparison of the effect of EB-101BIb with aflibercept, a recombinant human VEGFR-3 containing 7 extracellular domains (7 ECDs) and a combination of aflibercept with the VEGFR-3 on VEGF-A, -C and -D mediated human lymphatic endothelial cell (HLEC) proliferation.

DETAILED DESCRIPTION OF THE INVENTION

[0049] In the present disclosure, the terms "fusion polypeptide," "fusion protein," "chimeric polypeptide," and "chimeric protein" are used interchangeably.

[0050] The present invention provides a binding construct that is capable of binding at least one member of the VEGF family ("VEGF family member"); thereby, rendering said VEGF family member substantially unavailable for binding to a VEGF receptor on endothelial cells. As a result, a binding construct of the present invention substantially inhibits biological activity of said VEGF family member in promoting angiogenesis; thereby, controlling a pathological condition having etiology in aberrant angiogenesis.

[0051] The binding construct of the present invention comprises or consists of a fusion or chimeric polypeptide or protein and comprises one or more binding units that are associated with each other by covalent or other forms of attachment. A binding construct of the present invention is capable of binding a VEGF family member or a portion thereof and does so with high affinity. A binding unit preferably comprises at least one peptide or polypeptide. While a binding unit preferably comprises a single polypeptide, it may comprise multiple polypeptides if a single polypeptide is not sufficient for binding a particular growth factor. When more than one binding unit or polypeptide segment is in a given binding construct, the binding units may be joined together directly or through a linker. A binding construct may further include a heterologous peptide or other chemical moieties. Such additions can modify binding construct

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properties such as stability, solubility, toxicity, serum half-life, immunogenicity, detectability, or other properties.

[0052] The term "high affinity" is used in a physiological context pertaining to the relative affinity of the binding construct for the VEGF family members in vivo in a mammal, such as a laboratory test animal, a domesticated farm or pet animal, or a human. The targeted VEGF members in the present invention have characteristic affinities for their receptors in vivo, typically measured in terms of sub-nanomolar dissociation constants (K_d). For the purposes of this invention, a binding construct of the present invention can bind to its target VEGF family member(s) with a K_d less than or equal to about 1, or about 5, or about 10, or about 50, or about 100, or about 500, or about 1000 times the K_d of the natural growth factor-receptor pair.

[0053] In one aspect, a fusion or chimeric polypeptide or protein of the present invention comprises a first polypeptide binding unit, a second polypeptide binding unit, and a third polypeptide binding unit, operatively linked together. A polypeptide binding unit may be referred to herein in an abbreviated way as "a binding unit." In general, the first, second, and third binding units may be linked together in any order, directly or through an intervening linker.

[0054] In another aspect, a targeted VEGF family member may bind to one or more binding units of the fusion or chimeric polypeptide.

[0055] In still another aspect, a binding unit comprises substantially an Ig-like domain of a VEGF receptor.

[0056] In another aspect, a fusion or chimeric polypeptide of the present invention comprises a first binding unit that comprises an Ig-like domain of a first VEGF receptor; a second binding unit that comprises an Ig-like domain of a second VEGF receptor; and a third binding unit that comprises at least an Ig-like domain of a third VEGF receptor.

[0057] In another aspect, the fusion or chimeric polypeptide of the present invention comprises an Ig-like domain of a first VEGF receptor, an Ig-like domain of a second VEGF receptor, and at least an Ig-like domain of a third VEGF receptor; wherein said third VEGF

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receptor comprises human VEGFR-3 (or flt-4) having an amino acid sequence listed in SEQ ID NO: 13 with the proviso that the amino acids at positions 105 and 106 of SEQ ID NO: 13 are substitutable such that the amino acid sequence at positions 104-106 of SEQ ID NO: 13 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

[0058] In still another aspect, the fusion polypeptide of the present invention comprises: (a) an Ig-like domain of a first VEGF receptor that comprises an amino acid sequence that is at least 90% identical to Ig-like domain 2 of human VEGFR-1; (b) an Ig-like domain of a second VEGF receptor that comprises an amino acid sequence that is at least 90% identical to Ig-like domain 3 of human VEGFR-2; and (c) at least an Ig-like domain of a third VEGF receptor that comprises an amino acid sequence that is at least 90% identical to Ig-like domains 1 and 2 of human VEGFR-3; wherein the human VEGFR-3 has an amino acid sequence listed in SEQ ID NO: 13 with the proviso that the amino acids at positions 105 and 106 of SEQ ID NO: 13 are substitutable such that the amino acid sequence at positions 104-106 of SEQ ID NO: 13 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

[0059] In still another aspect, the fusion polypeptide of the present invention comprises: (a) an Ig-like domain of a first VEGF receptor that comprises an amino acid sequence that is at least 95% identical to Ig-like domain 2 of human VEGFR-1; (b) an Ig-like domain of a second VEGF receptor that comprises an amino acid sequence that is at least 95% identical to Ig-like domain 3 of human VEGFR-2; and (c) at least an Ig-like domain of a third VEGF receptor that comprises an amino acid sequence that is at least 95% identical to Ig-like domains 1 and 2 of human VEGFR-3; wherein the human VEGFR-3 has an amino acid sequence listed in SEQ ID NO: 13 with the proviso that the amino acids at positions 105 and 106 of SEQ ID NO: 13 are substitutable such that the amino acid sequence at positions 104-106 of SEQ ID NO: 13 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

[0060] In still another aspect, a fusion or chimeric polypeptide of the present invention comprises: (a) Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1 (or flt-1);

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(b) Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2 (or KDR); and (c) Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3 (or flt-4); or Ig-like domains 1, 2, and 3, or substantially Ig-like domains 1, 2, and 3, of human VEGFR-3.

[0061] In still another aspect, a fusion or chimeric polypeptide of the present invention comprises: (a) Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1; (b) Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2; and (c) Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3; or Ig-like domains 1, 2, and 3, or substantially Ig-like domains 1, 2, and 3, of human VEGFR-3; wherein the human VEGFR-3 has the amino acid sequence listed in SEQ ID NO: 13 with the proviso that the amino acids at positions 105 and 106 of SEQ ID NO: 13 are substitutable such that the amino acid sequence at positions 104-106 of SEQ ID NO: 13 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

[0062] In still another aspect, a fusion or chimeric polypeptide of the present invention comprises: (a) Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1; (b) Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2; and (c) Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3; or Ig-like domains 1, 2, and 3, or substantially Ig-like domains 1, 2, and 3, of human VEGFR-3; wherein the human VEGFR-3 has the amino acid sequence listed in SEQ ID NO: 13 with the proviso that the amino acid sequence at positions 104-106 of SEQ ID NO: 13 is unsubstituted and remains NDT.

[0063] In still another aspect, a fusion or chimeric polypeptide of the present invention comprises: (a) Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1; (b) Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2; and (c) Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3; or Ig-like domains 1, 2, and 3, or substantially Ig-like domains 1, 2, and 3, of human VEGFR-3; wherein the Ig-like domain 1 of the human VEGFR-3 has an amino acid sequence listed in SEQ ID NO: 14; wherein the amino acids at positions 81 and 82 of SEQ ID NO: 14 are substitutable such that the amino acid sequence at positions 80-82 of SEQ ID NO: 14 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

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[0064] In still another aspect, a fusion or chimeric polypeptide of the present invention comprises: (a) Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1; (b) Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2; and (c) Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3; or Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3; wherein the Ig-like domain 1 of the human VEGFR-3 has an amino acid sequence listed in SEQ ID NO: 14; wherein the amino acids at positions 81 and 82 of SEQ ID NO: 14 are substitutable such that the amino acid sequence at positions 80-82 of SEQ ID NO: 14 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

[0065] In still another aspect, a fusion or chimeric polypeptide of the present invention comprises: (a) Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1; (b) Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2; and (c) Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3; or Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3; wherein the Ig-like domain 1 of the human VEGFR-3 has an amino acid sequence listed in SEQ ID NO: 14; wherein the amino acid sequence at positions 80-82 of SEQ ID NO: 14 is unsubstituted and remains NDT.

[0066] In still another aspect, a fusion or chimeric polypeptide of the present invention comprises: (a) Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1; (b) Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2; and (c) Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3; or Ig-like domains 1, 2, and 3, or substantially Ig-like domains 1, 2, and 3, of human VEGFR-3; wherein the Ig-like domain 1 of human VEGFR-3 included in the fusion or chimeric polypeptide has an amino acid sequence listed in SEQ ID NO: 14.

[0067] In still another aspect, a fusion or chimeric polypeptide of the present invention comprises: (a) Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1; (b) Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2; and (c) Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3; or Ig-like domains 1, 2, and 3, or substantially Ig-like domains 1, 2, and 3, of human VEGFR-3; wherein the Ig-like domain 1

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of human VEGFR-3 included in the fusion or chimeric polypeptide has an amino acid sequence listed in SEQ ID NO: 14, and the amino acid sequence at positions is unsubstituted and remains NDT.

[0068] In yet another aspect, the fusion or chimeric polypeptide of the present invention comprises: (a) Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1; (b) Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2; and (c) Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3; linked to a multimerizing component; wherein the human VEGFR-3 has the amino acid sequence listed in SEQ ID NO: 13 with the proviso that the amino acids at positions 105 and 106 of SEQ ID NO: 13 are substitutable such that the amino acid sequence at positions 104-106 of SEQ ID NO: 13 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

[0069] In one embodiment, said multimerizing component comprises substantially a portion of an Fc domain of human IgG1.

[0070] In another embodiment, said multimerizing component comprises substantially a portion of an Fc domain of the heavy chain of human IgG1. In one embodiment, such portion of an Fc domain of the heavy chain of human IgG1 comprises the last two domains at the carboxy terminus of the IgG1 heavy chain.

[0071] In some embodiments, two or more binding units act together to bind a single ligand molecule (wherein the ligand may comprise a monomer or dimer). In some other embodiments, the binding units act independently, i.e., each binding unit binds a separate ligand molecule.

[0072] In one aspect, the fusion or chimeric protein comprises (a) Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1 operatively linked to (b) Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2, which is operatively linked to (c)(1) Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, or (c)(2) Ig-like domains 1, 2 and 3, or substantially Ig-like domains 1, 2, and 3, of human VEGFR-3, which is operatively linked to a portion of the Fc domain of IgG1.

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[0073] In one embodiment, a polypeptide linker is inserted between the last binding unit and said portion of the Fc domain of IgG1.

[0074] In one aspect, a fusion or chimeric protein comprises (a) Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1 operatively linked to (b) Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2, which is operatively linked to (c) (1) Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, or (c)(2) Ig-like domains 1, 2, and 3, or substantially Ig-like domains 1, 2, and 3, of human VEGFR-3, which is operatively linked to a portion of the Fc domain of IgG1; wherein the Ig-like domain 2 of human VEGFR-1 ("VEGFR-1-D2") has an amino acid sequence listed in SEQ ID NO: 2; the Ig-like domain 3 of human VEGFR-2 ("VEGFR-2-D3") has an amino acid sequence listed in SEQ ID NO: 4; the Ig-like domains 1 and 2 of human VEGFR-3 ("VEGFR-3-D1D2") have an amino acid sequence listed in SEQ ID NO: 6; the Ig-like domains 1, 2, and 3 of human VEGFR-3 ("VEGFR-3-D1D2D3") have an amino acid sequence listed in SEQ ID NO: 15; and the portion of the Fc domain of IgG1 ("IgG1Fc") has an amino acid sequence listed in SEQ ID NO: 8.

[0075] In another aspect, a fusion or chimeric polypeptide or protein of the present invention further comprises a polypeptide linker that is inserted between the carboxy terminus of VEGFR-3-D1D2 and the amino terminus of IgG1Fc; wherein said linker has an amino acid sequence listed in SEQ ID NO: 10.

[0076] In still another aspect, a fusion or chimeric polypeptide or protein of the present invention further can comprise a polypeptide leader sequence that precedes the amino terminus of VEGFR-1-D2.

[0077] In yet another aspect, a fusion or chimeric polypeptide or protein of the present invention comprises amino acid sequences that are at least 90% identical to each of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 15, SEQ ID NO: 8, and SEQ ID NO: 10; wherein the amino acids at positions 81 and 82 of SEQ ID NO: 6 and SEQ ID NO: 15 are substitutable such that the amino acid sequence at positions 80-82 of SEQ ID NO: 6 or SEQ ID NO: 15 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

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[0078] In still another aspect, a fusion or chimeric polypeptide or protein of the present invention comprises amino acid sequences that are at least 95% identical to SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 15, SEQ ID NO: 8, and SEQ ID NO: 10; wherein the amino acids at positions 81 and 82 of SEQ ID NO: 6 and SEQ ID NO: 15 are substitutable such that the amino acid sequence at positions 80-82 of SEQ ID NO: 6 or SEQ ID NO: 15 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

[0079] In still another aspect, a fusion or chimeric polypeptide or protein of the present invention comprises the amino acid sequence listed in SEQ ID NO: 12 or SEQ ID NO: 16. The fusion proteins having SEQ ID NO: 12 and SEQ ID NO: 16 were prepared for testing and are referred to herein as "EB-101" and "EB-101BIb," respectively.

[0080] In still another aspect, a fusion or chimeric polypeptide or protein of the present invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 12 or SEQ ID NO: 16; wherein the amino acids at positions 286 and 287 of SEQ ID NO: 12 or SEQ ID NO: 16 are substitutable such that the amino acid sequence at positions 285-287 of SEQ ID NO: 12 or SEQ ID NO: 16 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

[0081] In still another aspect, a fusion or chimeric polypeptide or protein of the present invention comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 12 or SEQ ID NO: 16; wherein the amino acids at positions 285-287 of SEQ ID NO: 12 or SEQ ID NO: 16 are unsubstituted.

[0082] In still another aspect, a fusion or chimeric polypeptide or protein of the present invention comprises an amino acid sequence that is at least 95% identical to SEQ ID NO: 12 or SEQ ID NO: 16; wherein the amino acids at positions 286 and 287 of SEQ ID NO: 12 or SEQ ID NO: 16 are substitutable such that the amino acid sequence at positions 285-287 of SEQ ID NO: 12 or SEQ ID NO: 16 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

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[0083] In still another aspect, a fusion or chimeric polypeptide or protein of the present invention comprises an amino acid sequence that is at least 95% identical to SEQ ID NO: 12 or SEQ ID NO: 16; wherein the amino acids at positions 285-287 of SEQ ID NO: 12 or SEQ ID NO: 16 are unsubstituted.

[0084] In still another aspect, one or more amino acid substitutions can be made in anyone of the above-described amino acid sequences. Preferably, such substitution is a conserved substitution, wherein an amino acid in one of the following groups is substituted with another in the same group: (1) A, S, T; (2) D, E; (3) N, Q; (4) R, K; (5) I, L, M, V; and (6) F, Y, W; and such substitution is selected so as to preserve substantially the binding activity of the fusion polypeptide. In one embodiment, the fusion polypeptide of the present invention having a conserved substitution has a K_d value less than about 120% of that before such substitution. Preferably, the K_d value is less than about 110% of that before such substitution. More preferably, the K_d value is less than about 105% of that before such substitution.

[0085] Most conserved substitutions are not expected to produce radical changes in the characteristics of the Ig-like domain or domains of the fusion polypeptide. However, when it is difficult to predict the exact effect of the substitution in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. For example, an Ig-like domain variant typically is made by site-specific mutagenesis of the nucleic acid encoding the intact fusion polypeptide, expression of the variant nucleic acid in recombinant cell culture, purification of the variant fusion polypeptide from the cell culture and detecting the ability of the variant fusion polypeptide to specifically bind to a VEGF ligand. An exemplary binding assay which can be employed to determine if a particular substitution or substitutions in an Ig-like domain or domains affect the capability of the fusion polypeptide to bind to and inhibit the activity of a VEGF family member is described in the article by Park et al., J. Biol. Chem. 269:25646-25654 (1994).

[0086] The VEGFR-1-D2 binding unit of the fusion protein is capable of binding free VEGF-A, VEGF-B, and PGF with high affinity (Davis-Smyth et al., EMBO J., 15(18):4919 (1996)). The VEGFR-2-D3 binding unit of the fusion protein is capable of binding free VEGF-A, VEGF-C, and VEGF-D with high affinity (Stuttfeld et al., 61(9):915 (2009)). The VEGFR-3-

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D1D2 or VEGFR-3-D1D2D3 (Ig-like domains 1, 2, and 3 of VEGFR-3) binding unit of the fusion protein is capable of binding free VEGF-C and VEGF-D with high affinity. Thus, a fusion protein of the present invention is capable of substantially inhibiting the angiogenic activity of these VEGF family members on endothelial cells at the site of the disease.

[0087] In still another aspect, the present invention provides an isolated nucleic acid molecule encoding said fusion or chimeric polypeptide.

[0088] In yet another aspect, the present invention provides an isolated nucleic acid molecule encoding said fusion or chimeric polypeptide; wherein said isolated nucleic acid molecule comprises: (a) a nucleic acid sequence encoding Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1 (or flt-1); (b) a nucleic acid sequence encoding Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2 (or KDR); and (c) a nucleic acid sequence encoding Ig-like domain 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3 (or flt-4).

[0089] In still another aspect, the present invention provides an isolated nucleic acid molecule encoding said fusion or chimeric polypeptide; wherein said isolated nucleic acid molecule comprises: (a) a nucleic acid sequence encoding Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1 (or flt-1); (b) a nucleic acid sequence encoding Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2 (or KDR); (c)(1) a nucleic acid sequence encoding Ig-like domains 1 and 2, or substantially Ig-like domains 1 and 2, or (c)(2) a nucleic acid sequence encoding Ig-like domains 1, 2, and 3, or substantially Ig-like domains 1, 2, and 3, of human VEGFR-3 (or flt-4); and (d) a nucleic acid sequence encoding a portion of the Fc domain of IgG1.

[0090] In still another aspect, the present invention provides an isolated nucleic acid molecule encoding said fusion or chimeric polypeptide; wherein said isolated nucleic acid molecule comprises: (a) a nucleic acid sequence encoding Ig-like domain 2, or substantially Ig-like domain 2, of human VEGFR-1 (or flt-1) and having a sequence listed in SEQ ID NO: 1; (b) a nucleic acid sequence encoding Ig-like domain 3, or substantially Ig-like domain 3, of human VEGFR-2 (or KDR) and having a sequence listed in SEQ ID NO: 3; (c) a nucleic acid sequence

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encoding Ig-like domain 1 and 2, or substantially Ig-like domains 1 and 2, of human VEGFR-3 (or flt-4) and having a sequence listed in SEQ ID NO: 5; and (d) a nucleic acid sequence encoding a portion of the Fc domain of IgG1 and having a sequence listed in SEQ ID NO: 7.

[0091] In still another aspect, the present invention provides an isolated nucleic acid molecule encoding said fusion or chimeric polypeptide; wherein said isolated nucleic acid molecule comprises: (a) a nucleic acid sequence encoding an amino acid sequence listed in SEQ ID NO: 2; (b) a nucleic acid sequence encoding an amino acid sequence listed in SEQ ID NO: 4; (c) a nucleic acid sequence encoding an amino acid sequence listed in SEQ ID NO: 6 or SEQ ID NO: 15; and (d) a nucleic acid sequence encoding an amino acid sequence of a portion of the Fc domain of IgG1 listed in SEQ ID NO: 8.

[0092] In yet another aspect, said isolated nucleic acid molecule further comprises a linking nucleic acid sequence encoding a polypeptide linker; wherein said linking nucleic acid has a sequence listed in SEQ ID NO: 9 and is inserted between SEQ ID NO: 5 and SEQ ID NO: 7.

[0093] In yet another aspect, said isolated nucleic acid further can comprise a leading nucleic acid sequence encoding a polypeptide leader sequence. When said leading nucleic acid sequence is present in said isolated nucleic acid, it precedes SEQ ID NO: 1.

[0094] In still another aspect, the present invention provides an isolated nucleic acid molecule encoding said fusion or chimeric polypeptide; wherein said isolated nucleic acid molecule comprises a nucleic acid sequence listed in SEQ ID NO: 11.

[0095] In still another aspect, the present invention provides an isolated nucleic acid molecule encoding a fusion or chimeric polypeptide or protein; wherein said fusion or chimeric polypeptide or protein has SEQ ID NO: 12 or SEQ ID NO: 16.

[0096] In another aspect, the present invention provides an isolated nucleic acid molecule encoding said fusion or chimeric polypeptide; wherein said isolated nucleic acid molecule comprises a nucleic acid sequence that, as a result of the degeneracy of the genetic code, differs in one or more codons from a nucleic acid sequence selected from the group consisting of SEQ

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ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, and SEQ ID NO: 11. Such different nucleic acid sequence is within the scope of the present invention.

[0097] In still another aspect, the present invention provides a vector that comprises any of said nucleic acid molecules, including an expression vector comprising any of said nucleic molecules operatively linked to an expression control sequence. Embodiments of the vector comprise a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, and SEQ ID NO: 11. In particular, a vector of the present invention comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, and SEQ ID NO: 11.

[0098] In still another aspect, the present invention provides a host-vector system for the production of said fusion or chimeric polypeptide that comprises the expression vector in a suitable host cell.

[0099] In one aspect, the present invention provides for the construction of a nucleic acid molecule encoding a fusion polypeptide disclosed herein, which nucleic acid is inserted into a vector that is able to express the fusion polypeptide when introduced into an appropriate host cell. Appropriate host cells include, but are not limited to, bacterial cells, yeast cells, insect cells, and mammalian cells. Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding the chimeric polypeptide molecules under control of transcriptional/translational control signals. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinations (genetic recombination) (See Sambrook, et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory; Current Protocols in Molecular Biology, Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY).

[00100] Expression of a nucleic acid molecule encoding a fusion polypeptide of the present invention may be regulated by a second nucleic acid sequence (a promoter) so that the fusion polypeptide is expressed in a host transformed with the nucleic acid molecule. For example, expression of the fusion polypeptide described herein may be controlled by any promoter/enhancer element known in the art.

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[00101] In general, plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences that are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see; e.g., Bolivar et al., Gene, 2:95 (1977)). The plasmid pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid or phage, must also contain, or be modified to contain, promoters that can be used by the microbial organism for expression of proteins.

[00102] Those promoters most commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems or a tryptophan (trp) promoter system (Goeddel et al., Nucleic Acids Res., 8:4057 (1980)). While these are the most commonly used, other microbial promoters have been discovered and utilized. For example, the tac promoter is a synthetically produced DNA promoter produced from the combination of promoters from the trp and lac operons (de Boer et al., PNAS, (1983-01-80 (1):21–25 (1983)). It is commonly used for protein production in *Escherichia coli*. (Amann et al., Gene, 25:167 (1983)). Any of these promoters may be used in connection with a method of producing a fusion polypeptide of the present invention.

[00103] In addition to prokaryotes, eukaryotic microbes, such as yeast cultures, may also be used. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example (Stinchcomb et al., Nature, 282:39 (1979)) is commonly used. Other plasmids are disclosed in US Patent 4,615,974; Struhl et al., PNAS,76(3):1035 (1979). The plasmid YRp7 contains the trp1 gene that provides a selection marker for a mutant strain of yeast lacking the ability to grow without tryptophan, for example, ATCC No. 44,076 or RH218 (Jones, Genetics, 85:23 (1977)). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

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[00104] Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem., 255:2073 (1980)) or other glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylas, and glucokinase (Romanos et al., Yeast, 8:423 (1992); Weinhandl et al., Microb. Cell factories, 13:5 (2014)). In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions, such as the promoter region for alcohol dehydrogenase 2, and enzymes responsible for maltose and galactose utilization (Romanos et al., Weinhandl et al., supra). Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

[00105] In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, much interest has been in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7, 293, and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences.

[00106] For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment that also contains the SV40 viral origin of replication (Fiers et al., Nature, 273:113 (1978)). Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250-bp sequence extending from the HindIII site toward the BglI site located in

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the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

[00107] Thus, according to the invention, expression vectors capable of being replicated in a bacterial, a yeast cell, an insect cell, or a mammalian cell host, comprising a fusion polypeptide-encoding nucleic acid as described herein, are used to transfect the host and thereby direct expression of such nucleic acids to produce the fusion polypeptide, which may then be recovered in a biologically active form. As used herein, a biologically active form includes a form capable of binding to at least a VEGF family member.

[00108] In some embodiments, the host cell can be *E. coli*, a COS cell, or a Chinese hamster ovary ("CHO") cell. In some other embodiments, the host cell can be HEK293 cell.

Vector Construction

[00109] Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and ligated in the form desired to form the plasmids required. The methods employed are not dependent on the DNA source or intended host. Cleavage is performed by treating with restriction enzyme (or enzymes) in suitable buffer.

[00110] A nucleic acid sequence substantially encoding one or more Ig-like domains of VEGFR-1, VEGFR-2, or VEGR-3 can be produced according to the method disclosed in U.S. Patent 6,897,294.

[00111] Nucleic acid sequences substantially encoding the Ig-like domain 2 of VEGFR-1, Ig-like domain 3 of VEGFR-2, and Ig-like domains 1 and 2 of VEGR-3 are ligated in tandem in the desired order. This construct is then ligated to the N-terminus of the nucleic acid sequence encoding the desired Fc portion of IgG1. The entire nucleic acid sequence is then positioned in a vector which contains a promoter in the reading frame with the gene and compatible with the proposed host cell. A number of plasmids, such as those described in U.S. Patents 4,456,748;

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5,460,811; 5,888,808; and 6,333,147, may be used in a production of a fusion polypeptide of the present invention.

[00112] In one embodiment, a fusion polypeptide of the present invention is produced according to the method described in U.S. Patent 7,070,959. The entire nucleic acid sequence of the fusion polypeptide ("VEGFR-1-D2-VEGFR-2-D3-VEGFR-3-D1D2-Fc" or "VEGFR-1-D2-VEGFR-2-D3-VEGFR-3-D1D2D3-Fc) is inserted into the expression vector pEE14.1 (Lonza Biologics) having the CMV promoter. CHO K1 cells are transfected with the pEE14.1/VEGFR-1-D2-VEGFR-2-D3-VEGFR-3-D1D2-Fc or pEE14.1/VEGFR-1-D2-VEGFR-2-D3-VEGFR-3-D1D2D3-Fc DNA construct and then grown. The fusion polypeptide obtained from the CHO or HEK293 cells is purified and characterized by binding assay, as described in U.S. Patent 7,070,959.

[00113] In one embodiment, a fusion polypeptide of the present invention can bind to VEGF family members with $K_d \leq 10^{-9}$ M. In another embodiment, a fusion polypeptide of the present invention can bind to VEGF family members with $K_d \leq 5x10^{-10}$ M. In still another embodiment, a fusion polypeptide of the present invention can bind to VEGF family members with $K_d \leq 10^{-10}$ M.

[00114] In one aspect, the present invention provides compounds, compositions, and methods for treating or controlling a disease, condition, or disorder having etiology in aberrant angiogenesis.

[00115] In another aspect, the present invention provides a method for treating or controlling at least an ocular disease, condition, or disorder, in a subject, which has etiology in aberrant angiogenesis. The method comprises administering to a subject in need of such treating or controlling a composition comprising a fusion polypeptide that comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 2; SEQ ID NO: 4; SEQ ID NO: 6; SEQ ID NO: 15, SEQ ID NO: 12, and SEQ ID NO: 16.

[00116] In still another aspect, the method comprises administering to a subject a composition comprising a fusion polypeptide that comprises an amino acid sequence that is at

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least 90% identical to the amino acid sequence listed in SEQ ID NO: 12 or SEQ ID NO: 16 with the proviso that the amino acids at positions 286 and 287 of SEQ ID NO: 12 or SEQ ID NO: 16 are substitutable such that the amino acid sequence at positions 285-287 of SEQ ID NO: 12 or SEQ ID NO: 16 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

[00117] In still another aspect, the method comprises administering to a subject a composition comprising a fusion polypeptide that comprises an amino acid sequence that is at least 90% identical to the amino acid sequence listed in SEQ ID NO: 12 or SEQ ID NO: 16 with the proviso that the amino acid sequence at positions 285-287 of SEQ ID NO: 12 or SEQ ID NO: 16 is unsubstituted and remains NDT.

[00118] In still another aspect, the method comprises administering to a subject a composition comprising a fusion polypeptide that comprises an amino acid sequence that is at least 95% identical to the amino acid sequence listed in SEQ ID NO: 12 or SEQ ID NO: 16 with the proviso that the amino acids at positions 286 and 287 of SEQ ID NO: 12 or SEQ ID NO: 16 are substitutable such that the amino acid sequence at positions 285-287 of SEQ ID NO: 12 or SEQ ID NO: 16 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

[00119] In still another aspect, the method comprises administering to a subject a composition comprising a fusion polypeptide that comprises an amino acid sequence that is at least 95% identical to the amino acid sequence listed in SEQ ID NO: 12 or SEQ ID NO: 16 with the proviso that the amino acid sequence at positions 285-287 of SEQ ID NO: 12 or SEQ ID NO: 16 is unsubstituted and remains NDT.

[00120] In still another aspect, said ocular disease, condition, or disorder is selected from the group consisting of: choroidal neovascularization (including myopic choroidal neovascularization), polypoidal choroidal vasculopathy, retinal neovascularization, vascular leak, retinal edema, diabetic macular edema, macular edema caused by a condition selected from the group consisting of retinal vein occlusion and non-infectious posterior uveitis, diabetic retinopathy, corneal neovascularization, corneal inflammation, myopic neovascularization,

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neovascular glaucoma, and neovascular age-related macular degeneration (wet age-related macular degeneration resulting from retinal neovascularization).

[00121] In another aspect, the present invention provides a use of a fusion polypeptide for a preparation or manufacture of a medicament for treating or controlling at least an ocular disease, condition, or disorder, in a subject, which has etiology in aberrant angiogenesis; wherein the fusion polypeptide comprises an amino acid sequence selected from the group consisting of: SEQ ID NO: 2; SEQ ID NO: 4; SEQ ID NO: 6; SEQ ID NO: 15, SEQ ID NO: 12, and SEQ ID NO: 16.

[00122] In still another aspect, the present invention provides a use of a fusion polypeptide for the preparation or manufacture of a medicament for treating or controlling at least an ocular disease, condition, or disorder, in a subject, which has etiology in aberrant angiogenesis; wherein the fusion polypeptide comprises an amino acid sequence that is at least 90% identical to the amino acid sequence listed in SEQ ID NO: 12 or SEQ ID NO: 16 with the proviso that the amino acids at positions 286 and 287 of SEQ ID NO: 12 or SEQ ID NO: 16 are substitutable such that the amino acid sequence at positions 285-287 of SEQ ID NO: 12 or SEQ ID NO: 16 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

[00123] In still another aspect, the fusion polypeptide for said use comprises an amino acid sequence that is at least 90% identical to the amino acid sequence listed in SEQ ID NO: 12 or SEQ ID NO: 16 with the proviso that the amino acid sequence at positions 285-287 of SEQ ID NO: 12 or SEQ ID NO: 16 is unsubstituted and remains NDT.

[00124] In still another aspect, the present invention provides a use of a fusion polypeptide for the preparation or manufacture of a medicament for treating or controlling at least an ocular disease, condition, or disorder, in a subject, which has etiology in aberrant angiogenesis; wherein the fusion polypeptide comprises an amino acid sequence that is at least 95% identical to the amino acid sequence listed in SEQ ID NO: 12 or SEQ ID NO: 16 with the proviso that the amino acids at positions 286 and 287 of SEQ ID NO: 12 or SEQ ID NO: 16 are substitutable such that the amino acid sequence at positions 285-287 of SEQ ID NO: 12 or SEQ ID NO: 16 is NDT,

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NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

[00125] In still another aspect, the fusion polypeptide for said use comprises an amino acid sequence that is at least 95% identical to the amino acid sequence listed in SEQ ID NO: 12 or SEQ ID NO: 16 with the proviso that the amino acid sequence at positions 285-287 of SEQ ID NO: 12 or SEQ ID NO: 16 is unsubstituted and remains NDT.

[00126] In still another aspect, said ocular disease, condition, or disorder is selected from the group consisting of the diseases, conditions, or disorders herein disclosed.

[00127] In one embodiment, the subject is administered with a dose of about 25-4000 micrograms of the fusion polypeptide. In another embodiment, the subject is administered with a dose of about 50-4000, about 100-4000, about 500-4000, about 1000-4000, about 2000-4000, about 50-3000, about 50-2000, about 50-1000, or about 50-500 micrograms of the fusion polypeptide.

[00128] In still another aspect, the composition comprising the fusion polypeptide is in the form of an eye drop or an ocular injection (such as intravitreal, intracameral, peri-orbital, subtenon, subretinal, or suprachoroidal injection). Such a composition comprises an ophthalmic composition. A fusion polypeptide of the present invention may also be incorporated in a medical device that is implantable into or near a diseased tissue.

[00129] In one embodiment, for treating or controlling an anterior-disease, condition, or disorder; such as corneal neovascularization, corneal inflammation, or neovascular glaucoma, the composition comprising the fusion polypeptide may be in the form of an eye drop or intracameral or subconjunctival injection. In another embodiment, for treating or controlling a posterior-segment disease, condition, or disorder; such as choroidal neovascularization (including myopic choroidal neovascularization), neovascular age-related macular degeneration, vascular leak, retinal edema, or diabetic retinopathy, the composition comprising the fusion polypeptide may be in the form of an intravitreal injection.

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[00130] In yet another aspect, an eye drop is administered to the subject at least once per day, at least once per week, or at least once per month until the disease, condition, or disorder is substantially treated or controlled.

[00131] In yet another aspect, the composition is administered to the subject for a period of at least one month, at least two months, at least three months, or at least six months.

[00132] In still another aspect, an intravitreal injection or an injection into, or near, a diseased tissue is administered to the subject according to a regimen recommended by a medical practitioner for a particular patient. For example, an injection may be administered at least once per month, at least once every two months, at least once every three months, or at least once every six months until the disease, condition, or disorder is substantially treated or controlled. In one embodiment, treatment may be administered more frequently at the beginning, and then less frequently after a period of time, as may be determined by a medical practitioner.

[00133] The concentration of a fusion polypeptide of the present invention in such an ophthalmic composition can be in the range from about 0.1 to about 200 mg/ml (or, alternatively, from about 0.25 to about 200 mg/ml, or from about 0.25 to about 160 mg/ml, or from about 0.5 to about 100 mg/ml, or from about 0.25 to about 50 mg/ml, or from about 0.5 to about 200 mg/ml, or from about 0.5 to about 160 mg/ml, or from about 0.5 to about 100 mg/ml, or from about 0.5 to about 50 mg/ml, or from about 1 to about 200 mg/ml, or from 1 to about 160 mg/ml, or from about 0.5 to about 100 mg/ml, or from about 1 to about 50 mg/ml).

[00134] In still another aspect, a method for preparing a composition of the present invention comprises combining: (a) an amount of a fusion polypeptide of the present invention; and (b) a physiologically acceptable carrier.

[00135] In one embodiment, such a physiologically acceptable carrier can be a sterile saline solution or a physiologically acceptable buffer. In another embodiment, such a carrier comprises a hydrophobic medium, such as a pharmaceutically acceptable oil. In still another embodiment, such as carrier comprises an emulsion of a hydrophobic material and water. In yet another

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embodiment, a fusion polypeptide of the present invention may be associated or linked with a high-molecular weight material to provide a long circulation time.

[00136] Physiologically acceptable buffers include, but are not limited to, a phosphate buffer or a Tris-HCl buffer (comprising tris(hydroxymethyl)aminomethane and HCl). For example, a Tris-HCl buffer having pH of 7.4 comprises 3 g/l of tris(hydroxymethyl)aminomethane and 0.76 g/l of HCl. In yet another aspect, the buffer is 10X phosphate buffer saline ("PBS") or 5X PBS solution.

[00137] Other buffers also may be found suitable or desirable in some circumstances, such as buffers based on HEPES (N-{2-hydroxyethyl}peperazine-N'-{2-ethanesulfonic acid}) having pK_a of 7.5 at 25 °C and pH in the range of about 6.8-8.2; BES (N,N-bis{2-hydroxyethyl}2-aminoethanesulfonic acid) having pK_a of 7.1 at 25°C and pH in the range of about 6.4-7.8; MOPS (3-{N-morpholino}propanesulfonic acid) having pK_a of 7.2 at 25°C and pH in the range of about 6.5-7.9; TES (N-tris{hydroxymethyl}-methyl-2-aminoethanesulfonic acid) having pK_a of 7.4 at 25°C and pH in the range of about 6.8-8.2; MOBS (4-{N-morpholino}butanesulfonic acid) having pK_a of 7.6 at 25°C and pH in the range of about 6.9-8.3; DIPSO (3-(N,N-bis{2-hydroxyethyl}amino)-2-hydroxypropane)) having pK_a of 7.52 at 25°C and pH in the range of about 7-8.2; TAPSO (2-hydroxy-3{tris(hydroxymethyl)methylamino}-1-propanesulfonic acid)) having pK_a of 7.61 at 25°C and pH in the range of about 7-8.2; TAPS ({(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino}-1-propanesulfonic acid)) having pK_a of 8.4 at 25°C and pH in the range of about 7.7-9.1; TABS (N-tris(hydroxymethyl)methyl-4-aminobutanesulfonic acid) having pK_a of 8.9 at 25°C and pH in the range of about 8.2-9.6; AMPSO (N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid)) having pK_a of 9.0 at 25°C and pH in the range of about 8.3-9.7; CHES (2-cyclohexylamino)ethanesulfonic acid) having pK_a of 9.5 at 25°C and pH in the range of about 8.6-10.0; CAPSO (3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid) having pK_a of 9.6 at 25°C and pH in the range of about 8.9-10.3; or CAPS (3-(cyclohexylamino)-1-propane sulfonic acid) having pK_a of 10.4 at 25°C and pH in the range of about 9.7-11.1.

[00138] In certain embodiments, a composition of the present invention is formulated in a buffer having an acidic pH, such as from about 4 to about 6.8, or alternatively, from about 5 to

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about 6.8. In such embodiments, the buffer capacity of the composition desirably allows the composition to come rapidly to a physiological pH after being administered into the patient.

[00139] In addition to a buffer, a composition of the present invention can comprise a material selected from the group consisting of surfactants, stabilizers, preservatives, co-solvent, humectants, emollients, chelating agents, tonicity-adjusting agents, and antioxidants.

[00140] In one aspect, any of these materials that may be used in a composition of the present invention is an ophthalmically acceptable material.

[00141] Water-soluble preservatives that may be employed include sodium bisulfite, sodium bisulfate, sodium thiosulfate, benzalkonium chloride, chlorobutanol, thimerosal, ethyl alcohol, methylparaben, polyvinyl alcohol, benzyl alcohol, and phenylethyl alcohol. These agents may be present in individual amounts of from about 0.001 to about 5% by weight (preferably, about 0.01% to about 2% by weight). Suitable water-soluble buffering agents that may be employed are sodium carbonate, sodium borate, sodium phosphate, sodium acetate, sodium bicarbonate, etc., as approved by the United States Food and Drug Administration ("US FDA") for the desired route of administration.

[00142] Non-limiting examples of surfactants include, but are not limited to, non-ionic surfactants, for example, polysorbates (such as polysorbate 20, polysorbate 80), 4-(1,1,3,3-tetramethylbutyl)phenol/poly(oxyethylene) polymers (such as the polymer sold under the trademark Tyloxapol), poly(oxyethylene)-poly(oxypropylene) block copolymers, glycolic esters of fatty acids and the like, and mixtures thereof.

[00143] In one aspect, the pH of the composition is in the range from about 4 to about 11. Alternatively, the pH of the composition is in the range from about 6 to about 8, or from about 6.5 to about 8.

[00144] In another aspect, the composition has a pH of about 7. Alternatively, the composition has a pH in a range from about 7 to about 7.5.

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[00145] In still another aspect, the composition has a pH of about 7.4.

[00146] In yet another aspect, a composition also can comprise a viscosity-modifying compound designed to facilitate the administration of the composition into the subject or to promote the bioavailability in the subject. In still another aspect, the viscosity-modifying compound may be chosen so that the composition is not readily dispersed after being administered into an environment of an eye. Such compounds may enhance the viscosity of the composition, and include, but are not limited to: monomeric polyols, such as, glycerol, propylene glycol, ethylene glycol; polymeric polyols, such as, polyethylene glycol; various polymers of the cellulose family, such as hydroxypropylmethyl cellulose ("HPMC"), carboxymethyl cellulose ("CMC") sodium, hydroxypropyl cellulose ("HPC"); polysaccharides, such as hyaluronic acid and its salts, chondroitin sulfate and its salts, dextrans, such as, dextran 70; water soluble proteins, such as gelatin; vinyl polymers, such as, polyvinyl alcohol, polyvinylpyrrolidone, povidone; carbomers, such as carbomer 934P, carbomer 941, carbomer 940, or carbomer 974P; and acrylic acid polymers. In general, a desired viscosity can be in the range from about 1 to about 400 centipoises ("cps") or mPa.s.

[00147] Non-limiting examples of chelating agents include ethylenediaminetetraacetic acid ("EDTA"), diethylenetriaminepentakis(methylphosphonic acid), etidronic acid, tetrasodium salt of etidronic acid (also known as "HAP").

[00148] While the buffer itself is a "tonicity-adjusting agent" and a "pH-adjusting agent" that broadly maintains the ophthalmic solution at a particular ion concentration and pH, additional "tonicity-adjusting agents" can be added to adjust the final tonicity of the solution. Such tonicity-adjusting agents are well known to those of skill in the art and include, but are not limited to, mannitol, sorbitol, dextrose, sucrose, urea, propylene glycol, and glycerin. Also, various salts, including halide salts of a monovalent cation (e.g., NaCl or KCl) can be utilized. Typically, the tonicity of a formulation of the present invention is in the range from about 200 to 400 mOsm/kg. Alternatively, the tonicity of a formulation of the present invention is in the range from about 220 to 400 mOsm/kg, or from about 220 to 350 mOsm/kg, or from about 220 to 300 mOsm/kg, or from about 250 to 350 mOsm/kg.

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[00149] Non-limiting examples of anti-oxidants include ascorbic acid (vitamin C) and its salts and esters; tocopherols (such as α-tocopherol) and tocotrienols (vitamin E), and their salts and esters (such as vitamin E TGPS (D-α-tocopheryl polyethylene glycol 1000 succinate)); glutathione; lipoic acid; uric acid; butylated hydroxyanisole ("BHA"); butylated hydroxytoluene ("BHT"); tertiary butylhydroquinone ("TBHQ"); and polyphenolic anti-oxidants (such as gallic acid, cinnanmic acid, flavonoids, and their salts, esters, and derivatives).

[00150] Non-limiting examples of stabilizers includes sucrose, mannitol, sorbitol, and trehalose.

[00151] It should be understood that the proportions of the various components or mixtures in the following examples may be adjusted for the appropriate circumstances.

[00152] In another aspect, a fusion polypeptide of the present invention and appropriate amounts of one or more desired excipients are incorporated into a formulation for topical administration or injection to a portion of the eye, such as the anterior or posterior segment, or the vitreous humor. An injectable formulation can desirably comprise a carrier that provides a sustained-release of the active ingredients, such as for a period longer than about 1 week (or longer than about 1, 2, 3, 4, 5, or 6 months).

[00153] In still another aspect, a composition comprising a fusion polypeptide of the present invention and desired excipients is lyophilized and is reconstituted with a physiologically acceptable liquid carrier substantially immediately before administration to a subject.

[00154] In one embodiment, a compound or composition of the present invention can be injected with a fine-gauge needle, such as 25-35 gauge. Typically, an amount from about 25 μl to about 100 μl of a composition comprising about 25-4000 μg of a fusion polypeptide of the present invention is administered into a patient. In one embodiment, the fusion polypeptide has an amino acid sequence that is substantially the same as that listed in SEQ ID NO:12 or SEQ ID NO: 16 with the proviso that the amino acids at positions 286 and 287 of SEQ ID NO: 12 or SEQ ID NO: 16 are substitutable such that the amino acid sequence at positions 285-287 of SEQ ID NO: 12 or SEQ ID NO: 16 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected

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from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V. In another embodiment, the fusion polypeptide has an amino acid sequence as listed in SEQ ID NO:12 or SEQ ID NO: 16. A concentration of such fusion polypeptide is selected from the ranges disclosed above.

[00155] In still another aspect, a fusion polypeptide of the present invention is incorporated into an ophthalmic device that comprises a biodegradable material, and the device is implanted into a posterior-segment tissue of a subject to provide a long-term (e.g., longer than about 1 week, or longer than about 1, 2, 3, 4, 5, or 6 months) treatment or control of an angiogenic disease, condition, or disorder. Such a device may be implanted by a skilled physician in the subject's ocular or periocular tissue. Non-limiting examples of ophthalmic implant systems or devices for the sustained-release of an active ingredient are disclosed in U.S. Patents 5,378,475; 5,773,019; 5,902,598; 6,001,386; 6,051,576; and 6,726,918.

[00156] In still another aspect, a method for treating or controlling an ophthalmic angiogenic disease, condition, or disorder comprises administering a composition comprising a fusion polypeptide of the present invention to a subject in need thereof.

[00157] In still another aspect, a method for treating or controlling an ophthalmic angiogenic disease, condition, or disorder comprises administering a composition comprising a fusion polypeptide having an amino acid sequence as listed in SEQ ID NO:12 or SEQ ID NO: 16 to a subject in need thereof.

[00158] In still another aspect, a method for treating or controlling an ophthalmic angiogenic disease, condition, or disorder having an etiology in aberrant angiogenesis of in the posterior segment of an eye comprises intravitreally injecting a composition comprising a fusion polypeptide having an amino acid sequence as listed in SEQ ID NO:12 or SEQ ID NO: 16 in a subject in need thereof.

[00159] In another embodiment, such disease, condition, or disorder is selected from the group consisting of choroidal neovascularization, polypoidal choroidal vasculopathy, myopic neovascularization, retinal neovascularization including retinopathy of prematurity, vascular leak,

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retinal edema including diabetic macular edema and macular edema caused by other retinal conditions such as retinal vein occlusion and non-infectious posterior uveitis, diabetic retinopathy including proliferative diabetic retinopathy, corneal neovascularization, and neovascular glaucoma

[00160] In yet another aspect, a composition of the present invention is administered once a week, once a month, once a year, twice a year, four times a year, or at a suitable frequency that is determined to be appropriate for treating or controlling an anterior-segment inflammatory disease, condition, or disorder.

[00161] In still another aspect, a fusion polypeptide or protein of the present invention can also be used for the treatment or control of other non-ocular diseases or conditions that are promoted by, or have etiology in, aberrant angiogenesis, such as cancers, psoriasis, rheumatoid arthritis, and atherosclerosis. Such treatment or control may be effected by, for example, systemic administration.

[00162] In yet another aspect, the present invention provides a use of a fusion polypeptide or protein for the preparation or manufacture of a medicament for treating or controlling of non-ocular diseases or conditions that are promoted by, or have etiology in, aberrant angiogenesis, such as cancers, psoriasis, rheumatoid arthritis, and atherosclerosis.

[00163] The cancer amenable for treatment by the present invention include, but not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer, lung cancer (including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung), cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer (including gastrointestinal cancer), pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL), small lymphocytic (SL) NHL, intermediate grade/follicular NHL,

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intermediate grade diffuse NHL, high grade immunoblastic NHL, high grade lymphoblastic NHL, high grade small non-cleaved cell NHL, bulky disease NHL, mantle cell lymphoma, AIDS-related lymphoma, and Waldenstrom's Macroglobulinemia), chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), Hairy cell leukemia, chronic myeloblastic leukemia, and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome. In some embodiments, the cancer is selected from the group consisting of breast cancer, colorectal cancer, rectal cancer, non-small cell lung cancer, non-Hodgkins lymphoma (NHL), renal cell cancer, prostate cancer, liver cancer, pancreatic cancer, soft-tissue sarcoma, kaposi's sarcoma, carcinoid carcinoma, head and neck cancer, melanoma, ovarian cancer, mesothelioma, and multiple myeloma. In another embodiment, the cancer is colorectal cancer. The cancerous conditions amenable for treatment of the invention include metastatic cancers. The method of the present invention is particularly suitable for the treatment of vascularized tumors.

EXAMPLE 1: Composition 1

[00164] A composition of the present invention is prepared by combining 10-100 mg/mL of the fusion protein EB-101, 10 mM histidine HCl buffer, 10% (by weight) α,α-trehalose, and 0.01% (by weight) polysorbate 20. In some embodiments, the composition may also include a material selected from the group consisting of an antioxidant, a chelating agent, and a preservative, and combinations thereof. The composition has a pH in the range of about 5-6 and is suitable for intraocular, including intravitreal, administration.

EXAMPLE 2: Composition 2

[00165] A composition of the present invention is prepared by combining 10-100 mg/mL of the fusion protein EB-101, 10 mM phosphate buffer, 40 mM NaCl, 5% (by weight) sucrose, and 0.03% (by weight) polysorbate 20. The composition has a pH in the range of about 5.5-6.5 and is suitable for intraocular, including intravitreal, administration.

EAMPLE 3: Transient expression of EB-101 in HEK293 cells

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[00166] Gene encoding the amino acid sequence of EB-101 as indicated by the SEQ ID NO: 12 was synthetized and a vector for expressing the protein was constructed. The expression vector was used to transiently transfect 293 cells with chemically defined culture media. The produced protein was purified by Protein A affinity column, ultrafiltration and then subjected to 0.2μm sterile filtration to get the bulk of high purity. Expression of EB-101 in HEK293 cells produced a protein with a MW ~200KDa (non-reduced form at SDS-PAGE) and a purity of 96% when analyzed by size exclusion -chromatography (SEC-HPLC). See Fig. 2.

EXAMPLE 4: Transient expression of EB-101 in CHO cells.

[00167] Gene encoding the amino acid sequence of EB-101 as indicated by the SEQ ID NO: 12 was synthetized and a vector for expressing the protein was constructed. The expression vector was used to transiently transfect CHO cells with chemically defined culture media. The produced protein was purified by Protein A affinity column, ultrafiltration and then subjected to 0.2μm sterile filtration to get the bulk of high purity. Expression of EB-101 in CHO cells produced a protein with a MW ~200KDa (non-reduced form at SDS-PAGE) and a purity of 100% when analyzed by SEC-HPLC. See Fig. 3.

[00168] EXAMPLE 5: Comparison of the binding affinity of EB-101 with aflibercept for human recombinant human VEGF-A_{165}, VEGF-$B_{167/189}$, VEGF-C, VEGF-D, and PlGF using SPR Biacore. See Figures 4-8.

[00169] SPR Biacore assays were performed by immobilization of the anti-human IgG (Fc) antibody onto the CM5 sensor chip surface and determined the level of ligand immobilization. The amount of anti-Fc antibody coupling onto the CM5 sensor chip was about 7,000~14,000 response unit ("RU"). Then, EB-101 or aflibercept were injected into the sample channel to reach a capture level of about 400 RU. Finally, different concentrations of the analyte (VEGF-A in this assay) diluted in running buffer were injected for affinity and kinetics measurements. The 1:1 binding model was used to measure the binding affinity and/or kinetics. The kinetic constants of VEGF-A_{165} binding to EB-101 were as follows: equilibrium dissociation constant $K_D = 7.51 \times 10^{-12}$ M (7.51 pM), association rate constant $K_a = 5.06 \text{ X } 10^7 \text{ M}^{-1}\text{s}^{-1}$, dissociation rate

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constant $K_d = 3.64 \times 10^{-4}\ s^{-1}$. The kinetic constants of VEGF-A_{165} binding to aflibercept were as follows: equilibrium dissociation constant $K_D = 3.36 \times 10^{-11}$ M (33.6 pM), association rate constant $K_a = 1.36 \times 10^{7}\ M^{-1}s^{-1}$, dissociation rate constant $K_d = 4.58 \times 10^{-4}\ s^{-1}$.

[00170] The kinetic constants of VEGF-$B_{167/189}$ binding to EB-101 were as follows: equilibrium dissociation constant $K_D = 1.38 \times 10^{-10}$ M (138 pM), association rate constant $K_a = 2.20 \times 10^{6}\ M^{-1}s^{-1}$, dissociation rate constant $K_d = 3.03 \times 10^{-4}\ s^{-1}$. The kinetic constants of VEGF-$B_{167/189}$ binding to aflibercept were as follows: equilibrium dissociation constant $K_D = 1.73 \times 10^{-11}$ M (17.3 pM), association rate constant $K_a = 3.27 \times 10^{6}\ M^{-1}s^{-1}$, dissociation rate constant $K_d = 5.67 \times 10^{-5}\ s^{-1}$.

[00171] The kinetic constants of VEGF-C binding to EB-101 were as follows: equilibrium dissociation constant $K_D = 4.64 \times 10^{-10}$ M (0.464 nM), association rate constant $K_a = 3.11 \times 10^{6}\ M^{-1}s^{-1}$, dissociation rate constant $K_d = 1.44 \times 10^{-3}\ s^{-1}$. The binding between VEGF-C and aflibercept cannot be detected.

[00172] The binding between VEGF-D and EB-101 or aflibercept cannot be detected.

[00173] The kinetic constants of PlGF binding to EB-101 were as follows: equilibrium dissociation constant $K_D = 2.09 \times 10^{-10}$ M (209 pM), association rate constant $K_a = 1.68 \times 10^{7}\ M^{-1}s^{-1}$, dissociation rate constant $K_d = 3.15 \times 10^{-3}\ s^{-1}$. The kinetic constants of PlGF binding to aflibercept were as follows: equilibrium dissociation constant $K_D = 4.84 \times 10^{-10}$ M (484 pM), association rate constant $K_a = 1.26 \times 10^{6}\ M^{-1}s^{-1}$, dissociation rate constant $K_d = 6.10 \times 10^{-4}\ s^{-1}$.

TABLE 1

Comparison of the binding affinities of EB-101 and aflibercept for VEGF-A_{165}, VEGF-$B_{167/189}$, VEGF-C, VEGF-D, and PlGF using SPR Biacore

K_D (SPR Biacore)	VEGFA_{165}	VEGF-$B_{167/189}$	VEGF-C	VEGF-D	PlGF
EB-101	7.51 pM	0.14 nM	0.46 nM	no binding	0.21 nM
Aflibercept	33.60 pM	17.30 pM	no binding	no binding	0.48 nM

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[00174] Thus, EB-101 binding to these VEGF family members compares more favorably than aflibercept.

EXAMPLE 6: Comparison of the effect of EB-101 with aflibercept and bevacizumab on inhibition of VEGF-A_{165} mediated VEGFR-2 signaling in VEGFR-2-NFAT-RE luciferase reporter cells.

[00175] Recombinant human VEGF-A_{165} were mixed with serial dilutions of the test compound, incubated at room temperature for 30 minutes, and then added into wells containing 4 x10^4 VEGFR-2 (KDR)-NFAT-RE luciferase reporter cells/well followed by an incubation at 37°C for 6 hours. Luciferase signal was detected by a plate reader after addition of 50 µl of Bright-Lite. The effect of EB-101 on inhibiting VEGF-A_{165} mediated VEGFR-2 signaling was comparable to that inhibited by aflibercept or bevacizumab. See Fig. 9.

EXAMPLE 7: Comparison of the effect of EB-101 with aflibercept on inhibition of VEGF-C mediated VEGFR2 signaling in VEGFR2-NFAT-RE luciferase reporter cells.

[00176] Recombinant human VEGF-C were mixed with serial dilutions of the test compound, incubated at room temperature for 30 minutes, and then added into wells containing 4 x10^4 VEGFR-2 (KDR)-NFAT-RE luciferase reporter cells/well followed by an incubation at 37°C for 6 hours. Luciferase signal was detected by a plate reader after addition of 50 µl of Bright-Lite. EB-101, but not aflibercept, had concentration-dependent inhibition of VEGF-C mediated VEGFR-2 signaling. See Fig. 10.

EXAMPLE 8: Comparison of EB-101 with aflibercept and a recombinant human VEGFR-3 containing 7 extracellular domains (rhVEGFR-3-7ECDs) on their inhibitory effect on VEGF-C mediated VEGFR-2 signaling in VEGFR-2-NFAT-RE luciferase reporter cells.

[00177] Recombinant human VEGF-C were mixed with serial dilutions of the test compound, incubated at room temperature for 30 minutes, and then added into wells containing 4 x10^4 VEGFR-2 (KDR)-NFAT-RE luciferase reporter cells/well followed by an incubation at 37°C for 6 hours. Luciferase signal was detected by a plate reader after addition of 50 µl of Bright-Lite.

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EB-101 and rhVEGFR-3, but not aflibercept, had dose-dependent inhibition of VEGF-C mediated VEGFR-2 signaling. See Fig. 11.

EXAMPLE 9: Quantitative analyses of the effect of EB-101 with aflibercept and a recombinant human VEGFR-3 containing 7 extracellular domains (rhVEGFR-3) on inhibition of VEGFR-2 signaling in VEGFR-2-NFAT-RE luciferase reporter cells stimulated by VEGF-A_{165} and VEGF-C.

[00178] The test compound was mixed with a cocktail containing 60 ng/ml of VEGF-A_{165} and 25 ng/ml of VEGF-C and incubated at room temperature for 30 minutes and then the resultant solution was added into wells containing 4 $x10^4$ VEGFR-2-NFAT-RE luciferase reporter cells/well, followed by an incubation at 37°C for 6 hours. Luciferase signal was detected by a plate reader after addition of 50 µl of Bright-Lite. EB-101 and a combination of aflibercept with rhVEGFR-3 effectively inhibited VEGF-A_{165} plus VEGF-C mediated VEGFR-2 signaling while either aflibercept or rhVEGFR3 alone showed only limited effect on inhibition of VEGF-A_{165} and VEGF-C-mediated VEGFR-2 signaling. See Fig. 12.

EXAMPLE 10: Comparison of the effect of EB-101 with aflibercept and bevacizumab on VEGF-A_{165}-mediated proliferation of human lymphatic endothelial cells (HLECs).

[00179] Primary HLECs were seeded into 96-well plates at a density of 2500 cells/well and cultured at 37°C for overnight. On the next day, recombinant human VEGF-A_{165} was mixed with serial dilutions of the test compound and incubate at room temperature for 30 minutes, and then the resultant solution was added into each well followed by an incubation at 37°C for 72 hours. Cell proliferation was evaluated by addition of 10% AlamarBlue dye into each well and incubated for 5 hours followed by fluorescent reading using a plate reader. The effect of EB-101 on inhibiting VEGF-A_{165}-mediated HLEC proliferation was comparable to that inhibited by aflibercept and bevacizumab. See Fig. 13.

[00180] EXAMPLE 11: Comparison of the effect of EB-101 with aflibercept and a recombinant human VEGFR-3 containing 7 extracellular domains (rhVEGFR-3 ECDs) on VEGF-C mediated HLEC proliferation.

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[00181] Primary HLECs were seeded into 96-well plates at a density of 2500 cells/well and cultured in an incubator at 37°C for overnight. On the next day, recombinant human VEGF-C were mixed with serial dilutions of the test compound and incubate at room temperature for 30 minutes, and then the resultant solution was added into each well followed by an incubation at 37°C for 72 hours. Cell proliferation was evaluated by addition of 10% AlamarBlue dye into each well and incubated for 5 hours followed by fluorescent reading using a plate reader. EB-101 and rhVEGFR-3, but not aflibercept, showed concentration-dependent inhibition of VEGF-C mediated HLEC proliferation. See Fig. 14.

EXAMPLE 12: Quantitative analyses of EB-101, aflibercept and a recombinant human VEGFR-3 containing 7 extracellular domains (rhVEGFR-3 ECDs), on VEGF-A_{165} and VEGF-C mediated human lymphatic endothelial cell (HLEC) proliferation.

[00182] Primary HLECs were seeded into 96-well plates at a density of 2500 cells/well and cultured in an incubator at 37°C for overnight. On the next day, a cocktail containing 60 ng/ml of VEGF-A_{165} and 25 ng/ml of VEGF-C were mixed with the test compound and incubated at room temperature for 30 minutes, and then the resultant solution was added into wells followed by an incubation at 37°C for 72 hours. Cell proliferation was evaluated by addition of 10% AlamarBlue dye and incubated for 5 hours followed by fluorescent reading using a plate reader. EB-101 and the combination of aflibercept with rhVEGFR-3 effectively inhibited VEGF-A_{165} and VEGF-C mediated VEGFR2 signaling, while either aflibercept or rhVEGFR3 alone had only limited inhibition in VEGF-A_{165} and VEGF-C mediated HLEC proliferation. See Fig. 15.

EXAMPLE 13: Molecular design and expression of EB-101BIb.

[00183] EB-101BIb is a recombinant human Fc fusion protein including the binding domain 2 of VEGFR-1, domain 3 of VEGFR-2, and domains 1-3 of VEGFR-3. Gene encoding the amino acid sequence of EB-101BIb as indicated by the SEQ ID NO: 16 was synthetized and a vector for expressing the protein was constructed. The expression vector was used to transiently transfect CHO cells with chemically defined culture media. The produced protein was purified by Protein A affinity column, ultrafiltration and then subjected to 0.2μm sterile filtration to get the bulk of high purity. Expression of EB-101BIb in CHO cells produced a protein with a MW

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~200KDa (non-reduced form at SDS-PAGE) and a purity of 88% when analyzed by SEC-HPLC. See Fig. 16.

EXAMPLE 14: Comparison of the effect of EB-101BIb with aflibercept, EB-101, EB-101BIa on VEGF-A mediated VEGFR-2 signaling in VEGFR2-NFAT-RE luciferase reporter cells.

[00184] Recombinant human VEGF-A_{165} were mixed with serial dilutions of the test compound, incubated at room temperature for 30 minutes, and then added into wells containing 4 x10^4 VEGFR2 (KDR)-NFAT-RE luciferase reporter cells/well followed by an incubation at 37°C for 6 hours. Luciferase signal was detected by a plate reader after addition of 50 µl of Bright-Lite. The effect of EB-101BIb on inhibiting VEGF-A_{165} mediated VEGFR-2 signaling was comparable to that inhibited by aflibercept, EB-101 and EB-101BIa. The latter two molecules contain binding domain 2 of VEGFR-1, domain 3 of VEGFR-2 and domains 1-2 but not domain 3 of VEGFR-3 and were expressed in HEK293 (EB-101) and CHO (EB-101BIa) cells, respectively. See Fig. 17.

EXAMPLE 15: Comparison of the effect of EB-101BIb with aflibercept, EB-101 and EB-101BIa on VEGF-C and -D mediated VEGFR-2 signaling in VEGFR-2-NFAT-RE luciferase reporter cells.

[00185] Recombinant human VEGF-C and -D were mixed with serial dilutions of the test compound, incubated at room temperature for 30 minutes, and then added into wells containing 4 x10^4 VEGFR-2 (KDR)-NFAT-RE luciferase reporter cells/well followed by an incubation at 37°C for 6 hours. Luciferase signal was detected by a plate reader after addition of 50 µl of Bright-Lite. EB-101BIb showed a concentration-dependent inhibition of VEGF-C and -D mediated VEGFR-2 signaling, while EB-101 and EB-101BIa showed limited inhibition of VEGF-C and -D mediated VEGFR-2 signaling. Aflibercept showed no effect on VEGF-C and -D mediated VEGFR-2 signaling. EB-101 and EB-101BIa contain the binding domain 2 of VEGFR-1, domain 3 of VEGFR-2 and domains 1-2 but not domain 3 of VEGFR-3 and were expressed in HEK293 (EB-101) and CHO (EB-101BIa) cells, respectively. See Fig. 18.

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EXAMPLE 16: Comparison of the effect of EB-101BIb with aflibercept, EB-101 and a combination of aflibercept with a recombinant human VEGFR-3 on VEGF-A, -C and -D mediated VEGFR-2 signaling in VEGFR-2-NFAT-RE luciferase reporter cells.

[00186] The test compound was mixed with a cocktail containing 60 ng/ml of VEGF-A_{165} and 25 ng/ml of VEGF-C and 150 ng/ml of VEGF-D followed by incubation at room temperature for 30 minutes, and then the resultant solution was added into wells containing 4 x10^4 VEGFR-2-NFAT-RE luciferase reporter cells/well, followed by an incubation at 37°C for 6 hours. Luciferase signal was detected by a plate reader after addition of 50 µl of Bright-Lite. Aflibercept showed little inhibition of VEGFR-2 signaling while EB-101BIb showed concentration-dependent inhibition of VEGFR-2 signaling in VEGFR-2-NFAT-RE luciferase reporter cells stimulated by a cocktail consisting of VEGF-A_{165}, -C and -D (Fig. 19 A). EB-101BIb completely inhibited VEGF-A_{165}, VEGF-C and VEGF-D mediated VEGFR-2 signaling. EB-101 showed limited inhibitory effect while aflibercept had the least inhibition of VEGF-A, -C, and -D mediated VEGFR-2 signaling compared with EB-101BIb and EB-101. The complete inhibition effected EB-101BIb was replicated by a combination of EB-101 with the recombinant human VEGFR-3 (Fig. 19 B). EB-101 contains the binding domain 2 of VEGFR-1, domain 3 of VEGFR-2 and domains 1-2 but not domain 3 of VEGFR-3. EB-101BIb contains the binding domain 2 of VEGFR-1, domain 3 of VEGFR-2, and domains 1-3 of VEGFR-3. See Figure 19.

EXAMPLE 17: Comparison of the effect of EB-101BIb with aflibercept and EB-101BIa on VEGF-A_{165}-mediated proliferation of human lymphatic endothelial cells (HLECs).

[00187] Primary HLECs were seeded into 96-well plates at a density of 2500 cells/well and cultured at 37°C for overnight. On the next day, recombinant human VEGF-A_{165} was mixed with serial dilutions of the test compound and incubate at room temperature for 30 minutes, and then the resultant solution was added into each well followed by an incubation at 37°C for 72 hours. Cell proliferation was evaluated by addition of 10% AlamarBlue dye into each well and incubated for 5 hours followed by fluorescent reading using a plate reader. The effect of EB-101BIb on inhibiting VEGF-A_{165}-mediated HLEC proliferation was comparable to that inhibited by aflibercept and EB-101BIa. See Fig. 20.

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EXAMPLE 18: Comparison of the effect of EB-101BIb with aflibercept, EB-101BIa and a recombinant human VEGFR-3 containing 7 extracellular domains on VEGF-C and VEGF-D mediated HLEC proliferation.

[00188] Primary HLECs were seeded into 96-well plates at a density of 2500 cells/well and cultured in an incubator at 37°C for overnight. On the next day, recombinant human VEGF-C (25 ng/ml) and VEGF-D (150 ng/ml) were mixed with serial dilutions of the test compound and incubated at room temperature for 30 minutes, and then the resultant solution was added into each well followed by an incubation at 37°C for 72 hours. Cell proliferation was evaluated by addition of 10% AlamarBlue dye into each well and incubated for 5 hours followed by fluorescent reading using a plate reader. EB-101BIb and rhVEGFR-3 showed more potent effect on inhibition of VEGF-C and VEGF-D mediated HLEC proliferation compared with EB-101Bia treated group. See Fig. 21.

EXAMPLE 19: Comparison of the effect of EB-101BIb with aflibercept, a recombinant human VEGFR-3 containing 7 extracellular domains (7 ECDs) and a combination of aflibercept with the VEGFR-3 on VEGF-A, -C and -D mediated human lymphatic endothelial cell (HLEC) proliferation.

[00189] Primary HLECs were seeded into 96-well plates at a density of 2500 cells/well and cultured in an incubator at 37°C for overnight. On the next day, a cocktail containing recombinant human VEGF-A_{165}, VEGF-C and VEGF-D was mixed with equal volume of serial dilutions of the test compound and incubated at room temperature for 30 minutes (final concentrations of VEGF-A_{165}, -C and -D: 60, 25 and 150 ng/ml, respectively). The resultant solutions were added into 9 wells in each group and then incubated at 37°C for 72 hours. Cell proliferation was evaluated by addition of 10% AlamarBlue dye into each well and incubated for 5 hours followed by fluorescent reading using a plate reader. Either aflibercept or VEGFR-3 alone showed limited inhibition while EB-101BIb or a combination of aflibercept with VEGFR-3 completely inhibited HLEC proliferation stimulated by VEGF-A_{165}, -C and -D. See Fig. 22.

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ESRHRQESGFSCKGPGQNVAVTRAHPDSQGRRRRPERGARGGQVFYNSEYGELSEPSEE DHCSPSARVTFFTDNSY (SEQ ID NO: 13)

[00203] YSMTPPTLNITEESHVIDTGDSLSISCRGQHPLEWAWPGAQEAPATGDKDSE DTGVVRDCEGTDARPYCKVLLLHEVHANDTGSYVCYYKYIKARIEGTTAAS (SEQ ID NO: 14)

[00204] YSMTPPTLNITEESHVIDTGDSLSISCRGQHPLEWAWPGAQEAPATGDKDSE DTGVVRDCEGTDARPYCKVLLLHEVHANDTGSYVCYYKYIKARIEGTTAASSYVFVRD FEQPFINKPDTLLVNRKDAMWVPCLVSIPGLNVTLRSQSSVLWPDGQEVVWDDRRGML VSTPLLHDALYLQCETTWGDQDFLSNPFLVHITGNELYDIQLLPRKSLELLVGEKLVLNC TVWAEFNSGVTFDWDYPGKQAERGKWVPERRSQQTHTELSSILTIHNVSQHDLGSYVC KANNGIQRFRESTEVIVHEN (SEQ ID NO: 15)

[00205] SDTGRPFVEMYSEIPEIIHMTEGRELVIPCRVTSPNITVTLKKFPLDTLIPDGKR IIWDSRKGFIISNATYKEIGLLTCEATVNGHLYKTNYLTHRQTNTIIDVVLSPSHGIELSVG EKLVLNCTARTELNVGIDFNWEYPSSKHQHKKLVNRDLKTQSGSEMKKFLSTLTIDGVT RSDQGLYTCAASSGLMTKKNSTFVRVHEKGGSGGGSGGGGSGGGGGSGGGGYSMTPP TLNITEESHVIDTGDSLSISCRGQHPLEWAWPGAQEAPATGDKDSEDTGVVRDCEGTDA RPYCKVLLLHEVHANDTGSYVCYYKYIKARIEGTTAASSYVFVRDFEQPFINKPDTLLV NRKDAMWVPCLVSIPGLNVTLRSQSSVLWPDGQEVVWDDRRGMLVSTPLLHDALYLQ CETTWGDQDFLSNPFLVHITGNELYDIQLLPRKSLELLVGEKLVLNCTVWAEFNSGVTF DWDYPGKQAERGKWVPERRSQQTHTELSSILTIHNVSQHDLGSYVCKANNGIQRFREST EVIVHENDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN NYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 16)

SEQUENCE LISTING

<110> Eluminex Biosciences Ltd.

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<120> Inhibitors of Angiogenic Factors

<130> EluminexBiosciences-01

<160> 16

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1 5 10 15

Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
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Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
35 40 45

Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
50 55 60

Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
65 70 75 80

Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg

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1 5 10 15

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20 25 30

Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu
35 40 45

Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe
50 55 60

Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu
65 70 75 80

Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr
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Phe Val Arg Val His Glu Lys
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20 25 30

Leu Glu Trp Ala Trp Pro Gly Ala Gln Glu Ala Pro Ala Thr Gly Asp
35 40 45

Lys Asp Ser Glu Asp Thr Gly Val Val Arg Asp Cys Glu Gly Thr Asp

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    50                  55                  60

Ala Arg Pro Tyr Cys Lys Val Leu Leu Leu His Glu Val His Ala Asn
65                  70                  75                  80

Asp Thr Gly Ser Tyr Val Cys Tyr Tyr Lys Tyr Ile Lys Ala Arg Ile
                85                  90                  95

Glu Gly Thr Thr Ala Ala Ser Ser Tyr Val Phe Val Arg Asp Phe Glu
            100                 105                 110

Gln Pro Phe Ile Asn Lys Pro Asp Thr Leu Leu Val Asn Arg Lys Asp
        115                 120                 125

Ala Met Trp Val Pro Cys Leu Val Ser Ile Pro Gly Leu Asn Val Thr
    130                 135                 140

Leu Arg Ser Gln Ser Ser Val Leu Trp Pro Asp Gly Gln Glu Val Val
145                 150                 155                 160

Trp Asp Asp Arg Arg Gly Met Leu Val Ser Thr Pro Leu Leu His Asp
                165                 170                 175

Ala Leu Tyr Leu Gln Cys Glu Thr Thr Trp Gly Asp Gln Asp Phe Leu
            180                 185                 190

Ser Asn Pro Phe Leu Val His Ile Thr Gly Asn Glu Leu
        195                 200                 205
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gggcagcccc gagaaccaca ggtgtacacc ctgcccccat cccgggatga gctgaccaag 420

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Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
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Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
35 40 45

Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val
50 55 60

His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr
65 70 75 80

Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly
85 90 95

Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile
100 105 110

Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val
115 120 125

Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser

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130 135 140

Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu
145 150 155 160

Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro
165 170 175

Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val
180 185 190

Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
195 200 205

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser
210 215 220

Pro Gly
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ttaaaaaagt ttccacttga cactttgatc cctgatggaa aacgcataat ctgggacagt 180

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atcatagatg tggttctgag tccgtctcat ggaattgaac tatctgttgg agaaaagctt 360

gtcttaaatt gtacagcaag aactgaacta aatgtgggga ttgacttcaa ctgggaatac 420

ccttcttcga agcatcagca taagaaactt gtaaaccgag acctaaaaac ccagtctggg 480

agtgagatga agaaattttt gagcacctta actatagatg gtgtaacccg gagtgaccaa 540

ggattgtaca cctgtgcagc atccagtggg ctgatgacca agaagaacag cacatttgtc 600

agggtccatg aaaagtactc catgaccccc ccgaccttga acatcacgga ggagtcacac 660

gtcatcgaca ccggtgacag cctgtccatc tcctgcaggg gacagcaccc cctcgagtgg 720

gcttggccag gagctcagga ggcgccagcc accggagaca aggacagcga ggacacgggg 780

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Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
20 25 30

Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
35 40 45

Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
50 55 60

Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
65 70 75 80

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Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
                85                  90                  95

Gln Thr Asn Thr Ile Ile Asp Val Val Leu Ser Pro Ser His Gly Ile
            100                 105                 110

Glu Leu Ser Val Gly Glu Lys Leu Val Leu Asn Cys Thr Ala Arg Thr
        115                 120                 125

Glu Leu Asn Val Gly Ile Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys
    130                 135                 140

His Gln His Lys Lys Leu Val Asn Arg Asp Leu Lys Thr Gln Ser Gly
145                 150                 155                 160

Ser Glu Met Lys Lys Phe Leu Ser Thr Leu Thr Ile Asp Gly Val Thr
                165                 170                 175

Arg Ser Asp Gln Gly Leu Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met
            180                 185                 190

Thr Lys Lys Asn Ser Thr Phe Val Arg Val His Glu Lys Tyr Ser Met
        195                 200                 205

Thr Pro Pro Thr Leu Asn Ile Thr Glu Glu Ser His Val Ile Asp Thr
    210                 215                 220

Gly Asp Ser Leu Ser Ile Ser Cys Arg Gly Gln His Pro Leu Glu Trp
225                 230                 235                 240

Ala Trp Pro Gly Ala Gln Glu Ala Pro Ala Thr Gly Asp Lys Asp Ser
                245                 250                 255

Glu Asp Thr Gly Val Val Arg Asp Cys Glu Gly Thr Asp Ala Arg Pro
            260                 265                 270

Tyr Cys Lys Val Leu Leu Leu His Glu Val His Ala Asn Asp Thr Gly
        275                 280                 285

Ser Tyr Val Cys Tyr Tyr Lys Tyr Ile Lys Ala Arg Ile Glu Gly Thr
    290                 295                 300
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Thr Ala Ala Ser Ser Tyr Val Phe Val Arg Asp Phe Glu Gln Pro Phe
305                 310                 315                 320

Ile Asn Lys Pro Asp Thr Leu Leu Val Asn Arg Lys Asp Ala Met Trp
                325                 330                 335

Val Pro Cys Leu Val Ser Ile Pro Gly Leu Asn Val Thr Leu Arg Ser
            340                 345                 350

Gln Ser Ser Val Leu Trp Pro Asp Gly Gln Glu Val Val Trp Asp Asp
        355                 360                 365

Arg Arg Gly Met Leu Val Ser Thr Pro Leu Leu His Asp Ala Leu Tyr
    370                 375                 380

Leu Gln Cys Glu Thr Thr Trp Gly Asp Gln Asp Phe Leu Ser Asn Pro
385                 390                 395                 400

Phe Leu Val His Ile Thr Gly Asn Glu Leu Gly Gly Ser Gly Gly Gly
                405                 410                 415

Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Gly Ser Gly Gly Gly Gly
            420                 425                 430

Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly
        435                 440                 445

Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
    450                 455                 460

Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
465                 470                 475                 480

Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val
                485                 490                 495

His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr
            500                 505                 510

Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly
        515                 520                 525
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Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile
    530                 535                 540

Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val
545                 550                 555                 560

Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser
                565                 570                 575

Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu
            580                 585                 590

Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro
        595                 600                 605

Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val
    610                 615                 620

Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
625                 630                 635                 640

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser
                645                 650                 655

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Leu Leu Asp Gly Leu Val Ser Gly Tyr Ser Met Thr Pro Pro Thr Leu
            20                  25                  30

Asn Ile Thr Glu Glu Ser His Val Ile Asp Thr Gly Asp Ser Leu Ser
        35                  40                  45
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Ile Ser Cys Arg Gly Gln His Pro Leu Glu Trp Ala Trp Pro Gly Ala
    50                  55                  60

Gln Glu Ala Pro Ala Thr Gly Asp Lys Asp Ser Glu Asp Thr Gly Val
65                  70                  75                  80

Val Arg Asp Cys Glu Gly Thr Asp Ala Arg Pro Tyr Cys Lys Val Leu
                85                  90                  95

Leu Leu His Glu Val His Ala Asn Asp Thr Gly Ser Tyr Val Cys Tyr
            100                 105                 110

Tyr Lys Tyr Ile Lys Ala Arg Ile Glu Gly Thr Thr Ala Ala Ser Ser
        115                 120                 125

Tyr Val Phe Val Arg Asp Phe Glu Gln Pro Phe Ile Asn Lys Pro Asp
    130                 135                 140

Thr Leu Leu Val Asn Arg Lys Asp Ala Met Trp Val Pro Cys Leu Val
145                 150                 155                 160

Ser Ile Pro Gly Leu Asn Val Thr Leu Arg Ser Gln Ser Ser Val Leu
                165                 170                 175

Trp Pro Asp Gly Gln Glu Val Val Trp Asp Asp Arg Arg Gly Met Leu
            180                 185                 190

Val Ser Thr Pro Leu Leu His Asp Ala Leu Tyr Leu Gln Cys Glu Thr
        195                 200                 205

Thr Trp Gly Asp Gln Asp Phe Leu Ser Asn Pro Phe Leu Val His Ile
    210                 215                 220

Thr Gly Asn Glu Leu Tyr Asp Ile Gln Leu Leu Pro Arg Lys Ser Leu
225                 230                 235                 240

Glu Leu Leu Val Gly Glu Lys Leu Val Leu Asn Cys Thr Val Trp Ala
                245                 250                 255

Glu Phe Asn Ser Gly Val Thr Phe Asp Trp Asp Tyr Pro Gly Lys Gln
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            260                 265                 270

Ala Glu Arg Gly Lys Trp Val Pro Glu Arg Arg Ser Gln Gln Thr His
        275                 280                 285

Thr Glu Leu Ser Ser Ile Leu Thr Ile His Asn Val Ser Gln His Asp
    290                 295                 300

Leu Gly Ser Tyr Val Cys Lys Ala Asn Asn Gly Ile Gln Arg Phe Arg
305                 310                 315                 320

Glu Ser Thr Glu Val Ile Val His Glu Asn Pro Phe Ile Ser Val Glu
                325                 330                 335

Trp Leu Lys Gly Pro Ile Leu Glu Ala Thr Ala Gly Asp Glu Leu Val
            340                 345                 350

Lys Leu Pro Val Lys Leu Ala Ala Tyr Pro Pro Pro Glu Phe Gln Trp
        355                 360                 365

Tyr Lys Asp Gly Lys Ala Leu Ser Gly Arg His Ser Pro His Ala Leu
    370                 375                 380

Val Leu Lys Glu Val Thr Glu Ala Ser Thr Gly Thr Tyr Thr Leu Ala
385                 390                 395                 400

Leu Trp Asn Ser Ala Ala Gly Leu Arg Arg Asn Ile Ser Leu Glu Leu
                405                 410                 415

Val Val Asn Val Pro Pro Gln Ile His Glu Lys Glu Ala Ser Ser Pro
            420                 425                 430

Ser Ile Tyr Ser Arg His Ser Arg Gln Ala Leu Thr Cys Thr Ala Tyr
        435                 440                 445

Gly Val Pro Leu Pro Leu Ser Ile Gln Trp His Trp Arg Pro Trp Thr
    450                 455                 460

Pro Cys Lys Met Phe Ala Gln Arg Ser Leu Arg Arg Arg Gln Gln Gln
465                 470                 475                 480
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Asp Leu Met Pro Gln Cys Arg Asp Trp Arg Ala Val Thr Thr Gln Asp
                485                 490                 495

Ala Val Asn Pro Ile Glu Ser Leu Asp Thr Trp Thr Glu Phe Val Glu
            500                 505                 510

Gly Lys Asn Lys Thr Val Ser Lys Leu Val Ile Gln Asn Ala Asn Val
        515                 520                 525

Ser Ala Met Tyr Lys Cys Val Val Ser Asn Lys Val Gly Gln Asp Glu
    530                 535                 540

Arg Leu Ile Tyr Phe Tyr Val Thr Thr Ile Pro Asp Gly Phe Thr Ile
545                 550                 555                 560

Glu Ser Lys Pro Ser Glu Glu Leu Leu Glu Gly Gln Pro Val Leu Leu
                565                 570                 575

Ser Cys Gln Ala Asp Ser Tyr Lys Tyr Glu His Leu Arg Trp Tyr Arg
            580                 585                 590

Leu Asn Leu Ser Thr Leu His Asp Ala His Gly Asn Pro Leu Leu Leu
        595                 600                 605

Asp Cys Lys Asn Val His Leu Phe Ala Thr Pro Leu Ala Ala Ser Leu
    610                 615                 620

Glu Glu Val Ala Pro Gly Ala Arg His Ala Thr Leu Ser Leu Ser Ile
625                 630                 635                 640

Pro Arg Val Ala Pro Glu His Glu Gly His Tyr Val Cys Glu Val Gln
                645                 650                 655

Asp Arg Arg Ser His Asp Lys His Cys His Lys Lys Tyr Leu Ser Val
            660                 665                 670

Gln Ala Leu Glu Ala Pro Arg Leu Thr Gln Asn Leu Thr Asp Leu Leu
        675                 680                 685

Val Asn Val Ser Asp Ser Leu Glu Met Gln Cys Leu Val Ala Gly Ala
    690                 695                 700
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```
His Ala Pro Ser Ile Val Trp Tyr Lys Asp Glu Arg Leu Leu Glu Glu
705                 710                 715                 720

Lys Ser Gly Val Asp Leu Ala Asp Ser Asn Gln Lys Leu Ser Ile Gln
                725                 730                 735

Arg Val Arg Glu Glu Asp Ala Gly Arg Tyr Leu Cys Ser Val Cys Asn
            740                 745                 750

Ala Lys Gly Cys Val Asn Ser Ser Ala Ser Val Ala Val Glu Gly Ser
        755                 760                 765

Glu Asp Lys Gly Ser Met Glu Ile Val Ile Leu Val Gly Thr Gly Val
    770                 775                 780

Ile Ala Val Phe Phe Trp Val Leu Leu Leu Leu Ile Phe Cys Asn Met
785                 790                 795                 800

Arg Arg Pro Ala His Ala Asp Ile Lys Thr Gly Tyr Leu Ser Ile Ile
                805                 810                 815

Met Asp Pro Gly Glu Val Pro Leu Glu Glu Gln Cys Glu Tyr Leu Ser
            820                 825                 830

Tyr Asp Ala Ser Gln Trp Glu Phe Pro Arg Glu Arg Leu His Leu Gly
        835                 840                 845

Arg Val Leu Gly Tyr Gly Ala Phe Gly Lys Val Val Glu Ala Ser Ala
    850                 855                 860

Phe Gly Ile His Lys Gly Ser Ser Cys Asp Thr Val Ala Val Lys Met
865                 870                 875                 880

Leu Lys Glu Gly Ala Thr Ala Ser Glu His Arg Ala Leu Met Ser Glu
                885                 890                 895

Leu Lys Ile Leu Ile His Ile Gly Asn His Leu Asn Val Val Asn Leu
            900                 905                 910

Leu Gly Ala Cys Thr Lys Pro Gln Gly Pro Leu Met Val Ile Val Glu
        915                 920                 925
```


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```
Phe Cys Lys Tyr Gly Asn Leu Ser Asn Phe Leu Arg Ala Lys Arg Asp
    930                 935                 940

Ala Phe Ser Pro Cys Ala Glu Lys Ser Pro Glu Gln Arg Gly Arg Phe
945                 950                 955                 960

Arg Ala Met Val Glu Leu Ala Arg Leu Asp Arg Arg Arg Pro Gly Ser
                965                 970                 975

Ser Asp Arg Val Leu Phe Ala Arg Phe Ser Lys Thr Glu Gly Gly Ala
            980                 985                 990

Arg Arg Ala Ser Pro Asp Gln Glu  Ala Glu Asp Leu Trp  Leu Ser Pro
        995                 1000                1005

Leu Thr  Met Glu Asp Leu Val  Cys Tyr Ser Phe Gln  Val Ala Arg
    1010                1015                1020

Gly Met  Glu Phe Leu Ala Ser  Arg Lys Cys Ile His  Arg Asp Leu
    1025                1030                1035

Ala Ala  Arg Asn Ile Leu Leu  Ser Glu Ser Asp Val  Val Lys Ile
    1040                1045                1050

Cys Asp  Phe Gly Leu Ala Arg  Asp Ile Tyr Lys Asp  Pro Asp Tyr
    1055                1060                1065

Val Arg  Lys Gly Ser Ala Arg  Leu Pro Leu Lys Trp  Met Ala Pro
    1070                1075                1080

Glu Ser  Ile Phe Asp Lys Val  Tyr Thr Thr Gln Ser  Asp Val Trp
    1085                1090                1095

Ser Phe  Gly Val Leu Leu Trp  Glu Ile Phe Ser Leu  Gly Ala Ser
    1100                1105                1110

Pro Tyr  Pro Gly Val Gln Ile  Asn Glu Glu Phe Cys  Gln Arg Leu
    1115                1120                1125

Arg Asp  Gly Thr Arg Met Arg  Ala Pro Glu Leu Ala  Thr Pro Ala
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    1130                1135                1140

Ile Arg  Arg Ile Met Leu Asn  Cys Trp Ser Gly Asp  Pro Lys Ala
    1145                1150                1155

Arg Pro  Ala Phe Ser Glu Leu  Val Glu Ile Leu Gly  Asp Leu Leu
    1160                1165                1170

Gln Gly  Arg Gly Leu Gln Glu  Glu Glu Glu Val Cys  Met Ala Pro
    1175                1180                1185

Arg Ser  Ser Gln Ser Ser Glu  Glu Gly Ser Phe Ser  Gln Val Ser
    1190                1195                1200

Thr Met  Ala Leu His Ile Ala  Gln Ala Asp Ala Glu  Asp Ser Pro
    1205                1210                1215

Pro Ser  Leu Gln Arg His Ser  Leu Ala Ala Arg Tyr  Tyr Asn Trp
    1220                1225                1230

Val Ser  Phe Pro Gly Cys Leu  Ala Arg Gly Ala Glu  Thr Arg Gly
    1235                1240                1245

Ser Ser  Arg Met Lys Thr Phe  Glu Glu Phe Pro Met  Thr Pro Thr
    1250                1255                1260

Thr Tyr  Lys Gly Ser Val Asp  Asn Gln Thr Asp Ser  Gly Met Val
    1265                1270                1275

Leu Ala  Ser Glu Glu Phe Glu  Gln Ile Glu Ser Arg  His Arg Gln
    1280                1285                1290

Glu Ser  Gly Phe Ser Cys Lys  Gly Pro Gly Gln Asn  Val Ala Val
    1295                1300                1305

Thr Arg  Ala His Pro Asp Ser  Gln Gly Arg Arg Arg  Arg Pro Glu
    1310                1315                1320

Arg Gly  Ala Arg Gly Gly Gln  Val Phe Tyr Asn Ser  Glu Tyr Gly
    1325                1330                1335
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Glu Leu Ser Glu Pro Ser Glu Glu Asp His Cys Ser Pro Ser Ala
1340 1345 1350

Arg Val Thr Phe Phe Thr Asp Asn Ser Tyr
1355 1360

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<212> PRT
<213> Homo sapiens

<400> 14

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1 5 10 15

Ile Asp Thr Gly Asp Ser Leu Ser Ile Ser Cys Arg Gly Gln His Pro
20 25 30

Leu Glu Trp Ala Trp Pro Gly Ala Gln Glu Ala Pro Ala Thr Gly Asp
35 40 45

Lys Asp Ser Glu Asp Thr Gly Val Val Arg Asp Cys Glu Gly Thr Asp
50 55 60

Ala Arg Pro Tyr Cys Lys Val Leu Leu Leu His Glu Val His Ala Asn
65 70 75 80

Asp Thr Gly Ser Tyr Val Cys Tyr Tyr Lys Tyr Ile Lys Ala Arg Ile
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Glu Gly Thr Thr Ala Ala Ser
100

<210> 15
<211> 306
<212> PRT
<213> Homo sapiens

<400> 15

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1 5 10 15

Ile Asp Thr Gly Asp Ser Leu Ser Ile Ser Cys Arg Gly Gln His Pro

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            20                  25                  30

Leu Glu Trp Ala Trp Pro Gly Ala Gln Glu Ala Pro Ala Thr Gly Asp
        35                  40                  45

Lys Asp Ser Glu Asp Thr Gly Val Val Arg Asp Cys Glu Gly Thr Asp
    50                  55                  60

Ala Arg Pro Tyr Cys Lys Val Leu Leu Leu His Glu Val His Ala Asn
65                  70                  75                  80

Asp Thr Gly Ser Tyr Val Cys Tyr Tyr Lys Tyr Ile Lys Ala Arg Ile
                85                  90                  95

Glu Gly Thr Thr Ala Ala Ser Ser Tyr Val Phe Val Arg Asp Phe Glu
            100                 105                 110

Gln Pro Phe Ile Asn Lys Pro Asp Thr Leu Leu Val Asn Arg Lys Asp
        115                 120                 125

Ala Met Trp Val Pro Cys Leu Val Ser Ile Pro Gly Leu Asn Val Thr
    130                 135                 140

Leu Arg Ser Gln Ser Ser Val Leu Trp Pro Asp Gly Gln Glu Val Val
145                 150                 155                 160

Trp Asp Asp Arg Arg Gly Met Leu Val Ser Thr Pro Leu Leu His Asp
                165                 170                 175

Ala Leu Tyr Leu Gln Cys Glu Thr Thr Trp Gly Asp Gln Asp Phe Leu
            180                 185                 190

Ser Asn Pro Phe Leu Val His Ile Thr Gly Asn Glu Leu Tyr Asp Ile
        195                 200                 205

Gln Leu Leu Pro Arg Lys Ser Leu Glu Leu Leu Val Gly Glu Lys Leu
    210                 215                 220

Val Leu Asn Cys Thr Val Trp Ala Glu Phe Asn Ser Gly Val Thr Phe
225                 230                 235                 240
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```
Asp Trp Asp Tyr Pro Gly Lys Gln Ala Glu Arg Gly Lys Trp Val Pro
                245                 250                 255

Glu Arg Arg Ser Gln Gln Thr His Thr Glu Leu Ser Ser Ile Leu Thr
            260                 265                 270

Ile His Asn Val Ser Gln His Asp Leu Gly Ser Tyr Val Cys Lys Ala
        275                 280                 285

Asn Asn Gly Ile Gln Arg Phe Arg Glu Ser Thr Glu Val Ile Val His
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Glu Asn
305
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<210> 16
<211> 759
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<213> Artificial

<220>
<223> Fusion protein constructed from extracellular domains of VEGFR-1, VEGFR-2, and VEGFR-3.

<400> 16

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1               5                   10                  15

Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
            20                  25                  30

Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
        35                  40                  45

Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
    50                  55                  60

Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
65                  70                  75                  80

Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
                85                  90                  95
```


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```
Gln Thr Asn Thr Ile Ile Asp Val Val Leu Ser Pro Ser His Gly Ile
            100                 105                 110

Glu Leu Ser Val Gly Glu Lys Leu Val Leu Asn Cys Thr Ala Arg Thr
        115                 120                 125

Glu Leu Asn Val Gly Ile Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys
    130                 135                 140

His Gln His Lys Lys Leu Val Asn Arg Asp Leu Lys Thr Gln Ser Gly
145                 150                 155                 160

Ser Glu Met Lys Lys Phe Leu Ser Thr Leu Thr Ile Asp Gly Val Thr
                165                 170                 175

Arg Ser Asp Gln Gly Leu Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met
            180                 185                 190

Thr Lys Lys Asn Ser Thr Phe Val Arg Val His Glu Lys Gly Gly Ser
        195                 200                 205

Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Gly Ser Gly
    210                 215                 220

Gly Gly Gly Tyr Ser Met Thr Pro Pro Thr Leu Asn Ile Thr Glu Glu
225                 230                 235                 240

Ser His Val Ile Asp Thr Gly Asp Ser Leu Ser Ile Ser Cys Arg Gly
                245                 250                 255

Gln His Pro Leu Glu Trp Ala Trp Pro Gly Ala Gln Glu Ala Pro Ala
            260                 265                 270

Thr Gly Asp Lys Asp Ser Glu Asp Thr Gly Val Val Arg Asp Cys Glu
        275                 280                 285

Gly Thr Asp Ala Arg Pro Tyr Cys Lys Val Leu Leu Leu His Glu Val
    290                 295                 300

His Ala Asn Asp Thr Gly Ser Tyr Val Cys Tyr Tyr Lys Tyr Ile Lys
305                 310                 315                 320
```


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```
Ala Arg Ile Glu Gly Thr Thr Ala Ala Ser Ser Tyr Val Phe Val Arg
                325                 330                 335

Asp Phe Glu Gln Pro Phe Ile Asn Lys Pro Asp Thr Leu Leu Val Asn
            340                 345                 350

Arg Lys Asp Ala Met Trp Val Pro Cys Leu Val Ser Ile Pro Gly Leu
        355                 360                 365

Asn Val Thr Leu Arg Ser Gln Ser Ser Val Leu Trp Pro Asp Gly Gln
    370                 375                 380

Glu Val Val Trp Asp Asp Arg Arg Gly Met Leu Val Ser Thr Pro Leu
385                 390                 395                 400

Leu His Asp Ala Leu Tyr Leu Gln Cys Glu Thr Thr Trp Gly Asp Gln
                405                 410                 415

Asp Phe Leu Ser Asn Pro Phe Leu Val His Ile Thr Gly Asn Glu Leu
            420                 425                 430

Tyr Asp Ile Gln Leu Leu Pro Arg Lys Ser Leu Glu Leu Leu Val Gly
        435                 440                 445

Glu Lys Leu Val Leu Asn Cys Thr Val Trp Ala Glu Phe Asn Ser Gly
    450                 455                 460

Val Thr Phe Asp Trp Asp Tyr Pro Gly Lys Gln Ala Glu Arg Gly Lys
465                 470                 475                 480

Trp Val Pro Glu Arg Arg Ser Gln Gln Thr His Thr Glu Leu Ser Ser
                485                 490                 495

Ile Leu Thr Ile His Asn Val Ser Gln His Asp Leu Gly Ser Tyr Val
            500                 505                 510

Cys Lys Ala Asn Asn Gly Ile Gln Arg Phe Arg Glu Ser Thr Glu Val
        515                 520                 525

Ile Val His Glu Asn Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala
    530                 535                 540
```


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```
Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
545                 550                 555                 560

Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
                565                 570                 575

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val
            580                 585                 590

Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
        595                 600                 605

Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
    610                 615                 620

Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
625                 630                 635                 640

Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
                645                 650                 655

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr
            660                 665                 670

Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
        675                 680                 685

Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
    690                 695                 700

Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr
705                 710                 715                 720

Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe
                725                 730                 735

Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys
            740                 745                 750

Ser Leu Ser Leu Ser Pro Gly
```


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755

[00206] While specific embodiments of the present invention have been described in the foregoing, it will be appreciated by those skilled in the art that many equivalents, modifications, substitutions, and variations may be made thereto without departing from the spirit and scope of the invention as defined in the appended claims.

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WHAT IS CLAIMED IS:

1. A recombinant human Fc fusion protein, comprising an Ig-like domain of a first VEGF receptor, an Ig-like domain of a second VEGF receptor, and at least an Ig-like domain of a third VEGF receptor; wherein said third VEGF receptor comprises human VEGFR-3 (flt-4) having an amino acid sequence listed in SEQ ID NO: 13 with the proviso that the amino acids at positions 105 and 106 of SEQ ID NO: 13 are substitutable such that the amino acid sequence at positions 104-106 of SEQ ID NO: 13 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

2. The fusion protein of claim 1; wherein said Ig-like domain of a first VEGF receptor comprises an amino acid sequence that is at least 90% identical to Ig-like domain 2 of human VEGFR-1 (flt-1); said Ig-like domain of a second VEGF receptor comprises an amino acid sequence that is at least 90% identical to Ig-like domain 3 of human VEGFR-2 (KDR); and said at least an Ig-like domain of a third VEGF receptor comprises an amino acid sequence that is at least 90% identical to Ig-like domains 1 and 2 of human VEGFR-3.

3. The fusion protein of claim 1; wherein said Ig-like domain of a first VEGF receptor comprises an amino acid sequence that is at least 95% identical to Ig-like domain 2 of human VEGFR-1 (flt-1); said Ig-like domain of a second VEGF receptor comprises an amino acid sequence that is at least 95% identical to Ig-like domain 3 of human VEGFR-2 (KDR); and said at least an Ig-like domain of a third VEGF receptor comprises an amino acid sequence that is at least 95% identical to Ig-like domains 1 and 2 of human VEGFR-3.

4. The fusion protein of claim 3; wherein said at least an Ig-like domain of a third VEGF receptor comprises an amino acid sequence that is at least 95% identical to Ig-like domains 1, 2, and 3 of human VEGFR-3.

5. The fusion protein of claim3, further comprising (d) a multimerizing component linked to said Ig-like domains.

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6. The fusion protein of claim 5; wherein said multimerizing component comprises a portion of an Fc domain of human IgG1.

7. The fusion protein of claim 3; wherein said Ig-like domain 2 of human VEGFR-1 comprises an amino acid sequence listed in SEQ ID NO: 2; said Ig-like domain 3 of human VEGFR-2 comprises an amino acid sequence listed in SEQ ID NO: 4; said Ig-like domains 1 and 2 of human VEGFR-3 comprises an amino acid sequence listed in SEQ ID NO: 6; and said portion of an Fc domain of human IgG1 comprises an amino acid sequence listed in SEQ ID NO: 8.

8. A fusion protein comprising an amino acid sequence that is at least 90% identical to the amino acid sequence listed in SEQ ID NO: 12 with the proviso that the amino acids at positions 286 and 287 of SEQ ID NO: 12 are substitutable such that the amino acid sequence at positions 285-287 of SEQ ID NO: 12 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

9. The fusion protein of claim 8, comprising an amino acid sequence that is at least 95% identical to the amino acid sequence listed in SEQ ID NO: 12.

10. The fusion protein of claim 8, comprising an amino acid sequence as listed in SEQ ID NO:12.

11. The fusion protein of claim 10; wherein said fusion polypeptide binds to a VEGF family member with dissociation constant $K_d \leq 10^{-9}$ M.

12. The fusion protein of claim 10; wherein said fusion polypeptide binds to a VEGF family member with dissociation constant $K_d \leq 10^{-10}$ M.

13. An isolated nucleic acid molecule encoding a fusion polypeptide of any one of claims 1-12.

14. The isolated nucleic acid molecule of claim 13, having a sequence substantially as listed in SEQ ID NO: 11.

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15. A vector comprising the nucleic acid molecule of claim 13 or 14.

16. The vector of claim 15, comprising said nucleic molecule operatively linked to an expression control sequence.

17. A host-vector system comprising the expression vector of claim 16 in a host cell.

18. A method of producing a substantially purified fusion polypeptide, which method comprises: (a) growing cells of the host-vector system of claim 17 under conditions permitting production of the fusion polypeptide; and (b) recovering the fusion or chimeric polypeptide to produce a recovered fusion polypeptide; and (c) purifying said recovered fusion polypeptide to produce the substantially purified fusion polypeptide.

19. A method for treating or controlling at least a disease, condition, or disorder, in a subject in need thereof, which has etiology in aberrant angiogenesis; said method comprising administering to said subject an amount of a composition of a fusion protein comprising an amino acid sequence that is at least 90% identical to the amino acid sequence listed in SEQ ID NO: 12 or SEQ ID NO: 16 with the proviso that the amino acids at positions 286 and 287 of SEQ ID NO: 12 or SEQ ID NO: 16 are substitutable such that the amino acid sequence at positions 285-287 of SEQ ID NO: 12 or SEQ ID NO: 16 is NDT, NDS, NXT, or NXS, wherein X is an amino acid selected from the group consisting of A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V.

20. The method of claim 19; wherein said fusion protein comprises an amino acid sequence that is at least 95% identical to the amino acid sequence listed in SEQ ID NO: 12 or SEQ ID NO: 16.

21. The method of claim 19; wherein said fusion protein has an amino acid sequence as listed in SEQ ID NO: 12 or SEQ ID NO: 16.

22. The method of claim 21; wherein said disease, condition, or disorder is selected from the group consisting of choroidal neovascularization, polypoidal choroidal vasculopathy, myopic neovascularization, retinal neovascularization, retinopathy of prematurity, vascular leak, retinal edema, diabetic macular edema, macular edema caused by

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retinal vein occlusion or non-infectious posterior uveitis, diabetic retinopathy, proliferative diabetic retinopathy, corneal neovascularization, and neovascular glaucoma.

23. The method of claim 22; wherein the subject is administered with a dose of about 25-4000 micrograms of the fusion polypeptide.

24. The method of claim 23; wherein the composition is administered to the subject as an eye drop, an intravitreal, subretinal, or suprachoroidal injection.

25. The method of claim 23; wherein the composition is administered to the subject for a period of at least one month if intraocularly administered.

26. The method of claim 23; wherein the composition is administered to the subject at a frequency of at least once per month if intraocularly administered.

27. The method of claim 21; wherein said disease, condition, or disorder is a non-ocular disease, condition, or disorder that has etiology in aberrant angiogenesis.

28. The method of claim 27; wherein said disease, condition, or disorder is selected from the group consisting of cancers, psoriasis, rheumatoid arthritis, and atherosclerosis.

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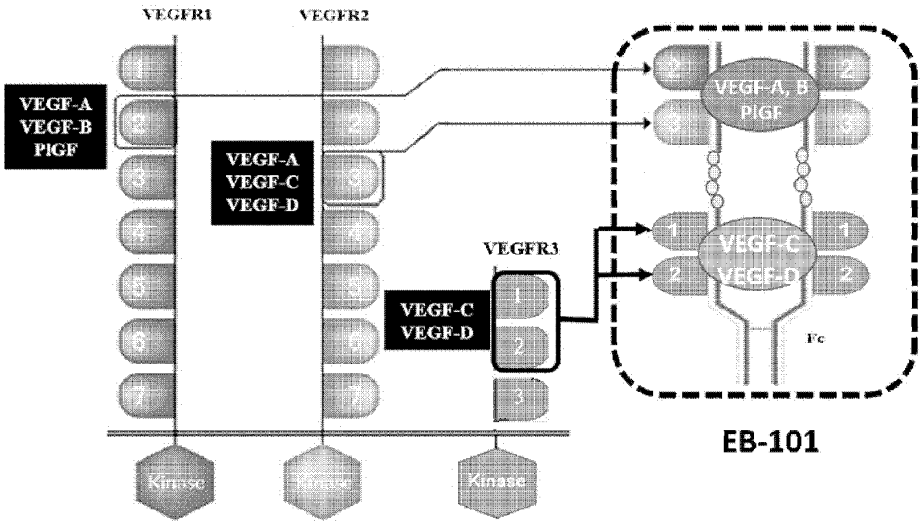


Figure 1

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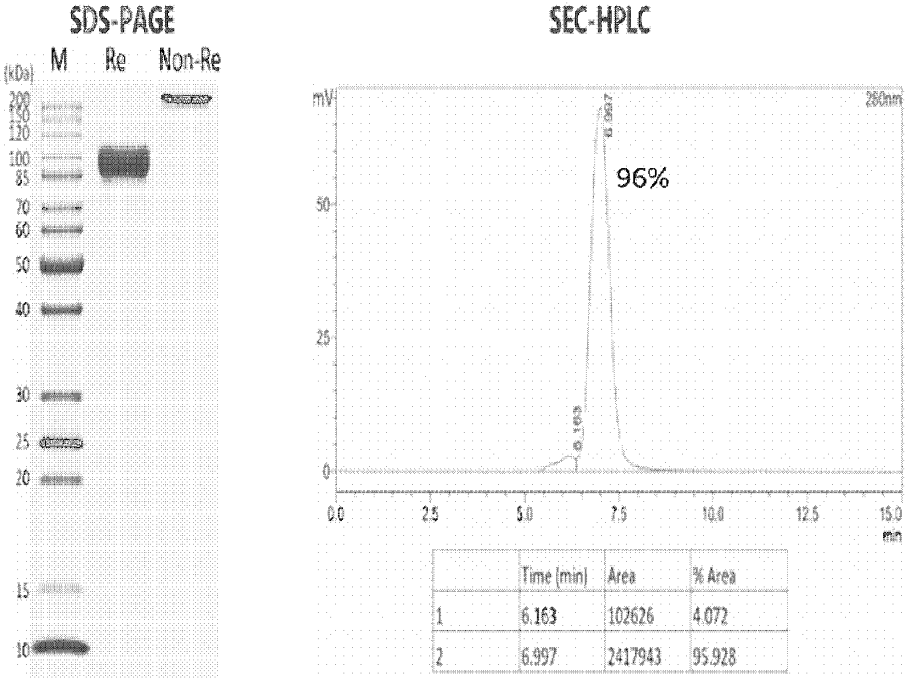
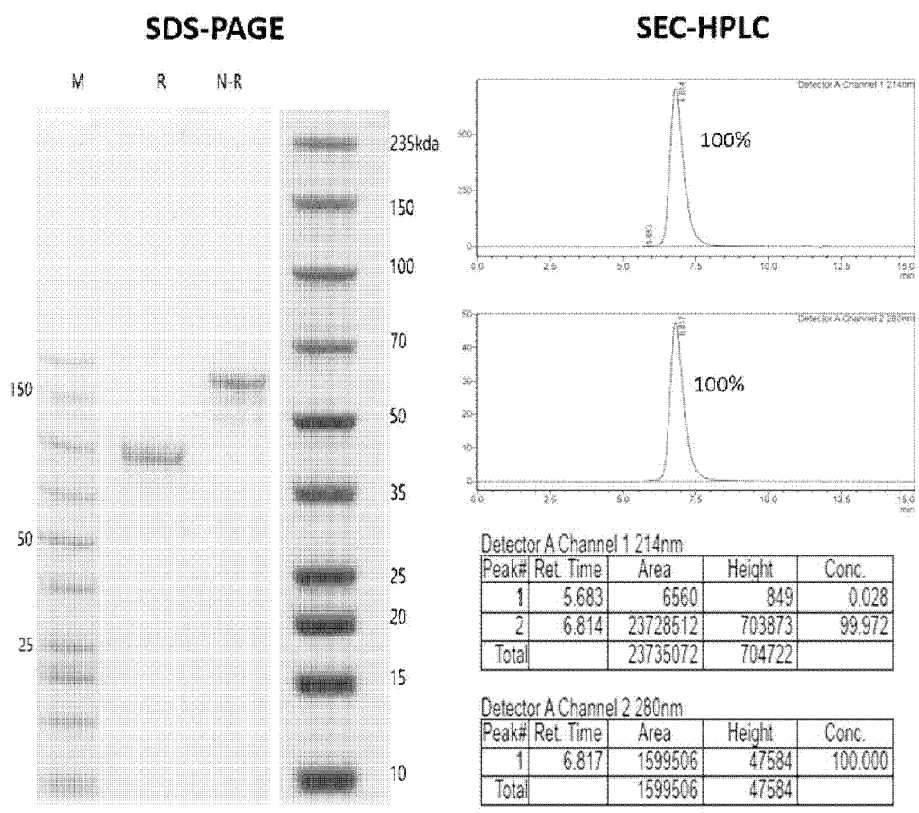


Figure 2

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Detector A Channel 1 214nm

Peak#	Ret. Time	Area	Height	Conc.
1	5.683	6560	849	0.028
2	6.814	23728512	703873	99.972
Total		23735072	704722	

Detector A Channel 2 280nm

Peak#	Ret. Time	Area	Height	Conc.
1	6.817	1599506	47584	100.000
Total		1599506	47584	

Figure 3

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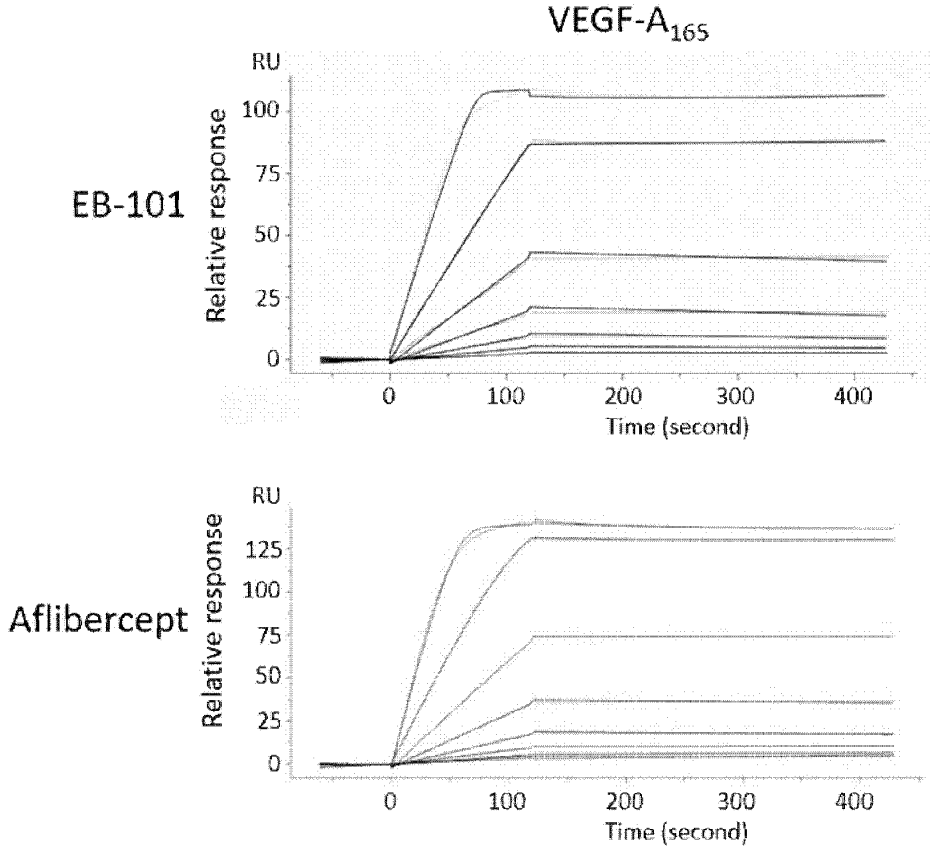


Figure 4

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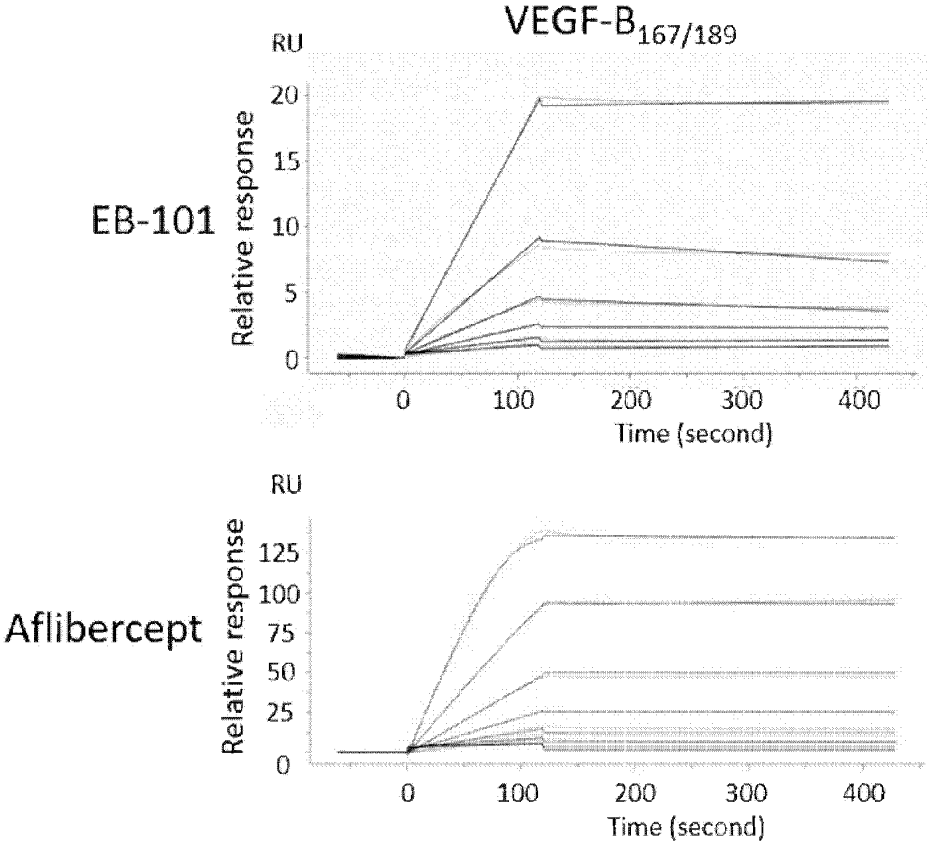


Figure 5

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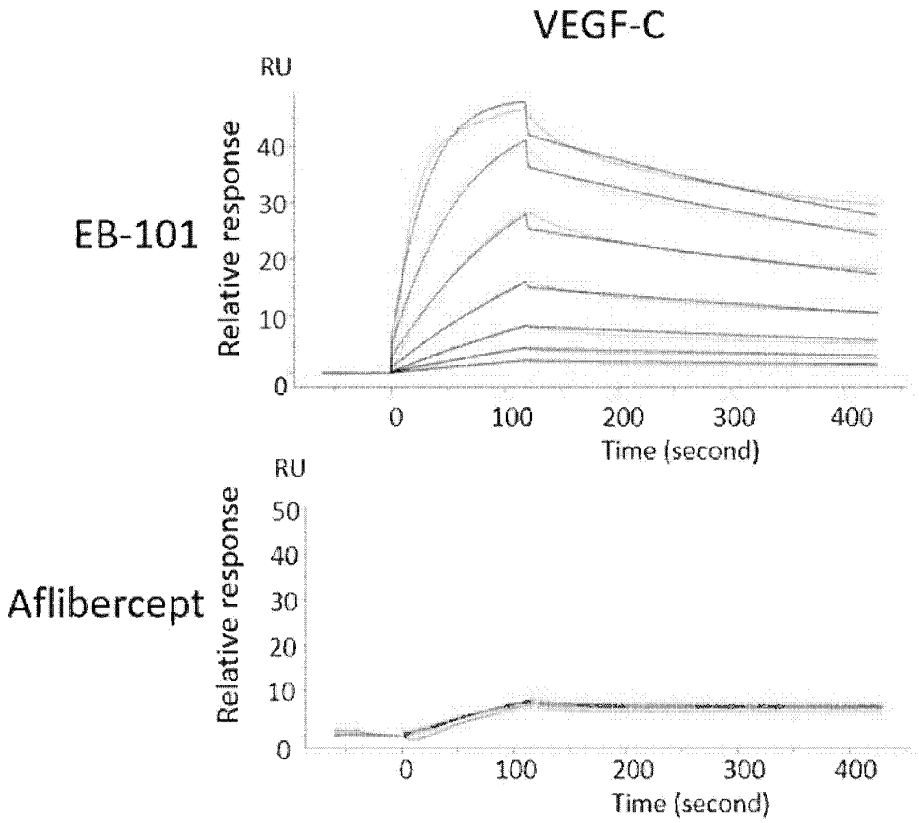


Figure 6

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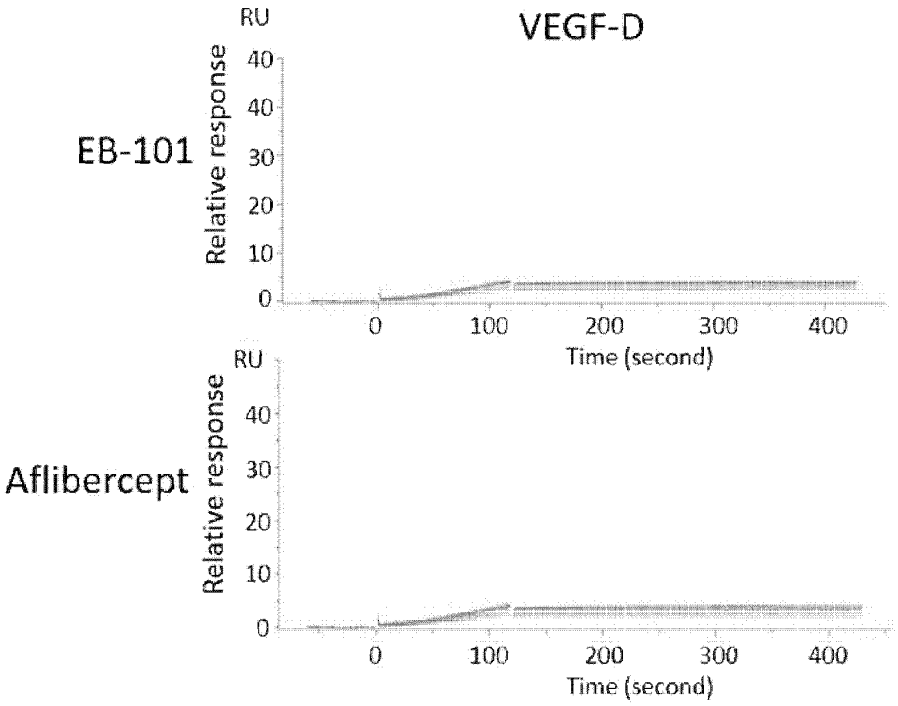


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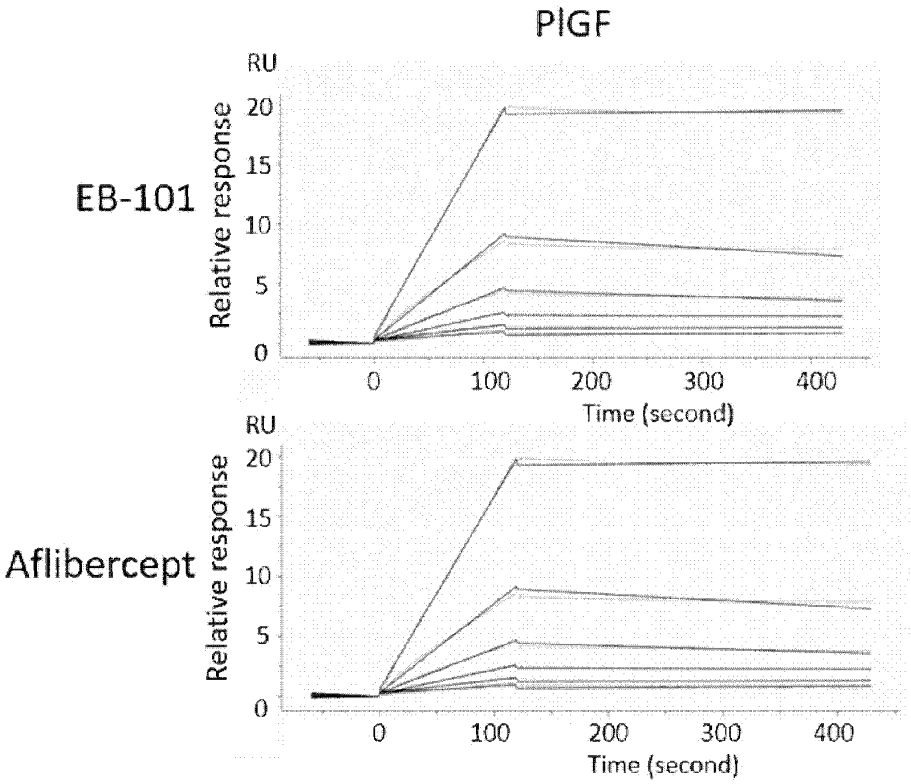


Figure 8

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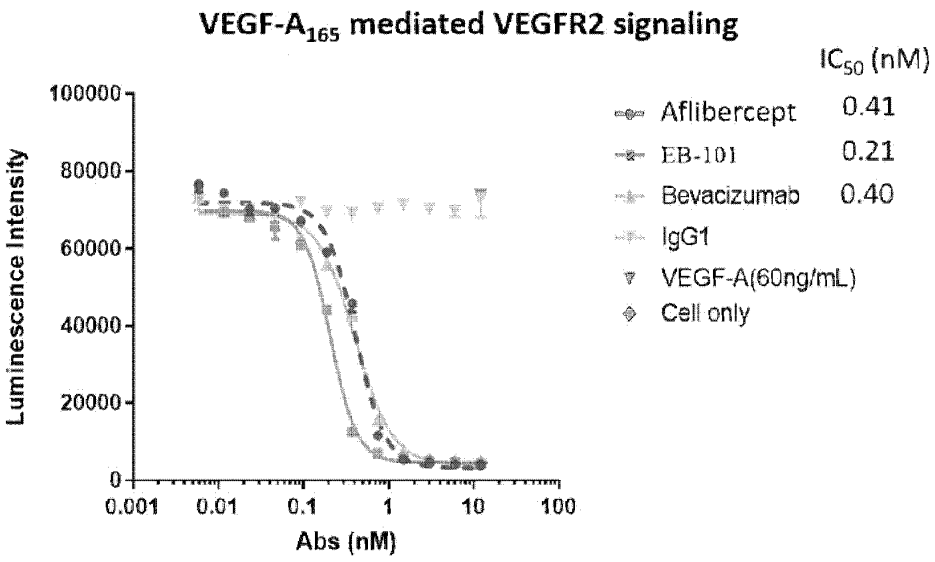


Figure 9

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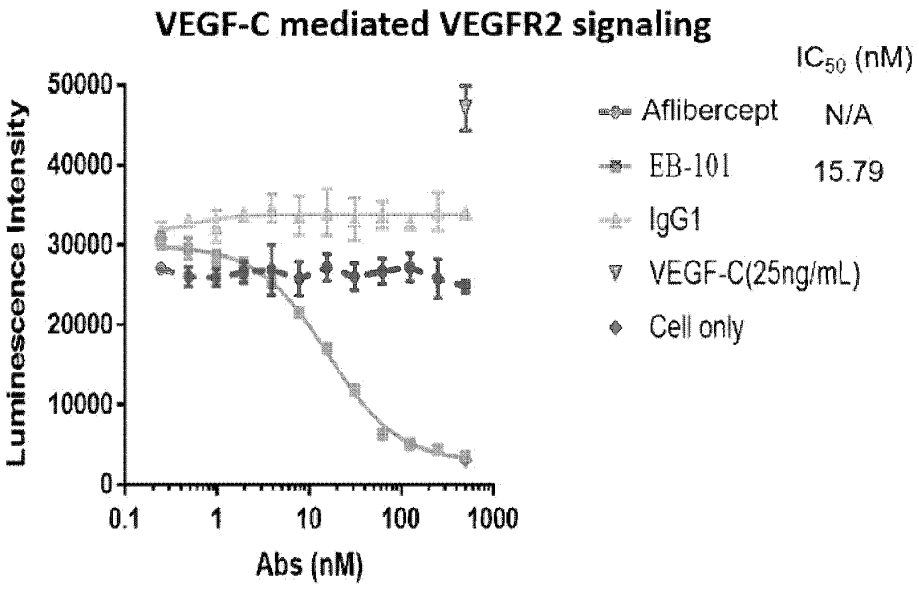


Figure 10

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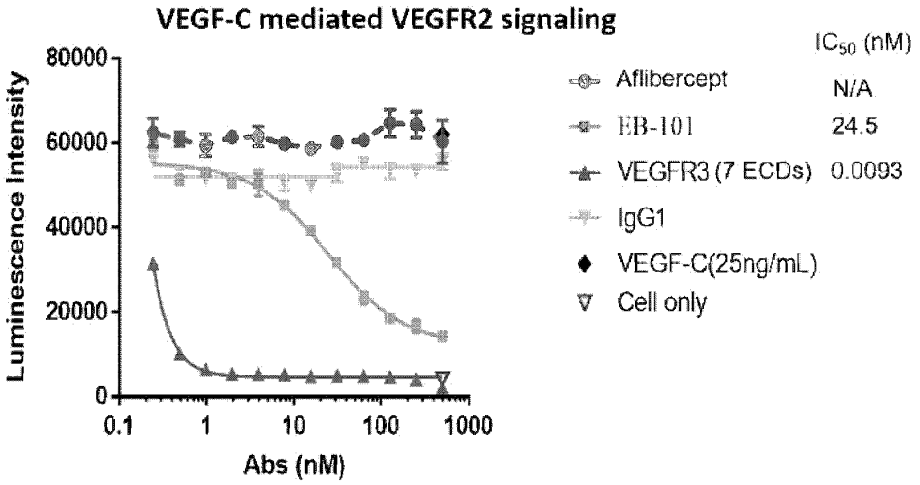


Figure 11

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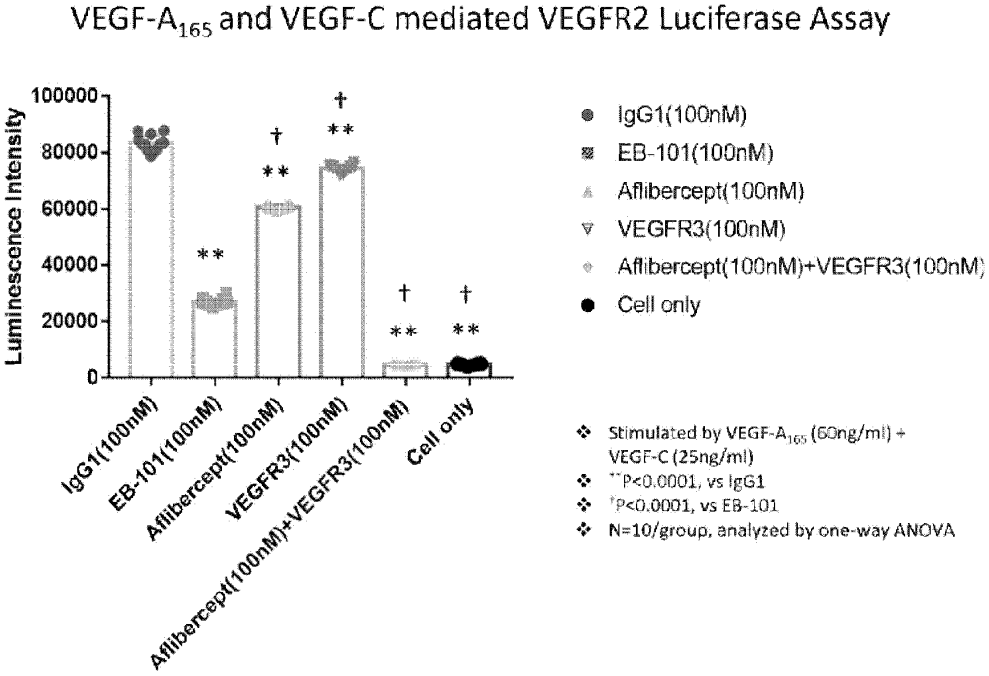


Figure 12

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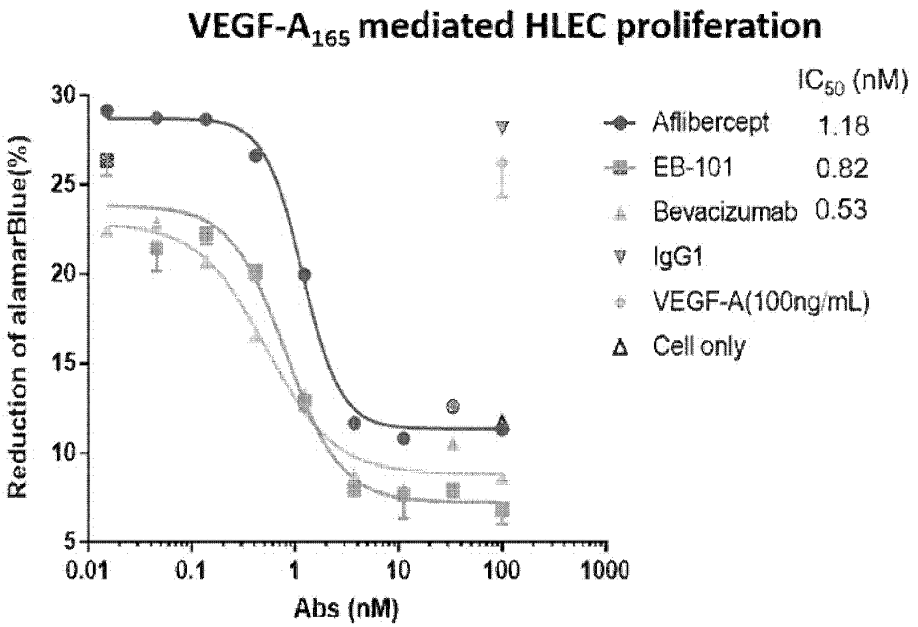


Figure 13

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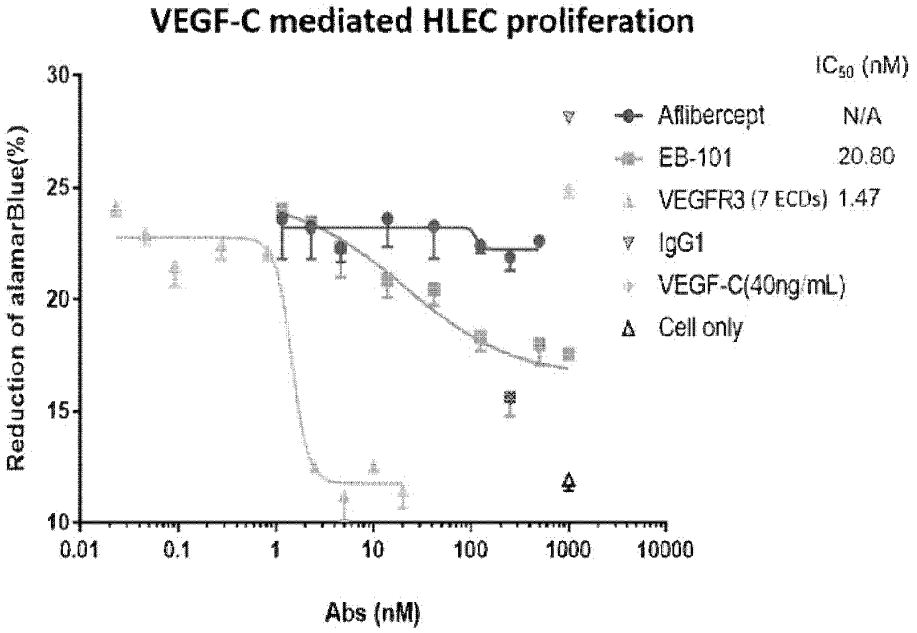


Figure 14

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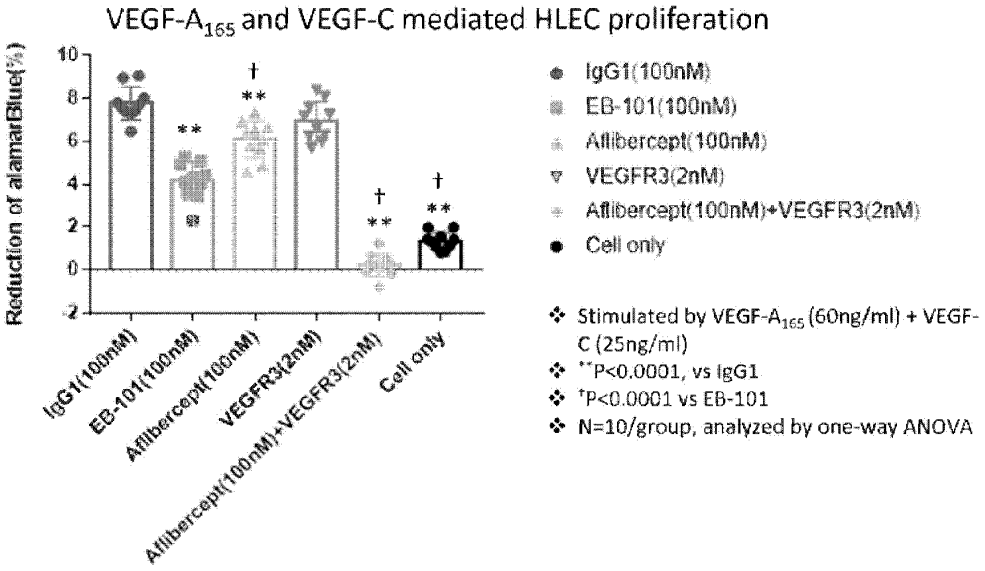


Figure 15

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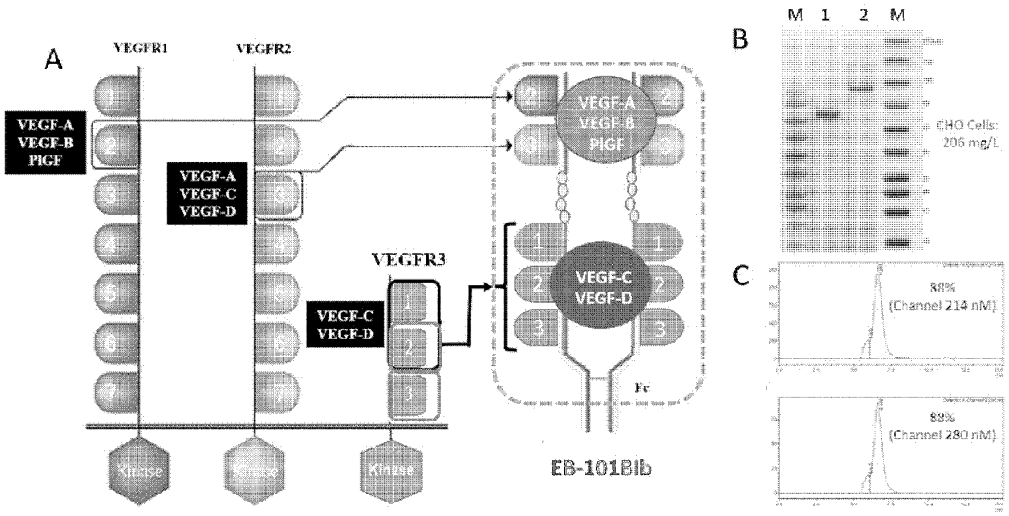


Figure 16

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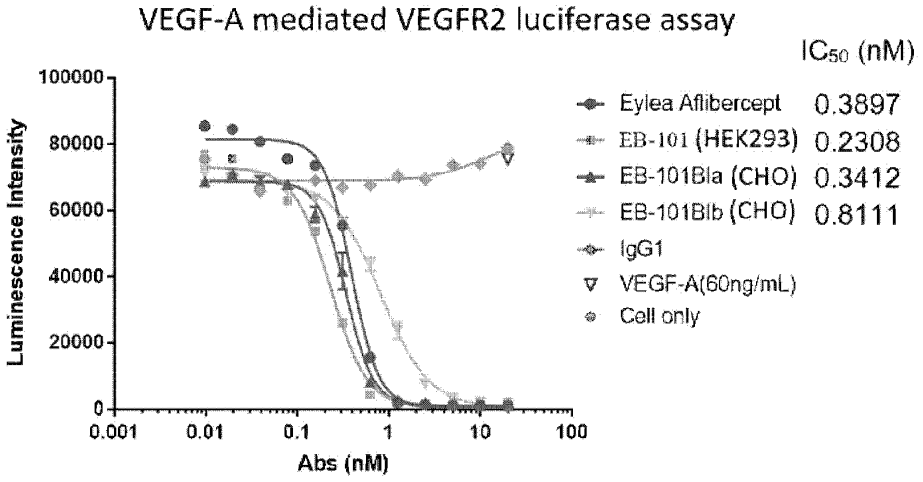


Figure 17

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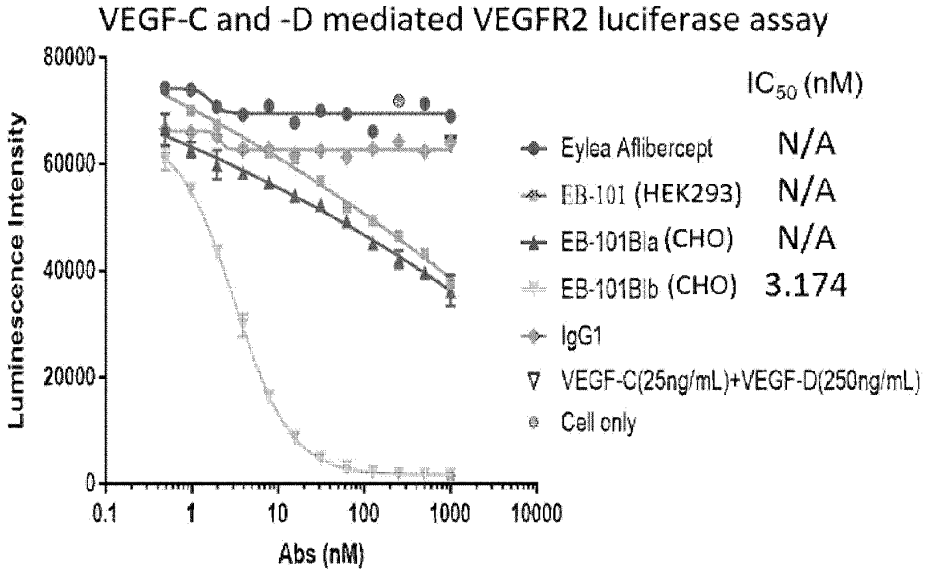


Figure 18

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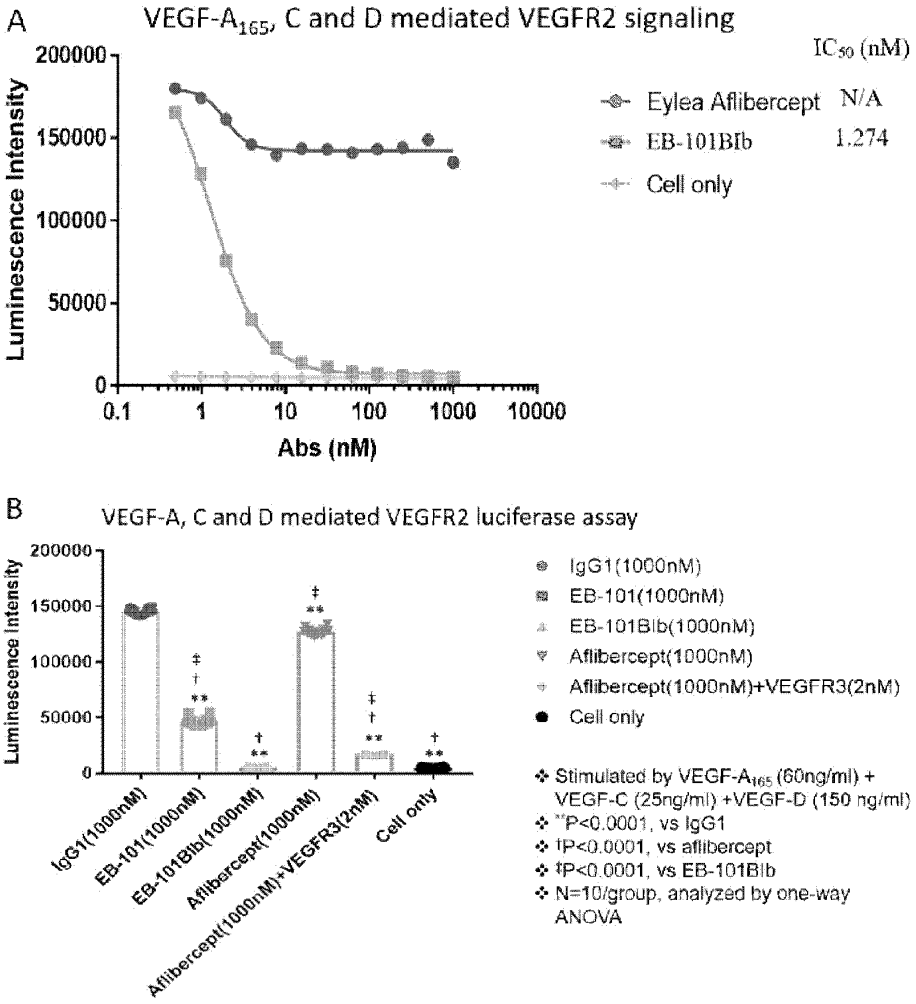


Figure 19

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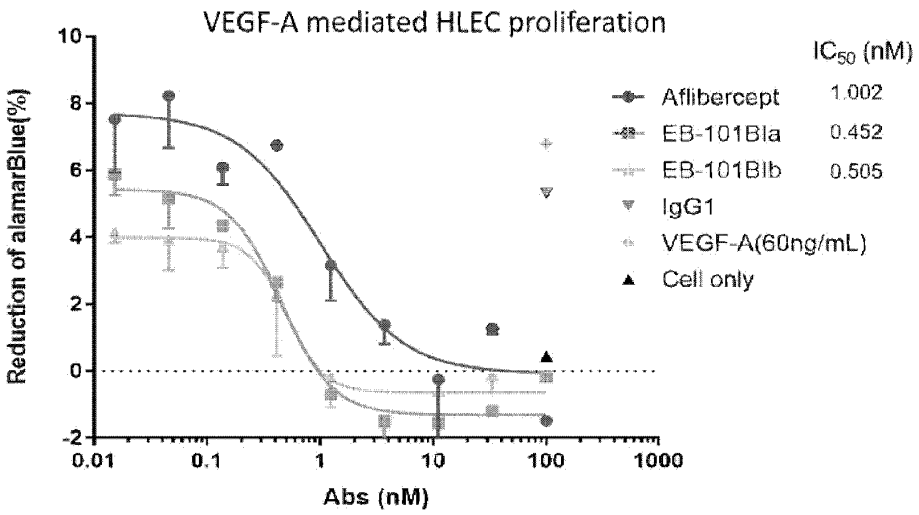


Figure 20

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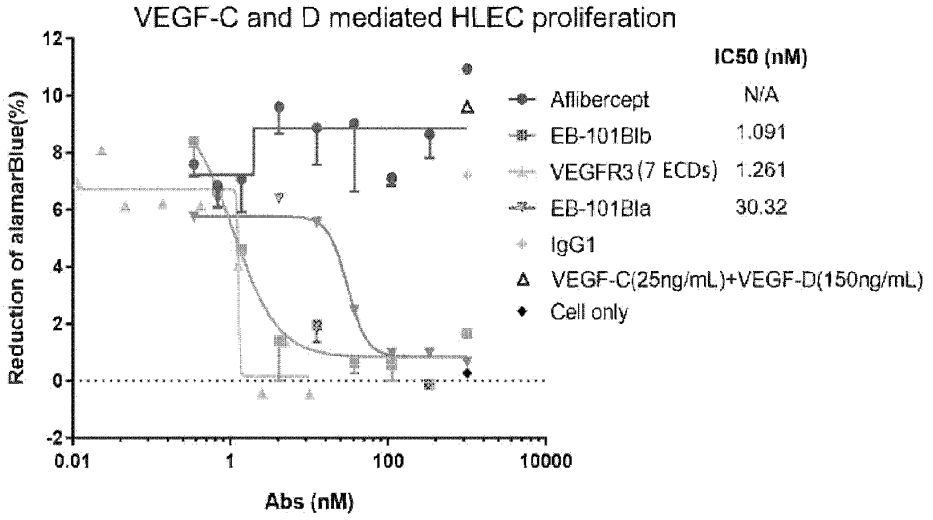


Figure 21

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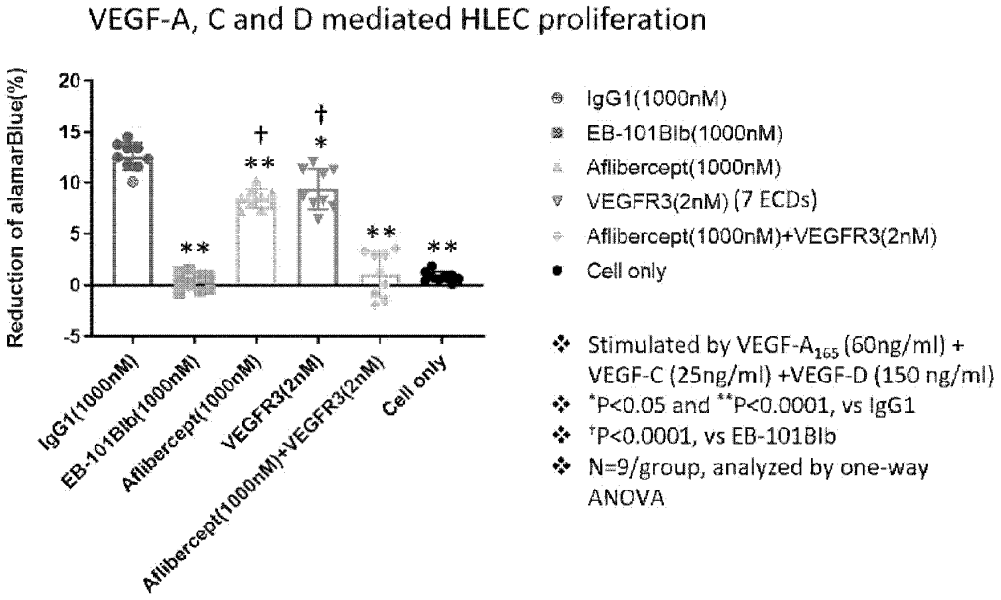


Figure 22

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(54) Title: METHODS FOR TREATING INFLAMMATORY OCULAR DISEASES WITH COMPLEMENT FACTOR H

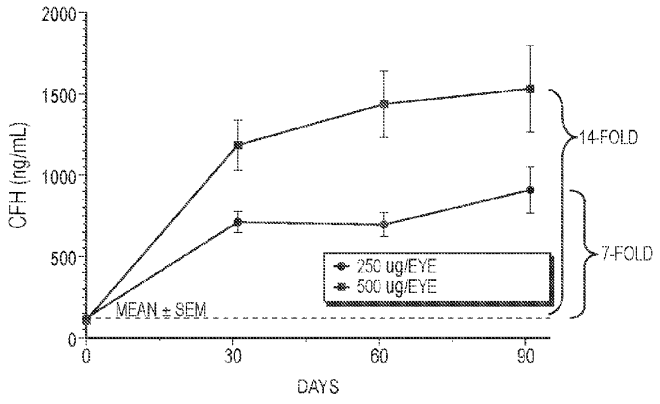


FIG. 6

(57) Abstract: The present disclosure relates to dosage regimens of complement factor H (CFH) protein for treating patients having inflammatory ocular diseases or conditions (e.g., age-related macular degeneration or early-onset macular dystrophies). The present disclosure also provides methods of treating patients having inflammatory ocular diseases, disorders, or conditions (*e.g.*, age-related macular degeneration) using complement factor H (CFH) based on the levels of certain protein biomarkers, such as complement components and inflammation markers, in ocular samples of the patients.

[Continued on next page]

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— *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))*

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— *with sequence listing part of description (Rule 5.2(a))*

METHODS FOR TREATING INFLAMMATORY OCULAR DISEASES WITH COMPLEMENT FACTOR H

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of and priority to U.S. Provisional Patent Application No. 63/107,736, filed October 30, 2020, the entire disclosure of which is hereby incorporated by reference herein in its entirety for all purposes.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on October 26, 2021, is named GEM-019WO_SL_ST25.txt and is 98,500 bytes in size.

FIELD OF THE INVENTION

[0003] The invention relates to dosage regimens of complement factor H (CFH) protein for treating patients having inflammatory ocular diseases or conditions (*e.g.*, age-related macular degeneration or early-onset macular dystrophies). The invention also relates to methods of treating patients having inflammatory ocular diseases or conditions using CFH based on the levels of certain protein biomarkers in ocular samples of the patients.

BACKGROUND OF THE DISCLOSURE

[0004] Age-related macular degeneration (AMD) is a medical condition and is the leading cause of legal blindness in Western societies. AMD typically affects older adults and results in a loss of central vision due to degenerative and neovascular changes to the macula, a pigmented region at the center of the retina which is responsible for visual acuity. There are four major AMD subtypes: early AMD; intermediate AMD; advanced non-neovascular AMD ("dry AMD"); and advanced neovascular AMD ("wet AMD"). Typically, AMD is identified by the focal hyperpigmentation of the retinal pigment epithelium (RPE) and accumulation of drusen deposits and/or geographic atrophy (GA). The size and number of drusen deposits and level of geographic atrophy typically correlates with AMD severity.

[0005] AMD occurs in up to 8% of individuals over the age of 60, and the prevalence of AMD continues to increase with age. The U.S. is anticipated to have nearly 22 million cases of

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AMD by the year 2050, while global cases of AMD are expected to be nearly 288 million by the year 2040. There is a need for novel treatments for preventing progression from early to intermediate and/or from intermediate to advanced stages of AMD to prevent loss of vision. In particular, there remains a need to identify patients that are likely to respond to such novel treatments and a need to monitor the response and efficacy of patients to such novel treatments.

SUMMARY OF THE DISCLOSURE

[0006] The disclosure relates to dosage regimens of complement factor H (CFH) protein for treating patients having inflammatory ocular diseases or conditions (e.g., age-related macular degeneration or early-onset macular dystrophies). The present disclosure also provides methods for treating, preventing, or inhibiting an inflammatory ocular disease, disorder, or condition comprising administering to a subject complement factor H (CFH).

[0007] Accordingly, in one aspect, the present disclosure provides a method of treating a subject having an inflammatory ocular disease, disorder, or condition, the method comprising administering to the subject 50 μg, 100 μg, 250 μg, or 500 μg of CFH protein per eye, thereby treating the inflammatory ocular disease, disorder, or condition. In certain embodiments, the CFH protein is administered to the subject by intravitreal (IVT) injection.

[0008] In certain embodiments, the inflammatory ocular disease, disorder, or condition is selected from the group consisting of macular degeneration, diabetic retinopathy, diabetic macular edema, retinal vein occlusion, uveitis, sterile conjunctivitis, keratitis, episcleritis, and Stargardt's Disease. In certain embodiments, the macular degeneration is AMD. In certain embodiments, the AMD is dry AMD. In certain embodiments, the inflammatory ocular disease, disorder, or condition comprises geographic atrophy secondary to dry AMD. In certain embodiments, the AMD is neovascular AMD. In certain embodiments, the subject has been treated with a VEGF-A antagonist. In certain embodiments, the uveitis is anterior uveitis.

[0009] In certain embodiments, the CFH is administered by intravitreal injection.

[0010] In certain embodiments, the CFH protein comprises a recombinant CFH protein. In certain embodiments, the CFH protein comprises the amino acid sequence of SEQ ID NO: 2.

[0011] In certain embodiments, the CFH protein is administered (*e.g.,* intravitreally) at a dose of 50 μg per eye. In certain embodiments, the CFH protein is administered (*e.g.,* intravitreally) at a dose of 100 μg per eye. In certain embodiments, the CFH protein is

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administered (*e.g.,* intravitreally) at a dose of 250 μg per eye. In certain embodiments, the CFH protein is administered (*e.g.,* intravitreally) at a dose of 500 μg per eye. In certain embodiments, the CFH protein is administered once every four weeks, once every eight weeks, once every month, once every two months, once every three months, once every four months, once every five months, or once every six months. In certain embodiments, the dose of CFH protein is 250 μg per eye, administered once every month or once every four weeks. In certain embodiments, the dose of CFH protein is 250 μg per eye, administered once every two months or once every eight weeks. In certain embodiments, the dose of CFH protein is 500 μg per eye, administered once every month or once every four weeks. In certain embodiments, the dose of CFH protein is 500 μg per eye, administered once every two months or once every eight weeks.

[0012] In certain embodiments, a lesion associated with the inflammatory ocular disease, disorder, or condition is observed in the subject, for example, by fundus imaging. In certain embodiments, the subject carries a genetic variant associated with the inflammatory ocular disease, disorder, or condition. In certain embodiments, the inflammatory ocular disease, disorder, or condition is geographic atrophy, and wherein the genetic variant results in a mutation of the Tyr at amino acid position 402 of human CFH protein and is present in both alleles of the genome. In certain embodiments, the genetic variant results in a mutation of the Tyr at amino acid position 402 of human CFH protein to His residue and is present in both alleles of the genome. In certain embodiments, the genetic variant comprises rs1061170 in both alleles of the human genome. In certain embodiments, the subject is homozygous for the Y402H mutation of the CFH gene. In certain embodiments, the subject is negative for any one of the missense CFH mutations selected from the group consisting of R2T, L3V, R53C, R53H, S58A, D90G, D130N, R175Q, R175P, I221V, R303W, R303Q, Q400K, P503A, R567G, G650V, S890I, T956M, G1194D, R1210C, and any combination(s) thereof. In certain embodiments, the subject is negative for a combination of homozygous CFH 62V, homozygous C3 102G, and homozygous CFB 32R. In certain embodiments, the subject is (a) homozygous for the Y402H mutation of CFH; (b) negative for any one of the missense CFH mutations selected from the group consisting of R2T, L3V, R53C, R53H, S58A, D90G, D130N, R175Q, R175P, I221V, R303W, R303Q, Q400K, P503A, R567G, G650V, S890I, T956M, G1194D, R1210C, and any combination(s) thereof; and (c) negative for a combination of homozygous CFH 62V, homozygous C3 102G, and homozygous CFB 32R. In certain embodiments, the subject is positive for a missense CFH

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mutation selected from the group consisting of R2T, L3V, R53C, R53H, S58A, D90G, D130N, R175Q, R175P, I221V, R303W, R303Q, Q400K, P503A, R567G, G650V, S890I, T956M, G1194D, R1210C, and any combination(s) thereof. In certain embodiments, the subject is positive for a combination of homozygous CFH 62V, homozygous C3 102G, and homozygous CFB 32R. In certain embodiments, the subject is at least 50 years old.

[0013] In certain embodiments, the method further comprises administering to the subject an effective amount of a VEGF antagonist. In certain embodiments, the subject has neovascular AMD. In certain embodiments, the VEGF antagonist comprises aflibercept. In certain embodiments, the effective amount of aflibercept is 2 mg per eye. In certain embodiments, the aflibercept is administered once every four weeks, once every eight weeks, once every month, or once every two months.

[0014] In certain embodiments, the treatment slows the progression of the inflammatory ocular disease, disorder, or condition in the subject. In certain embodiments, the treatment reduces the severity of the inflammatory ocular disease, disorder, or condition in the subject. In certain embodiments, the progression or severity of the inflammatory ocular disease, disorder, or condition is assessed by one or more methods selected from:

(a) best corrected visual acuity (BCVA) score;

(b) low luminance visual acuity (LLVA) score;

(c) AREDS 9-step severity scale score;

(d) area of geographic atrophy assessed by color fundus photography, fundus autofluorescence, optical coherence tomography, optical coherence tomography – angiography, near infrared imaging, and/or fluorescein angiography;

(e) drusen volume; and

(f) one or more retinal architecture parameters assessed by optical coherence tomography, selected from total retinal and choroidal thickness, photoreceptor layer thickness, features of nascent geographic atrophy, retinal pigment epithelium thickening, and integrity of retinal pigment epithelium layer.

[0015] Accordingly, in another aspect, the present disclosure provides a method for treating a subject having an inflammatory ocular disease, disorder, or condition, the method comprising:

(a) obtaining or having obtained a measurement of the protein level of at least one biomarker in an ocular sample of the subject, wherein the biomarker is selected from the group

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consisting of (i) complement component C3, complement factor B (CFB), complement component C5, cleavage fragments thereof, and complement factor H (CFH), (ii) proteins associated with ocular inflammation, and (iii) proteins associated with choroidal neovascularization;

(b) determining whether the protein level of the biomarker is greater than or lower than a predetermined threshold; and

(c) administering to the subject an effective amount of CFH if the protein level of the biomarker is (i) greater than or equal to the threshold if the biomarker is positively correlated with activation of the complement pathway or with inflammation or (ii) lower than or equal to the threshold if the biomarker is negatively correlated with activation of the complement pathway or with inflammation,

thereby treating the inflammatory ocular disease, disorder, or condition.

[0016] In certain embodiments, the inflammatory ocular disease, disorder, or condition is selected from the group consisting of macular degeneration, diabetic retinopathy, diabetic macular edema, retinal vein occlusion, uveitis, sterile conjunctivitis, keratitis, episcleritis, and Stargardt's Disease. In certain embodiments, the macular degeneration is age-related macular degeneration (AMD). In certain embodiments, the AMD is dry AMD. In certain embodiments, the inflammatory ocular disease, disorder, or condition comprises geographic atrophy secondary to dry AMD. In certain embodiments, the AMD is neovascular AMD. In certain embodiments, the subject has been treated with a VEGF (*e.g.*, VEGF-A) antagonist. In certain embodiments, the uveitis is anterior uveitis.

[0017] In certain embodiments, the ocular sample comprises aqueous humor.

[0018] In certain embodiments, the at least one biomarker comprises a cleavage fragment of C3, CFB, or C5, and the protein level of the cleavage fragment is positively correlated with activation of the complement pathway. In certain embodiments, the cleavage fragment is selected from the group consisting of C3a, Ba, and C5a. In certain embodiments, the cleavage fragment is C3a. In certain embodiments, the threshold of C3a is 2 ng/mL. In certain embodiments, the cleavage fragment is Ba. In certain embodiments, the threshold of Ba is 8 ng/mL. In certain embodiments, the at least one biomarker comprises CFH, and the protein level of CFH is negatively correlated with activation of the complement pathway. In certain embodiments, the threshold of CFH is 60 ng/mL.

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[0019] In certain embodiments, the at least one biomarker comprises a protein associated with ocular inflammation. In certain embodiments, the protein associated with ocular inflammation is selected from the group consisting of IL-1β, IL-6, IL-8, IL-10, IL-18, TNF-α, CCL2, CXCL5, and Eotaxin-2, and the protein level of the biomarker is positively correlated with ocular inflammation.

[0020] In certain embodiments, the at least one biomarker comprises a protein associated with choroidal neovascularization. In certain embodiments, the protein associated with choroidal neovascularization is VEGF-A, and the protein level of the biomarker is positively correlated with choroidal neovascularization.

[0021] In certain embodiments, the CFH is administered by intravitreal injection.

[0022] In certain embodiments, the CFH comprises a CFH protein. In certain embodiments, the CFH protein comprises a recombinant CFH protein. In certain embodiments, the CFH protein is administered at a dose of 50 μg, 100 μg, 250 μg, or 500 μg per eye. In certain embodiments, the CFH protein is administered (*e.g.,* intravitreally) at a dose of 50 μg per eye. In certain embodiments, the CFH protein is administered (*e.g.,* intravitreally) at a dose of 100 μg per eye. In certain embodiments, the CFH protein is administered (*e.g.,* intravitreally) at a dose of 250 μg per eye. In certain embodiments, the CFH protein is administered (*e.g.,* intravitreally) at a dose of 500 μg per eye. In certain embodiments, the CFH protein is administered once every four weeks, once every eight weeks, once every month, once every two months, once every three months, once every four months, once every five months, or once every six months. In certain embodiments, the dose of CFH protein is 250 μg per eye, administered once every month or once every four weeks. In certain embodiments, the dose of CFH protein is 250 μg per eye, administered once every two months or once every eight weeks. In certain embodiments, the dose of CFH protein is 500 μg per eye, administered once every month or once every four weeks. In certain embodiments, the dose of CFH protein is 500 μg per eye, administered once every two months or once every eight weeks.

[0023] In certain embodiments, the CFH comprises a vector encoding a CFH protein. In certain embodiments, the vector is an adeno-associated virus vector.

[0024] In certain embodiments, the CFH protein comprises the amino acid sequence of SEQ ID NO: 2.

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[0025] In certain embodiments, the steps (a)-(c) are conducted after an initial administration of CFH to the subject.

[0026] In certain embodiments, a lesion associated with the inflammatory ocular disease, disorder, or condition is observed in the subject, for example, by fundus imaging. In certain embodiments, the subject carries a genetic variant associated with the inflammatory ocular disease, disorder, or condition. In certain embodiments, the inflammatory ocular disease, disorder, or condition is geographic atrophy, and wherein the genetic variant results in a mutation of the Tyr at amino acid position 402 of human CFH protein and is present in both alleles of the genome. In certain embodiments, the genetic variant results in a mutation of the Tyr at amino acid position 402 of human CFH protein to His residue and is present in both alleles of the genome. In certain embodiments, the genetic variant comprises rs1061170 in both alleles of the human genome. In certain embodiments, the subject is homozygous for the Y402H mutation of the CFH gene. In certain embodiments, the subject is negative for any one of the missense CFH mutations selected from the group consisting of R2T, L3V, R53C, R53H, S58A, D90G, D130N, R175Q, R175P, I221V, R303W, R303Q, Q400K, P503A, R567G, G650V, S890I, T956M, G1194D, R1210C, and any combination(s) thereof. In certain embodiments, the subject is negative for a combination of homozygous CFH 62V, homozygous C3 102G, and homozygous CFB 32R. In certain embodiments, the subject is (a) homozygous for the Y402H mutation of CFH; (b) negative for any one of the missense CFH mutations selected from the group consisting of R2T, L3V, R53C, R53H, S58A, D90G, D130N, R175Q, R175P, I221V, R303W, R303Q, Q400K, P503A, R567G, G650V, S890I, T956M, G1194D, R1210C, and any combination(s) thereof; and (c) negative for a combination of homozygous CFH 62V, homozygous C3 102G, and homozygous CFB 32R. In certain embodiments, the subject is positive for a missense CFH mutation selected from the group consisting of R2T, L3V, R53C, R53H, S58A, D90G, D130N, R175Q, R175P, I221V, R303W, R303Q, Q400K, P503A, R567G, G650V, S890I, T956M, G1194D, R1210C, and any combination(s) thereof. In certain embodiments, the subject is positive for a combination of homozygous CFH 62V, homozygous C3 102G, and homozygous CFB 32R. In certain embodiments, the subject is at least 50 years old.

[0027] In certain embodiments, the method further comprises administering to the subject an effective amount of a VEGF antagonist. In certain embodiments, the subject has neovascular AMD. In certain embodiments, the VEGF antagonist comprises aflibercept. In certain

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embodiments, the effective amount of aflibercept is 2 mg per eye. In certain embodiments, the aflibercept is administered once every four weeks, once every eight weeks, once every month, or once every two months.

[0028] In certain embodiments, the treatment slows the progression of the inflammatory ocular disease, disorder, or condition in the subject. In certain embodiments, the treatment reduces the severity of the inflammatory ocular disease, disorder, or condition in the subject. In certain embodiments, the progression or severity of the inflammatory ocular disease, disorder, or condition is assessed by one or more methods selected from:

(a) best corrected visual acuity (BCVA) score;

(b) low luminance visual acuity (LLVA) score;

(c) AREDS 9-step severity scale score;

(d) area of geographic atrophy assessed by color fundus photography, fundus autofluorescence, optical coherence tomography, optical coherence tomography – angiography, near infrared imaging, and/or fluorescein angiography;

(e) drusen volume; and

(f) one or more retinal architecture parameters assessed by optical coherence tomography, selected from total retinal and choroidal thickness, photoreceptor layer thickness, features of nascent geographic atrophy, retinal pigment epithelium thickening, and integrity of retinal pigment epithelium layer.

[0029] In another aspect, the present disclosure provides a method for selecting a subject for treatment of an inflammatory ocular disease, disorder, or condition, the method comprising:

(a) obtaining or having obtained a measurement of the protein level of at least one biomarker in an ocular sample of the subject, wherein the biomarker is selected from the group consisting of (i) complement component C3, CFB, complement component C5, cleavage fragments thereof, and CFH, (ii) proteins associated with ocular inflammation, and (iii) proteins associated with choroidal neovascularization;

(b) determining whether the protein level of the biomarker is greater than or lower than a predetermined threshold; and

(c) selecting the subject for treatment of the inflammatory ocular disease, disorder, or condition if the protein level of the biomarker is (i) greater than or equal to the threshold if the biomarker is positively correlated with activation of the complement pathway, ocular

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inflammation, or choroidal neovascularization; or (ii) lower than or equal to the threshold if the biomarker is negatively correlated with activation of the complement pathway, ocular inflammation, or choroidal neovascularization,

wherein the treatment comprises administering to the subject CFH.

[0030] In certain embodiments, step (a) comprises measuring the protein level of the biomarker in an ocular sample of the subject.

[0031] In certain embodiments, the inflammatory ocular disease, disorder, or condition is selected from the group consisting of macular degeneration, diabetic retinopathy, diabetic macular edema, retinal vein occlusion, uveitis, sterile conjunctivitis, keratitis, episcleritis, and Stargardt's Disease. In certain embodiments, the macular degeneration is AMD. In certain embodiments, the AMD is dry AMD. In certain embodiments, the inflammatory ocular disease, disorder, or condition comprises geographic atrophy secondary to dry AMD. In certain embodiments, the AMD is neovascular AMD. In certain embodiments, the subject has been treated with a VEGF (*e.g.*, VEGF-A) antagonist. In certain embodiments, the uveitis is anterior uveitis.

[0032] In certain embodiments, the ocular sample comprises aqueous humor.

[0033] In certain embodiments, the at least one biomarker comprises a cleavage fragment of C3, CFB, or C5, and the protein level of the cleavage fragment is positively correlated with activation of the complement pathway. In certain embodiments, the biomarker is selected from the group consisting of C3a, Ba, and C5a. In certain embodiments, the biomarker is C3a. In certain embodiments, the threshold of C3a is 2 ng/mL. In certain embodiments, the biomarker is Ba. In certain embodiments, the threshold of Ba is 8 ng/mL. In certain embodiments, the at least one biomarker comprises CFH, and the protein level of CFH is negatively correlated with activation of the complement pathway. In certain embodiments, the threshold of CFH is 60 ng/mL.

[0034] In certain embodiments, the at least one biomarker comprises a protein associated with ocular inflammation. In certain embodiments, the protein associated with ocular inflammation is selected from the group consisting of IL-1β, IL-6, IL-8, IL-10, IL-18, TNF-α, CCL2, CXCL5, and Eotaxin-2, and the protein level of the biomarker is positively correlated with ocular inflammation.

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[0035] In certain embodiments, the at least one biomarker comprises a protein associated with choroidal neovascularization. In certain embodiments, the protein associated with choroidal neovascularization is VEGF-A, and the protein level of the biomarker is positively correlated with choroidal neovascularization.

[0036] In certain embodiments, the CFH is administered by intravitreal injection.

[0037] In certain embodiments, the CFH comprises a CFH protein. In certain embodiments, the CFH protein comprises a recombinant CFH protein. In certain embodiments, the CFH protein is administered at a dose of 50 μg, 100 μg, 250 μg, or 500 μg per eye. In certain embodiments, the CFH protein is administered (*e.g.*, intravitreally) at a dose of 50 μg per eye. In certain embodiments, the CFH protein is administered (*e.g.*, intravitreally) at a dose of 100 μg per eye. In certain embodiments, the CFH protein is administered (*e.g.*, intravitreally) at a dose of 250 μg per eye. In certain embodiments, the CFH protein is administered (*e.g.*, intravitreally) at a dose of 500 μg per eye. In certain embodiments, the CFH protein is administered once every four weeks, once every eight weeks, once every month, once every two months, once every three months, once every four months, once every five months, or once every six months. In certain embodiments, the dose of CFH protein is 250 μg per eye, administered once every month or once every four weeks. In certain embodiments, the dose of CFH protein is 250 μg per eye, administered once every two months or once every eight weeks. In certain embodiments, the dose of CFH protein is 500 μg per eye, administered once every month or once every four weeks. In certain embodiments, the dose of CFH protein is 500 μg per eye, administered once every two months or once every eight weeks.

[0038] In certain embodiments, the CFH comprises a vector encoding a CFH protein. In certain embodiments, the vector is an adeno-associated virus vector.

[0039] In certain embodiments, the CFH protein comprises the amino acid sequence of SEQ ID NO: 2.

[0040] In certain embodiments, the steps (a)-(c) are conducted after an initial administration of CFH to the subject.

[0041] In certain embodiments, a lesion associated with the inflammatory ocular disease, disorder, or condition is observed in the subject, for example, by fundus imaging. In certain embodiments, the subject carries a genetic variant associated with the inflammatory ocular disease, disorder, or condition. In certain embodiments, the inflammatory ocular disease,

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disorder, or condition is geographic atrophy, and wherein the genetic variant results in a mutation of the Tyr at amino acid position 402 of human CFH protein and is present in both alleles of the genome. In certain embodiments, the genetic variant comprises rs1061170 in both alleles of the human genome. In certain embodiments, the genetic variant results in a mutation of the Tyr at amino acid position 402 of human CFH protein to His residue and is present in both alleles of the genome. In certain embodiments, the genetic variant comprises rs1061170 in both alleles of the human genome. In certain embodiments, the subject is homozygous for the Y402H mutation of the CFH gene. In certain embodiments, the subject is negative for any one of the missense CFH mutations selected from the group consisting of R2T, L3V, R53C, R53H, S58A, D90G, D130N, R175Q, R175P, I221V, R303W, R303Q, Q400K, P503A, R567G, G650V, S890I, T956M, G1194D, R1210C, and any combination(s) thereof. In certain embodiments, the subject is negative for a combination of homozygous CFH 62V, homozygous C3 102G, and homozygous CFB 32R. In certain embodiments, the subject is (a) homozygous for the Y402H mutation of CFH; (b) negative for any one of the missense CFH mutations selected from the group consisting of R2T, L3V, R53C, R53H, S58A, D90G, D130N, R175Q, R175P, I221V, R303W, R303Q, Q400K, P503A, R567G, G650V, S890I, T956M, G1194D, R1210C, and any combination(s) thereof; and (c) negative for a combination of homozygous CFH 62V, homozygous C3 102G, and homozygous CFB 32R. In certain embodiments, the subject is positive for a missense CFH mutation selected from the group consisting of R2T, L3V, R53C, R53H, S58A, D90G, D130N, R175Q, R175P, I221V, R303W, R303Q, Q400K, P503A, R567G, G650V, S890I, T956M, G1194D, R1210C, and any combination(s) thereof. In certain embodiments, the subject is positive for a combination of homozygous CFH 62V, homozygous C3 102G, and homozygous CFB 32R. In certain embodiments, the subject is at least 50 years old.

[0042] In certain embodiments, the method further comprises administering to the subject an effective amount of a VEGF antagonist. In certain embodiments, the subject has neovascular AMD. In certain embodiments, the VEGF antagonist comprises aflibercept. In certain embodiments, the effective amount of aflibercept is 2 mg per eye. In certain embodiments, the aflibercept is administered once every four weeks, once every eight weeks, once every month, or once every two months.

[0043] In certain embodiments, the treatment slows the progression of the inflammatory ocular disease, disorder, or condition in the subject. In certain embodiments, the treatment

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reduces the severity of the inflammatory ocular disease, disorder, or condition in the subject. In certain embodiments, the progression or severity of the inflammatory ocular disease, disorder, or condition is assessed by one or more methods selected from:

(a) best corrected visual acuity (BCVA) score;

(b) low luminance visual acuity (LLVA) score;

(c) AREDS 9-step severity scale score;

(d) area of geographic atrophy assessed by color fundus photography, fundus autofluorescence, optical coherence tomography, optical coherence tomography – angiography, near infrared imaging, and/or fluorescein angiography;

(e) drusen volume; and

(f) one or more retinal architecture parameters assessed by optical coherence tomography, selected from total retinal and choroidal thickness, photoreceptor layer thickness, features of nascent geographic atrophy, retinal pigment epithelium thickening, and integrity of retinal pigment epithelium layer.

[0044] In another aspect,.

BRIEF DESCRIPTION OF THE DRAWINGS

[0045] **FIGs. 1A-1C** are a set of graphs showing reduction of C3bBb deposition (FIG. 1A), reduction of C3a generation (FIG. 1B), and reduction of Ba generation (FIG. 1C) in a retinal pigment epithelium (RPE) cell culture as a result of exogenous supplementation of recombinant CFH protein (rCFH) in a dose dependent manner.

[0046] **FIG. 2** is a diagram of a clinical study design for a single-ascending dose (SAD) of recombinant CFH illustrating a cohort patient flow. DLTs = dose limiting toxicity; MTD = maximum tolerated dose; n = number of subjects; TBD = to be determined; wk = week.

[0047] **FIGs. 3A-3B** are a set of graphs showing the levels of Ba (FIG. 3A) and C3a (FIG. 3B) in aqueous humor samples obtained from patients on the days indicated after the rCFH treatment relative to the respective levels prior to the rCFH treatment (Day 0). The p values were calculated under the two tailed t-test, which makes no assumption of expected direction of change. Where an assumption of reduced Ba and C3a levels is made, the p values should be reduced by half.

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[0048] **FIGs. 4A-4B** are a set of graphs showing the best corrected visual acuity (BCVA) (FIG. 4A) and low luminance visual acuity (LLVA) (FIG. 4B) scores of the patients enrolled in the phase 1 clinical study on the days indicated after the rCFH treatment relative to the respective scores prior to the rCFH treatment (Day 0).

[0049] **FIG. 5** is a diagram of a clinical study design for a Phase 2 multiple dose study of recombinant CFH (rCFH) in patients with dry AMD. BL=baseline; min=minimum; PD=pharmacodynamics; PK=pharmacokinetics; q30d=every 30 days; q60d=every 60 days; *In Version 1.0 of the protocol, eligible subjects with non-central GA were enrolled in Cohort 1 and those with central GA were enrolled in Cohort 2.

[0050] **FIG. 6** is a graph showing total CFH levels in aqueous humor samples obtained from patients on the days indicated after the rCFH treatment relative to the respective levels prior to the rCFH treatment (Day 0). By the indicated time points, the patients were administered either 250 μg of rCFH monthly or 500 μg of rCFH monthly.

[0051] **FIGs. 7A-7C** are a set of graphs showing Ba concentration levels in aqueous humor after administration of either 250 μg of rCFH monthly or 500 μg of rCFH monthly (FIG. 7A); 250 μg of rCFH monthly (FIG. 7B); or 500 μg of rCFH monthly(FIG. 7C). "Healthy" Ba levels in FIGs. 7A-7C range from 7 ng/mL to 21 ng/mL (with an average of 11.7 ng/mL). Quartiles 1-4 (abbreviated as Q1-Q4) denote Ba levels between 5.4 ng/mL and 16.65 ng/mL; between 16.91 ng/mL and 25.87 ng/mL; between 26.25 ng/mL and 44.22 ng/mL; and between 44.46 ng/mL and 165.6 ng/mL, respectively.

[0052] **FIG. 8** is a graph showing C3a concentration levels in aqueous humor after administration of either 250 μg of rCFH monthly or 500 μg of rCFH monthly. "Healthy" C3a levels in range from 1300 pg/mL to 4100 pg/mL (with an average of 2700 pg/mL). Quartiles 1-4 denote C3a levels between 1097 pg/mL and 3442 pg/mL; between 3510 pg/mL and 4950 pg/mL; between 5040 pg/mL and 6968 pg/mL; and between 7022 pg/mL and 17882 pg/mL, respectively.

[0053] **FIG. 9** is a scatter dot plot showing correlation Ba and C3a concentration levels at baseline and after administration of rCFH.

[0054] **FIG. 10** is a diagram of a clinical study design for Phase 2 multiple dose study of recombinant CFH (rCFH) as an adjunct to standard of care Aflibercept therapy in patients with neovascular AMD (nAMD).

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DETAILED DESCRIPTION OF THE DISCLOSURE

[0055] The disclosure provides methods for treating, preventing, or inhibiting an inflammatory ocular disease, disorder, or condition comprising administering to a subject complement factor H (CFH). In one aspect, the disclosure provides dosage regimens of CFH protein for treating an ocular disease or disorder. In another aspect, the disclosure provides a method of treating, preventing, or inhibiting an inflammatory ocular disease, disorder, or condition in a patient who is selected based on the protein level of one or more biomarkers in an ocular sample, relative to a predetermined threshold, and intraocularly (*e.g.*, intravitreally) administering an effective amount of CFH (*e.g.*, a recombinant CFH protein or biologically active fragments and/or variants thereof).

[0056] A wide variety of diseases of the eye associated with the presence of selected biomarkers may be treated or prevented using the methods provided herein. Inflammatory ocular diseases, disorders, or conditions that may be treated or prevented using the methods of the disclosure include but are not limited to glaucoma diabetic retinopathies, inherited retinal degeneration such as retinitis pigmentosa, retinal detachment or injury and retinopathies (such as retinopathies that are inherited, induced by surgery, trauma, an underlying aetiology such as severe anemia, SLE, hypertension, blood dyscrasias, systemic infections, or underlying carotid disease, a toxic compound or agent, or photically). Inflammatory ocular diseases, disorders, or conditions include, but is not limited to, macular degeneration, *e.g.*, age-related macular degeneration (AMD), dry AMD, geographic atrophy (GA) secondary to dry AMD, neovascular AMD, uveitis (*e.g.*, anterior uveitis), sterile conjunctivitis, keratitis, episcleritis, Stargardt's Disease, diabetic retinopathy, diabetic macular edema, and retinal vein occlusion.

Definitions

[0057] The following terms, unless otherwise indicated, shall be understood to have the following meanings:

[0058] As used herein, and unless otherwise stated, the term "complement factor H" or "CFH" encompasses all the isoforms of complement factor H, including the canonical isoform (the human protein identified as P08603-1 in UniProt) and a second isoform called factor-H-like protein 1 (FHL1) (the human protein identified as P08603-2 in UniProt), and functional fragments and/or variants thereof. Exemplary amino acid sequences of CFH are provided in

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SEQ ID NOs: 1-10. Unless indicated otherwise expressly or by context (*e.g.*, "recombinant CFH" or "rCFH"), this term also refers to a vector encoding a CFH protein.

[0059] As used herein, the term "biomarker" refers to a substance, *e.g.*, a protein or a nucleic acid, where the presence, absence or concentration of the biomarker in a biological sample from a subject indicates the subject's disease status and/or potential response or lack of response to a therapeutic treatment. For example, the concentration of a biomarker in an ocular or blood sample from a subject having an inflammatory ocular disease, disorder, or condition, relative to the concentration of the biomarker in a control sample of the same type (*e.g.*, a sample of the same type from a subject not having the inflammatory ocular disease, disorder, or condition), may be used to select a subject for a given therapeutic treatment and/or to monitor the progress of the treatment.

[0060] As used herein, the term "cleavage fragment" refers to a peptide fragment of a larger precursor protein, wherein the cleavage fragment is produced as a consequence of a proteolytic cleavage event, *e.g.*, cleavage by a protease or self-cleavage.

[0061] As used herein, the term "proteins associated with ocular inflammation" refers to any protein that is associated with increased inflammation, either with inducing inflammation or as a result of inflammatory responses in an ocular tissue. Examples of proteins associated with inflammation include, but are not limited to inflammatory cytokines (*e.g.*, interleukin-1 (IL-1), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin 12 (IL-12), interleukin 18 (IL-18), tumor necrosis factor alpha (TNFα, a.k.a. TNF), interferon gamma (INF-γ), granulocyte-macrophage colony stimulating factor (GMCSF)), chemokines (*e.g.*, (C-Cmotif) ligand 2 (CCL2)/monocyte chemoattractant protein 1 (MCP1), C-X-C motif chemokine 5 (CXCL5), C-C motif chemokine 24 (CCL24, a.k.a. Eotaxin-2)), and other factors (*e.g.*, uncleaved complement component C3).

[0062] As used herein, the terms "patient," "subject," and "individual" are used interchangeably herein and refer to either a human or a non-human animal. These terms include mammals, such as humans, non-human primates, laboratory animals, livestock animals (including bovines, porcines, camels, etc.), companion animals (*e.g.*, canines, felines, other domesticated animals, etc.) and rodents (*e.g.*, mice and rats).

[0063] As used herein, the terms "treat," "treating," and "treatment," with respect to a disease, disorder or condition in a patient, refers to taking steps to obtain beneficial or desired

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results such as clinical results in the patient. Exemplary beneficial or desired clinical results include reduction or amelioration of the progression, severity, and/or duration of (a) the disease, disorder or condition, (b) one or more symptoms associated therewith, or (c) one or more symptoms resulting from the administration of one or more therapies (including, without limitation, administration of one or more prophylactic or therapeutic agents) directed to the disease, disorder or condition. It is understood that treatment of a disease, disorder or condition in a patient does not require that the patient manifests clinical symptoms. Rather, a patient can be identified as having an increased risk of developing clinical symptoms or progressing to an advanced stage of disease by retinal imaging and/or by one or more genetic markers and/or biomarkers, and the treatment can be used prophylactically for preventing or slowing clinical development of the disease, disorder or condition. For example, a patient who is diagnosed with intermediate AMD and identified as having an increased risk of progressing to geographic atrophy or neovascular AMD can be treated prophylactically for preventing or slowing the clinical progression.

[0064] As used herein, the terms "prevent," "preventing," and "prevention" refer to the prevention of the onset or recurrence of (a) a disease, disorder or condition, (b) one or more symptoms associated therewith, or (c) one or more symptoms resulting from the administration of one or more therapies (including, without limitation, administration of one or more prophylactic or therapeutic agents) directed to the disease, disorder or condition.

[0065] As used herein, the term "effective amount" refers to the amount of a substance (*e.g.*, a protein of the present disclosure) sufficient to effect beneficial or desired results. 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, administration route, or frequency of administration.

[0066] As used herein, the term "ocular cell" refers to any cell in, or associated with the function of, the eye. The term may refer to any one or more of photoreceptor cells, including rod, cone and photosensitive ganglion cells, retinal pigment epithelium (RPE) cells, glial cells, Muller cells, bipolar cells, horizontal cells, amacrine cells. In one embodiment, the ocular cells are bipolar cells. In another embodiment, the ocular cells are horizontal cells. In another embodiment, the ocular cells are ganglion cells. In yet another embodiment, the ocular cells are RPE cells.

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[0067] The terms "polypeptide," "oligopeptide," "peptide" and "protein" are used interchangeably herein to refer to chains of amino acids of any length. The chain may be linear or branched, it may comprise modified amino acids, and/or may be interrupted by non-amino acids. The terms also encompass an amino acid chain that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. It is understood that the polypeptides can occur as single chains or associated chains.

[0068] As known in the art, "polynucleotide," or "nucleic acid," as used interchangeably herein, refer to chains of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a chain by DNA or RNA polymerase. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the chain. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, "caps," substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (*e.g.*, nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (*e.g.*, acridine, psoralen, etc.), those containing chelators (*e.g.*, metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (*e.g.*, alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted

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with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, alpha- or beta-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), (O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

[0069] Percent (%) sequence identity of a given amino acid sequence or nucleotide sequence (the "query sequence"), with respect to a reference sequence, is defined as the percentage of amino acid residues or nucleotides in the query sequence that are identical with the amino acid residues or nucleotides in the reference sequence after aligning the sequences (and introducing gaps, if necessary) to achieve the maximum percent sequence identity, not considering any conservative substitutions as part of the 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 or Megalign (DNASTAR) 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.

[0070] Unless otherwise defined herein, scientific and technical terms used in this application shall have the meanings that are commonly understood by those of ordinary skill in the art. Generally, nomenclature used in connection with, and techniques of, pharmacology, cell and tissue culture, molecular biology, cell and cancer biology, neurobiology, neurochemistry, virology, immunology, microbiology, genetics and protein and nucleic acid chemistry, described herein, are those well-known and commonly used in the art. In case of conflict, the present specification, including definitions, will control.

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[0071] The practice of the present disclosure will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, Molecular Cloning: A Laboratory Manual, second edition (Sambrook et al., 1989) Cold Spring Harbor Press; Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook (J.E. Cellis, ed., 1998) Academic Press; Animal Cell Culture (R.I. Freshney, ed., 1987); Introduction to Cell and Tissue Culture (J.P. Mather and P.E. Roberts, 1998) Plenum Press; Cell and Tissue Culture: Laboratory Procedures (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., 1993-1998) J. Wiley and Sons; Methods in Enzymology (Academic Press, Inc.); Gene Transfer Vectors for Mammalian Cells (J.M. Miller and M.P. Calos, eds., 1987); Current Protocols in Molecular Biology (F.M. Ausubel et al., eds., 1987); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (2001); Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, NY (2002); Harlow and Lane Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1998); Coligan et al., Short Protocols in Protein Science, John Wiley & Sons, NY (2003); Short Protocols in Molecular Biology (Wiley and Sons, 1999). The skilled worker would recognize many additional sources describing conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology.

[0072] Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein. The nomenclatures used in connection with, and the laboratory procedures and techniques of, analytical chemistry, biochemistry, immunology, molecular biology, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art. Standard techniques are used for chemical syntheses, and chemical analyses.

[0073] Throughout this specification and embodiments, the word "comprise," or variations such as "comprises" or "comprising," will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. It is

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understood that wherever embodiments are described herein with the language "comprising," otherwise analogous embodiments described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[0074] The term "including" is used to mean "including but not limited to." "Including" and "including but not limited to" are used interchangeably.

[0075] Any example(s) following the term "*e.g.*" or "for example" is not meant to be exhaustive or limiting.

[0076] Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[0077] The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

[0078] Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example, description referring to "about X" includes description of "X." Similarly, where description refers to a number "X," description referring to "about X" is also contemplated. The term "about" or "approximately" can mean within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *e.g.*, the limitations of the measurement system. For example, "about" can mean plus or minus 10%, per the practice in the art. Alternatively, "about" can mean a range of plus or minus 20%, plus or minus 10%, plus or minus 5%, or plus or minus 1% of a given value. When a value includes associated error measurements; the term "about" typically includes values encompassed within a range of +/- the stated error or +/- twice the stated error, or +/- three times the stated error. In case of doubt, encompassed within the term "about" are numbers that are insignificantly different from the stated number. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, within 5-fold, or within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated, the term "about" meaning within an acceptable error range for the particular value can be assumed. Also, where ranges and/or subranges of values are provided, the ranges and/or subranges can include the endpoints of the ranges and/or subranges.

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[0079] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the disclosure are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements. Moreover, all ranges disclosed herein are to be understood to encompass any and all subranges subsumed therein. For example, a stated range of "1 to 10" should be considered to include any and all subranges between (and inclusive of) the minimum value of 1 and the maximum value of 10; that is, all subranges beginning with a minimum value of 1 or more, *e.g.*, 1 to 6.1, and ending with a maximum value of 10 or less, *e.g.*, 5.5 to 10.

[0080] Where aspects or embodiments of the disclosure are described in terms of a Markush group or other grouping of alternatives, the present disclosure encompasses not only the entire group listed as a whole, but each member of the group individually and all possible subgroups of the main group, but also the main group absent one or more of the group members. The present disclosure also envisages the explicit exclusion of one or more of any of the group members in the disclosure.

[0081] Each embodiment described herein may be used individually or in combination with any other embodiment described herein.

Methods of Treatment

[0082] Disclosed herein are methods of treating, preventing, and/or ameliorating the progression of an ocular inflammatory disease, disorder, or condition, or retinal changes associated therewith, the method comprising administering CFH (*e.g.*, a CFH protein or a vector encoding a CFH protein) to a subject in need thereof.

[0083] In certain embodiments, the subject has, or is at risk of developing a disease of the eye. A disease of the eye, includes, without limitation, AMD, retinitis pigmentosa, rod-cone dystrophy, Leber's congenital amaurosis, Usher's syndrome, Bardet-Biedl Syndrome, early-onset macular dystrophy, Best disease, retinoschisis, uveitis (*e.g.*, anterior uveitis), sterile conjunctivitis, keratitis, Stargardt's disease (autosomal dominant or autosomal recessive), untreated retinal detachment, pattern dystrophy, cone-rod dystrophy, achromatopsia, ocular albinism, enhanced S cone syndrome, diabetic retinopathy, diabetic macular edema, retinopathy of prematurity, sickle cell retinopathy, Congenital Stationary Night Blindness, glaucoma, or retinal vein occlusion. In another embodiment, the subject has, or is at risk of developing

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glaucoma, Leber's hereditary optic neuropathy, lysosomal storage disorder, or peroxisomal disorder. In another embodiment, the subject has shown clinical signs of a disease of the eye.

[0084] An inflammatory ocular disease, disorder, or condition may exist simultaneously with a renal disease or complication in the same subject. In some embodiments, the subject has, or is at risk of developing a renal disease or complication. In some embodiments, the renal disease or complication is associated with AMD or atypical hemolytic uremic syndrome (aHUS). In some embodiments, the subject has, or is at risk of developing AMD or aHUS.

[0085] Clinical signs of a disease of the eye include, but are not limited to, decreased peripheral vision, decreased central (reading) vision, decreased night vision, loss of color perception, reduction in visual acuity, decreased photoreceptor function, and pigmentary changes. In one embodiment, the subject shows degeneration of the outer nuclear layer (ONL). In another embodiment, the subject has been diagnosed with a disease of the eye. In yet another embodiment, the subject has not yet shown clinical signs of a disease of the eye.

[0086] In certain embodiments, the inflammatory ocular disease, disorder, or condition is AMD. In certain embodiments, the AMD is dry AMD. In certain embodiments, the AMD is geographic atrophy (GA) secondary to dry AMD. In certain embodiments, the GA is multifocal. In certain embodiments, the GA that affects the foveal center point. In certain embodiments, the GA has a total size within 0.5 to 15.0 disk areas.

[0087] In some embodiments, the subject is a mammal. In some embodiments, the subject is a human. In some embodiments, the human is an adult. In some embodiments, the human is an elderly adult. In some embodiments, the human (*e.g.*, the human who has or is at risk of age-related macular degeneration) is at least 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, or 95 years of age. In particular embodiments, the human is at least 50 years of age.

[0088] In some embodiments, the method disclosed herein is used in treatment of a patient having dry AMD. In certain embodiments, the patient has geographic atrophy. In certain embodiments, the area of geographic atrophy in an eye of the patient is from 2.5 to 17.5 mm^2 as determined by a method recognized by a clinician, for example, by screening images of fundus autofluorescence (FAF). In certain embodiments, the patient has multifocal geographic atrophy and at least one focal lesion has a size of 1.25 mm^2 or larger.

[0089] In certain embodiments, the subject has a best corrected visual acuity (BCVA) in the affected eye using Early Treatment Diabetic Retinopathy Study (ETDRS) chart VAS of 5-45

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letters (equivalent to Snellen VA of approximately 20/800-20/125). In certain embodiments, the subject does not have any active ocular disease, disorder, or condition that could be a contraindication to intravitreal (IVT) injection.

[0090] The method disclosed herein may be used to prevent the occurrence of retinal damage or to rescue eyes having mild or advanced disease, i.e., to prevent progression of the disease to total blindness, prevent spread of damage to uninjured ocular cells, improve damage in injured ocular cells, or to provide enhanced vision. In one embodiment, the composition is administered before the disease becomes symptomatic or prior to photoreceptor loss. By symptomatic is meant onset of any of the various retinal changes described above or vision loss. In another embodiment, the composition is administered after disease becomes symptomatic. In yet another embodiment, the composition is administered after initiation of photoreceptor loss. In another embodiment, the composition is administered after outer nuclear layer (ONL) degeneration begins. In some embodiments, it is desirable that the composition is administered while bipolar cell circuitry to ganglion cells and optic nerve remains intact.

[0091] In another embodiment, the composition is administered after initiation of photoreceptor loss. In yet another embodiment, the composition is administered when less than 90% of the photoreceptors are functioning or remaining, as compared to a non-diseased eye. In another embodiment, the composition is administered when less than 80% of the photoreceptors are functioning or remaining. In another embodiment, the composition is administered when less than 70% of the photoreceptors are functioning or remaining. In another embodiment, the composition is administered when less than 60% of the photoreceptors are functioning or remaining. In another embodiment, the composition is administered when less than 50% of the photoreceptors are functioning or remaining. In another embodiment, the composition is administered when less than 40% of the photoreceptors are functioning or remaining. In another embodiment, the composition is administered when less than 30% of the photoreceptors are functioning or remaining. In another embodiment, the composition is administered when less than 20% of the photoreceptors are functioning or remaining. In another embodiment, the composition is administered when less than 10% of the photoreceptors are functioning or remaining. In one embodiment, the composition is administered only to one or more regions of the eye. In another embodiment, the composition is administered to the entire eye.

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[0092] The present disclosure provides dosing regimens of CFH protein for treating the ocular inflammatory disease, disorder, or condition, or retinal changes associated therewith. The present disclosure also provides biomarkers for monitoring the response to a treatment of CFH (*e.g.*, a CFH protein or a vector encoding a CFH protein) or for selecting a subject for such treatment. These aspects of the disclosure are provided in the "Dosage Regimen" section and the "Biomarkers" section below, respectively. A combination of these two aspects, directed to a method of treating an ocular inflammatory disease, disorder, or condition, or retinal changes associated therewith, following a dosing regimen disclosed herein based on one or more biomarker levels disclosed herein, is also contemplated.

Dosing Regimen

[0093] The CFH or pharmaceutical composition disclosed herein is administered at an effective amount. The effective amount can be achieved by one or more doses of administration. In one aspect, the present disclosure provides a method of treating a subject having an inflammatory ocular disease, disorder, or condition, the method comprising administering to the subject an effective dose of CFH protein per eye, thereby treating the inflammatory ocular disease, disorder, or condition.

[0094] Doses of the CFH or pharmaceutical composition can be selected based on the route of administration. Routes of intraocular administration include but are not limited to intravitreal, subretinal, suprachoroidal, subconjunctival, retrobulbar, peribulbar, and intracameral administration. The specific doses disclosed herein are suitable for at least intravitreal administration. In some embodiments, the dose of a single administration in the eye is in the range of 10-1,000 μg/eye (*e.g.*, 10-500 μg/eye, 10-250 μg/eye, 50-1,000 μg/eye, 50-500 μg/eye, 50-250 μg/eye, 100-1,000 μg/eye, 100-500 μg/eye, or 100-250 μg/eye). Informed by toxicological studies in cynomolgus monkeys and clinical and pre-clinical data from human patients, a new dosing regimen for the administration of CFH has been created to achieve less frequent dosing. In some embodiments, the dose for a single administration in the eye is about 50 μg/eye, about 100 μg/eye, about 250 μg/eye, or about 500 μg/eye. In some embodiments, the dose for a single administration in the eye is 50 μg/eye, 100 μg/eye, 250 μg/eye, or 500 μg/eye. It is understood that a higher dose, to the extent meeting safety criteria, is more desirable in clinical use given the expected advantage of less frequent dosing.

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[0095] In certain embodiments, the CFH or pharmaceutical composition disclosed herein is administered at a fixed frequency. For example, doses may be administered in the eye (*e.g.*, intravitreal administration or subretinal injection) weekly, biweekly, once every three weeks, monthly (or once every 30 days), once every two months (or once every 60 days), once every three months (or once every 90 days), once every four months, once every five months, once every six months, once every seven months, once every eight months, once every nine months, once every ten months, once every eleven months, or yearly, or even once every 2 to 20 years. In certain embodiments, the CFH or pharmaceutical composition disclosed herein is administered to the patient for at least two, at least three, at least four, at least five, or at least six doses.

[0096] In certain embodiments, a dose of 250 μg is administered once every month, once every 4 weeks, once every two months, or once every 8 weeks. In certain embodiments, a dose of 500 μg is administered once every month, once every 4 weeks, once every two months, or once every 8 weeks.

[0097] In certain embodiments, the CFH or pharmaceutical composition disclosed herein is to be administered at a variable frequency. Persons of ordinary skill in the art can estimate repetition rates for dosing based on measured residence times and concentrations of the active agent and/or biomarkers described herein in bodily fluids (*e.g.*, ocular sample or plasma) or tissues.

[0098] For example, the protein level of one or more biomarkers disclosed herein relative to a predetermined threshold can inform the investigator/physician of the presence of the disease, disorder, or condition, whether the subject is a candidate for treatment with CFH, the severity of the disease, disorder, or condition, whether prior treatment with CFH is effective, and whether the dose, frequency, and/or mode of administration of a prior treatment with CFH is adequate or should be altered. Thus, the method disclosed herein includes obtaining or having obtained a measurement of the protein level of at least one biomarker for the purpose of diagnosis, treating, monitoring treatment, reducing progression, and/or selecting subjects for treatment with CFH. In certain embodiments, the subject is first administered complement factor H (CFH) in one or more initial doses, and afterwards, the protein level of at least one biomarker of a sample from the subject is obtained.

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[0099] In certain embodiments, the concentration of CFH is also determined or obtained for assessing the patient's need for the next dose. In certain embodiments, CFH concentrations (*e.g.*, total CFH concentration or concentration of the administered CFH) in an ocular sample are determined prior to or after treatment with CFH.

[0100] In certain embodiments, the method increases the level of CFH protein in an ocular sample (*e.g.*, aqueous humor) of the subject. In certain embodiments, the CFH protein concentration in aqueous humor remains at the level of at least 100 ng/mL, at least 200 ng/mL, at least 300 ng/mL, at least 400 ng/mL, at least 500 ng/mL, at least 600 ng/mL, at least 700 ng/mL, at least 800 ng/mL, at least 900 ng/mL, or at least 1,000 ng/mL one month or 30 days after administration. In certain embodiments, the CFH protein concentration in aqueous humor remains at the level of at least 100 ng/mL, at least 200 ng/mL, at least 300 ng/mL, at least 400 ng/mL, at least 500 ng/mL, at least 600 ng/mL, at least 700 ng/mL, at least 800 ng/mL, at least 900 ng/mL, or at least 1,000 ng/mL two months or 60 days after administration.

[0101] Additional methods of assessing the progression or severity of the inflammatory ocular disease, disorder, or condition, for the purpose of assessing the patient's need for the next dose, are described in the "therapeutic efficacy" subsection *infra*.

Biomarkers

[0102] Disclosed herein are methods of treating, preventing, ameliorating, diagnosing, and/or monitoring the progression of an ocular inflammatory disease, disorder, or condition or retinal changes associated therewith, based on the levels of one or more biomarkers.

[0103] In one aspect, the present disclosure provides a method for treating a subject having an inflammatory ocular disease, disorder, or condition, the method comprising: (a) obtaining or having obtained a measurement of the protein level of at least one biomarker disclosed herein in a sample (*e.g.*, an ocular sample) of the subject; (b) determining whether the measurement is greater than or lower than a predetermined threshold; and (c) administering to the subject CFH if the protein level of the biomarker is (i) greater than or equal to the threshold if the biomarker is positively correlated with activation of the complement pathway or with inflammation or (ii) is lower than or equal to the threshold if the biomarker is negatively correlated with activation of the complement pathway or with inflammation, thereby treating the inflammatory ocular disease, disorder, or condition.

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[0104] In one aspect, the present disclosure provides a method for identifying a subject having an inflammatory ocular disease, disorder, or condition suitable for treatment with CFH, the method comprising: (a) obtaining or having obtained a measurement of the protein level of at least one biomarker disclosed herein in a sample (*e.g.*, an ocular sample) of the subject; and (b) determining whether the measurement is greater than or lower than a predetermined threshold, wherein the subject is suitable for treatment with CFH if the protein level of the biomarker is (i) greater than or equal to the threshold if the biomarker is positively correlated with activation of the complement pathway or with inflammation or (ii) is lower than or equal to the threshold if the biomarker is negatively correlated with activation of the complement pathway or with inflammation.

[0105] In another aspect, the present disclosure provides a method for treating a subject having an inflammatory ocular disease, disorder, or condition, the method comprising: (a) selecting a subject whose protein level of at least one biomarker disclosed herein in a sample (*e.g.*, an ocular sample) of the subject is (i) greater than or equal to a predetermined threshold if the biomarker is positively correlated with activation of the complement pathway or with inflammation or (ii) is lower than or equal to a predetermined threshold if the biomarker is negatively correlated with activation of the complement pathway or with inflammation; and (b) administering to the subject CFH, thereby treating the inflammatory ocular disease, disorder, or condition.

[0106] In another aspect, the present disclosure provides a method for selecting a subject for treatment of an inflammatory ocular disease, disorder, or condition, the method comprising: (a) obtaining or having obtained a measurement of the protein level of at least one biomarker disclosed herein in a sample (*e.g.*, an ocular sample) of the subject; (b) determining whether the protein level of the biomarker is greater than or lower than a predetermined threshold; and (c) selecting the subject for treatment of the inflammatory ocular disease, disorder, or condition if the protein level of the biomarker is (i) greater than or equal to the threshold if the biomarker is positively correlated with activation of the complement pathway or with inflammation or (ii) is lower than or equal to the threshold if the biomarker is negatively correlated with activation of the complement pathway or with inflammation, wherein the treatment comprises administering to the subject CFH.

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[0107] The protein level of a biomarker selected from the group consisting of (i) complement component C3, complement factor B (CFB), complement component C5, and a cleavage fragment thereof, (ii) proteins associated with inflammation; and (iii) proteins associated with choroidal neovascularization may be used to monitor the response to a treatment disclosed herein and/or to determine when to provide the next dose of treatment. In certain embodiments, the protein levels of one or more biomarkers (*e.g.*, one or more aqueous humor complement protein and/or complement split products) are used to evaluate the efficacy of administration of CFH (*e.g.*, a CFH protein or a vector encoding a CFH protein) of the present disclosure to a subject diagnosed with an ocular disease or disorder. In certain embodiments, the protein levels of one or more biomarkers (*e.g.*, one or more aqueous humor complement protein and/or complement split products) are evaluated before and after treatment from aqueous humor samples obtained from the subjects who are administered the CFH or protein of the present disclosure. In certain embodiments, the protein levels of one or more biomarkers (*e.g.*, one or more aqueous humor complement protein and/or complement split products) are evaluated before and after treatment from plasma samples obtained from the subjects who are administered the CFH (*e.g.*, a CFH protein or a vector encoding a CFH protein) of the present disclosure.

[0108] In certain embodiments, the subject selected for the treatment based on the biomarker profile has been diagnosed of an inflammatory ocular disease, disorder, or condition by one or more clinical standards. In certain embodiments, the subject selected for the treatment based on the biomarker profile does not meet all the clinical standards of an inflammatory ocular disease, disorder, or condition, but is otherwise identified to be at high risk of developing such inflammatory ocular disease, disorder, or condition (*e.g.*, according to genetic test results).

[0109] The alternative pathway of the complement system must be tightly controlled by a group of alternative pathway regulators to prevent host cell and tissue destruction. One endogenous mechanism often employed to regulate excessive alternative pathway activity is to prevent convertase formation by degrading the core component C3b to a proteolytic by-product that is incapable of forming the convertase. The activity of the alternative pathway is indicated, for example, by the formation and protease activity of C3-convertase, serine protease factor D (CFD), C5-convertase, which cleave many proteins in the complement system. For example, C3 is a substrate of C3-convertase and the cleavage products include C3a and C3b. CFB is a substrate of CFD and the cleavage products include Ba and Bb. C5 is a substrate of C5-

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convertase and the cleavage products include C5a and C5b. It is contemplated that the activity of the alternative pathway can be used as a criterion to select a subject who is more likely to respond to a CFH therapy.

[0110] In some embodiments, the biomarker is a cleavage fragment of C3, CFB, or C5. In some embodiments, the biomarker is selected from the group consisting of C3a, Ba, Bb, C5a, C5b, and sC5b-9. The protein levels of these biomarkers are positively correlated with activation of the complement pathway. Therefore, in these embodiments, the subject is treated with CFH or selected for the treatment if the protein level of the biomarker is greater than or equal to the threshold. Median Ba and C3a concentration levels of patients diagnosed with dry AMD are ~2-fold greater than that of healthy patients (*see* Schick et al., "Local complement activation in aqueous humor in patients with age-related macular degeneration," Eye, 1-4 (2017) and Altay et al., "Early local activation of complement in aqueous humor in patients with age-related macular degeneration," Eye (Lond), 33(12):1859-1864 (2019).

[0111] In certain embodiments, the biomarker is C3a and the predetermined threshold is 1 ng/mL, 1.1 ng/mL, 1.2 ng/mL, 1.3 ng/mL, 1.4 ng/mL, 1.5 ng/mL, 1.6 ng/mL, 1.7 ng/mL, 1.8 ng/mL, 1.9 ng/mL, 2 ng/mL, 2.1 ng/mL, 2.2 ng/mL, 2.3 ng/mL, 2.4 ng/mL, 2.5 ng/mL, 2.6 ng/mL, 2.7 ng/mL, 2.8 ng/mL, 2.9 ng/mL, 3 ng/mL, 3.1 ng/mL, 3.2 ng/mL, 3.3 ng/mL, 3.4 ng/mL, 3.5 ng/mL, 3.6 ng/mL, 3.7 ng/mL, 3.8 ng/mL, 3.9 ng/mL, 4 ng/mL, 4.1 ng/mL, 4.2 ng/mL, 4.3 ng/mL, 4.4 ng/mL, 4.5 ng/mL, 4.6 ng/mL, 4.7 ng/mL, 4.8 ng/mL, 4.9 ng/mL, 5 ng/mL, 6 ng/mL, 7 ng/mL, 8 ng/mL, 9 ng/mL, or 10 ng/mL. In certain embodiments, subjects with elevated C3a concentration levels showed reduction in C3a concentration levels after administration of CFH or the protein of present disclosure. In certain embodiments, C3a concentration levels are reduced 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% in patients with elevated C3a levels after administration of CFH or the protein of present disclosure. In certain embodiments, C3a concentration levels do not reduce below 3000 pg/mL as measured by a MicroVue C3a Plus Enzyme Immunoassay Kit after administration of CFH or the protein of present disclosure.

[0112] In certain embodiments, the biomarker is Ba and the predetermined threshold is 6 ng/mL, 7 ng/mL, 8 ng/mL, 9 ng/mL, 10 ng/mL, 11 ng/mL, 12 ng/mL, 13 ng/mL, 14 ng/mL, 15 ng/mL, 16 ng/mL, 17 ng/mL, 18 ng/mL, 19 ng/mL, 20 ng/mL, 21 ng/mL, 22 ng/mL, 23 ng/mL, 24 ng/mL, 25 ng/mL, 30 ng/mL, 40 ng/mL, or 50 ng/mL. In certain embodiments, subjects with

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elevated Ba concentration levels showed reduction in Ba concentration levels after administration of CFH or the protein of present disclosure. In certain embodiments, Ba concentration levels are reduced 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% in patients with elevated Ba levels after administration of CFH or the protein of present disclosure. In certain embodiments, Ba concentration levels do not reduce below 10 ng/mL, below 9 ng/mL, below 8 ng/mL, below 7 ng/mL, or below 6 ng/mL as measured by a MicroVue Ba fragment EIA Kit after administration of CFH or the protein of present disclosure.

[0113] In certain embodiments, the biomarker is C5a and the predetermined threshold is C5a and the predetermined threshold is 100 pg/mL, 150 pg/mL, 200 pg/mL, 250 pg/mL, 300 pg/mL, 400 pg/mL, 500 pg/mL, 600 pg/mL, 700 pg/mL, 800 pg/mL, 900 pg/mL, or 1 ng/mL.

[0114] In some embodiments, the biomarker is uncleaved CFB, or C5. The protein levels of these biomarkers are negatively correlated with activation of the complement pathway. Therefore, in these embodiments, the subject is treated with CFH or selected for the treatment if the protein level of the biomarker is lower than or equal to the threshold. In certain embodiments, the biomarker is CFB and the predetermined threshold is 10 ng/mL, 20 ng/mL, 30 ng/mL, 40 ng/mL, 50 ng/mL, 60 ng/mL, 70 ng/mL, 80 ng/mL, 90 ng/mL, 100 ng/mL, 200 ng/mL, 300 ng/mL, 400 ng/mL, 500 ng/mL, 600 ng/mL, 700 ng/mL, 800 ng/mL, 900 ng/mL, or 1 μg/mL. In certain embodiments, the biomarker is C5 and the predetermined threshold is 10 ng/mL, 20 ng/mL, 30 ng/mL, 40 ng/mL, 50 ng/mL, 60 ng/mL, 70 ng/mL, 80 ng/mL, 90 ng/mL, 100 ng/mL, 110 ng/mL, 120 ng/mL, 130 ng/mL, 140 ng/mL, 150 ng/mL, 160 ng/mL, 170 ng/mL, 180 ng/mL, 190 ng/mL, 200 ng/mL, or 250 ng/mL.

[0115] In some embodiments, the biomarker is CFH. The protein level of this biomarker is negatively correlated with activation of the complement pathway. Therefore, in these embodiments, the subject is treated with CFH or selected for the treatment if the protein level of the biomarker is lower than or equal to the threshold. In certain embodiments, the biomarker is CFH and the predetermined threshold is 10 ng/mL, 15 ng/mL, 20 ng/mL, 25 ng/mL, 30 ng/mL, 35 ng/mL, 40 ng/mL, 45 ng/mL, 50 ng/mL, 55 ng/mL, or 60 ng/mL. In certain embodiments, a subject with a reduced CFH concentration in an ocular sample (*e.g.*, aqueous humor) shows an increase in CFH concentration after administration of CFH. In certain embodiments, the CFH concentration in the ocular sample (*e.g.*, aqueous humor) is increased at least 2-fold, at least 3-

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fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, at least 10-fold, at least 11-fold, at least 12-fold, at least 13-fold, at least 14-fold, at least 15-fold, at least 20-fold, at least 25-fold in the patient with reduced CFH level after administration of CFH. In certain embodiments, the CFH concentration in aqueous humor remains at the level of at least 50 ng/mL, at least 100 ng/mL, at least 200 ng/mL, at least 300 ng/mL, at least 400 ng/mL, at least 500 ng/mL, at least 600 ng/mL, at least 700 ng/mL, at least 800 ng/mL, at least 900 ng/mL, or at least 1,000 ng/mL one month or 30 days after administration. In certain embodiments, the CFH concentration in aqueous humor remains at the level of at least 50 ng/mL, at least 100 ng/mL, at least 200 ng/mL, at least 300 ng/mL, at least 400 ng/mL, at least 500 ng/mL, at least 600 ng/mL, at least 700 ng/mL, at least 800 ng/mL, at least 900 ng/mL, or at least 1,000 ng/mL two months or 60 days after administration. It is understood that reduced levels of CFH protein in ocular samples are often observed in patients having an ocular disease, disorder, or condition associated with choroidal neovascularization (CNV) who have been treated with VEGF (*e.g.*, VEGF-A) antagonists. Exemplary ocular diseases, disorders, and conditions associated with CNV include neovascular AMD (*i.e.*, wet AMD) and diabetic retinopathy (*e.g.*, diabetic macular edema). Such patients are susceptible to developing macular atrophy and are suitable for treatment with a CFH therapy. Accordingly, in certain embodiments, CFH is used as a biomarker to select a patient having an ocular disease, disorder, or condition associated with CNV and who has been treated with an VEGF (*e.g.*, VEGF-A) antagonist. In certain embodiments, the patient has received a VEGF antagonist treatment by ocular administration. In certain embodiments, the patient received a VEGF antagonist treatment within one month, two months, three months, four months, five months, six months, or a year before the administration of the CFH therapy. In certain embodiments, the patient continues to receive a VEGF antagonist during the treatment with the CFH therapy. In certain embodiments, the patient received a VEGF antagonist treatment 10 minutes, 15 minutes, 20 minutes, 25 minutes, 30 minutes, 35 minutes, 40 minutes, 50 minutes, 60 minutes, 120 minutes, 140 minutes, 240 minutes before the administration of the CFH therapy.

[0116] Patients having or at risk of developing an ocular disease, disorder, or condition associated with CNV, such as neovascular AMD (*i.e.*, wet AMD) and diabetic retinopathy (*e.g.*, diabetic macular edema), can also be selected based on the protein level of one or more biomarkers associated with CNV. Exemplary biomarkers positively associated with CNV

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include VEGF-A. In certain embodiments, the biomarker is VEGF-A and the predetermined threshold of VEGF-A is 100 pg/mL, 110 pg/mL, 120 pg/mL, 130 pg/mL, 140 pg/mL, 150 pg/mL, 160 pg/mL, 170 pg/mL, 180 pg/mL, 190 pg/mL, 200 pg/mL, 250 pg/mL, 300 pg/mL, 350 pg/mL, 400 pg/mL, 450 pg/mL, or 500 pg/mL. It is contemplated that CFH is suitable for treating such patients without causing choroidal neovascularization as many other treatments for geographic atrophy or macular atrophy do. In certain embodiments, the patient has not received a VEGF antagonist treatment. In other embodiments, the patient has received a VEGF antagonist treatment but still exhibit an elevated level of VEGF-A relative to the threshold level.

[0117] It is contemplated that patients having or at risk of developing an ocular disease, disorder, or condition associated with CNV, including but not limited to patient selected based on the protein levels of CFH and/or VEGF-A, can be treated by a combination therapy of CFH and a VEGF antagonist without causing choroidal neovascularization. Accordingly, in certain embodiments, the method disclosed herein comprises (a) obtaining or having obtained a measurement of the protein level of CFH and/or a protein associated with choroidal neovascularization (*e.g.*, VEGF-A); (b) determining whether the protein level of the biomarker is greater than or lower than a predetermined threshold as disclosed herein; and (c) administering to the subject an effective amount of CFH and an effective amount of a VEGF antagonist if the protein level of the biomarker is (i) greater than or equal to the threshold if the biomarker is positively correlated with activation of the complement pathway or with inflammation or (ii) lower than or equal to the threshold if the biomarker is negatively correlated with activation of the complement pathway or with inflammation, thereby treating the inflammatory ocular disease, disorder, or condition. Combination therapies of CFH and a VEGF antagonist are disclosed in more detail in the Combination Therapy subsection below. In certain embodiments, the patient has an ocular disease, disorder, or condition associated with CNV, such as neovascular AMD (*i.e.*, wet AMD) and diabetic retinopathy (*e.g.*, diabetic macular edema). In certain embodiments, the patient is selected for the therapy if the protein level of a protein associated with choroidal neovascularization meets the selection standard (*e.g.*, the protein level of VEGF-A is greater than or equal to a predetermined threshold disclosed herein). In certain embodiments, the patient is selected for the therapy if the protein level of CFH is lower than or equal to a predetermined threshold disclosed herein. It is understood that a combination of CFH and a VEGF antagonist can be administered to a patient prophylactically before its ocular CFH

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protein level reaches or falls below the threshold. Accordingly, in certain embodiments, the patient is selected for the therapy if the protein level of CFH is greater than the threshold. In certain embodiments, obtaining the protein level of CFH is dispensable.

[0118] It is understood that activation of the complement system may result in release of host cell components, which may in turn enhance inflammation. Therefore, it is contemplated that the activity of inflammation can be used as a criterion to select a subject who is more likely to respond to a CFH therapy. Exemplary biomarkers positively associated with ocular inflammation include interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-18, TNF-α, C-C motif chemokine ligand 2 (CCL2, a.k.a. monocyte chemotactic protein 1 or MCP-1), C-X-C motif chemokine 5 (CXCL5), C-C motif chemokine 24 (CCL24, a.k.a. Eotaxin-2), and uncleaved C3. Accordingly, in certain embodiments, the protein associated with ocular inflammation is selected from the group consisting of IL-1β, IL-6, IL-8, IL-10, IL-18, TNF-α, CCL2, CXCL5, Eotaxin-2, and uncleaved C3, and the subject is treated with CFH or selected for the treatment if the protein level of the biomarker is greater than or equal to the threshold.

[0119] In certain embodiments, the biomarker is IL-1β and the predetermined threshold of IL-1β is 0.2 pg/mL, 0.3 pg/mL, 0.4 pg/mL, 0.5 pg/mL, 0.6 pg/mL, 0.7 pg/mL, 0.8 pg/mL, 0.9 pg/mL, or 1 pg/mL. In certain embodiments, the biomarker is IL-6 and the predetermined threshold of IL-6 is 4 pg/mL, 5 pg/mL, 6 pg/mL, 7 pg/mL, 8 pg/mL, 9 pg/mL, 10 pg/mL, 15 pg/mL, or 20 pg/mL. In certain embodiments, the biomarker is IL-8 and the predetermined threshold of IL-8 is 9 pg/mL, 10 pg/mL, 11 pg/mL, 12 pg/mL, 13 pg/mL, 14 pg/mL, 15 pg/mL, 20 pg/mL, 25 pg/mL, 30 pg/mL, 40 pg/mL, 50 pg/mL, 60 pg/mL, 70 pg/mL, 80 pg/mL, 90 pg/mL, or 100 pg/mL. In certain embodiments, the biomarker is IL-10 and the predetermined threshold of IL-10 is 1.2 pg/mL, 1.3 pg/mL, 1.4 pg/mL, 1.5 pg/mL, 1.6 pg/mL, 1.7 pg/mL, 1.8 pg/mL, 1.9 pg/mL, 2 pg/mL, 2.5 pg/mL, 3 pg/mL, 3.5 pg/mL, 4 pg/mL, or 5 pg/mL. In certain embodiments, the biomarker is IL-18 and the predetermined threshold of IL-18 is 5 pg/mL, 10 pg/mL, 15 pg/mL, 20 pg/mL, 25 pg/mL, 30 pg/mL, 35 pg/mL, 40 pg/mL, 45 pg/mL, 50 pg/mL, 60 pg/mL, 70 pg/mL, or 80 pg/mL. In certain embodiments, the biomarker is TNF-α and the predetermined threshold of TNF-α is 0.3 pg/mL, 0.35 pg/mL, 0.4 pg/mL, 0.45 pg/mL, or 0.5 pg/mL. In certain embodiments, the biomarker is CCL2 and the predetermined threshold of CCL2 is 2 ng/mL, 2.5 ng/mL, 3 ng/mL, 3.5 ng/mL, 4 ng/mL, 4.5 ng/mL, 5 ng/mL, 5.5 ng/mL, 6 ng/mL, 6.5 ng/mL, 7 ng/mL, 7.5 ng/mL, 8 ng/mL, 8.5 ng/mL, 9 ng/mL, 9.5 ng/mL, or 10 ng/mL.

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In certain embodiments, the biomarker is CXCL5 and the predetermined threshold of CXCL5 is 1 pg/mL, 2 pg/mL, 3 pg/mL, 4 pg/mL, 5 pg/mL, 6 pg/mL, 7 pg/mL, 8 pg/mL, 9 pg/mL, 10 pg/mL, 15 pg/mL, 20 pg/mL, 25 pg/mL, 30 pg/mL, 40 pg/mL, or 50 pg/mL. In certain embodiments, the biomarker is Eotaxin-2 and the predetermined threshold of Eotaxin-2 is 10 pg/mL, 20 pg/mL, 30 pg/mL, 40 pg/mL, 50 pg/mL, 60 pg/mL, 70 pg/mL, 80 pg/mL, 90 pg/mL, 100 pg/mL, 110 pg/mL, 120 pg/mL, 130 pg/mL, 140 pg/mL, 150 pg/mL, 160 pg/mL, 170 pg/mL, 180 pg/mL, 190 pg/mL, or 200 pg/mL. In certain embodiments, the biomarker is uncleaved C3 and the predetermined threshold of uncleaved C3 is 100 ng/mL, 200 ng/mL, 300 ng/mL, 400 ng/mL, 500 ng/mL, 600 ng/mL, 700 ng/mL, 800 ng/mL, 900 ng/mL, 1 μg/mL, 1.1 μg/mL, 1.2 μg/mL, 1.3 μg/mL, 1.4 μg/mL, or 1.5 μg/mL.

[0120] A plurality of biomarkers disclosed herein can be used for the patient selection. In certain embodiments, the method comprises obtaining or determining the protein levels of 2, 3, 4, 5, 6, 7, 8, 9 , 10, 11, 12, 13, 14, 15 or more biomarkers disclosed herein.

[0121] In certain embodiments, the method comprises obtaining or determining the protein levels of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more complement components or fragments thereof. In certain embodiments, the method described herein comprises obtaining or determining the protein levels of a panel of biomarkers comprising a complement component and a cleavage fragment thereof. In certain embodiments, the method described herein comprises obtaining or determining the protein levels of a panel of biomarkers comprising two or more (*e.g.*, three or more) biomarkers selected from C5, CFB, C3a, and Ba. In certain embodiments, the method described herein comprises obtaining or determining the protein levels of a panel of biomarkers comprising C3a and Ba. In certain embodiments, the method described herein comprises obtaining or determining the protein levels of a panel of biomarkers comprising C5, CFB, C3a, and Ba.

[0122] In certain embodiments, the method comprises obtaining or determining the protein levels of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more proteins associated with ocular inflammation. In certain embodiments, the method comprises obtaining or determining the protein levels of a panel of biomarkers comprising two or more (*e.g.*, three or more, four or more, five or more, six or more, seven or more, eight or more, or nine or more) biomarkers selected from TNF, IL-6, IL-8, IL-1β, IL-18, IL-10, CCL2, CXCL5, Eotaxin-2, and C3.

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[0123] In certain embodiments, the method comprises obtaining or determining the protein levels of one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more) complement components or fragments thereof, one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more) proteins associated with ocular inflammation, and/or one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more) proteins associated with ocular inflammation proteins associated with ocular neovascularization. In certain embodiments, the method comprises obtaining or determining the protein levels of one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more) complement components or fragments thereof and one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more) proteins associated with ocular inflammation, and optionally VEGF-A. In certain embodiments, the method comprises obtaining or determining the protein levels of a panel of biomarkers comprising two or more (*e.g.*, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, 11 or more, 12 or more, 13 or more, or 14 or more) biomarkers selected from C3, C5, B, C3a, Ba, TNF, IL-6, IL-8, IL-1β, IL-18, IL-10, CCL2, CXCL5, Eotaxin-2, and VEGF-A. In certain embodiments, the method comprises obtaining or determining the protein levels of a panel of biomarkers comprising C3, C5, B, C3a, Ba, TNF, IL-6, IL-8, IL-1β, IL-18, IL-10, CCL2, CXCL5, Eotaxin-2, and VEGF-A. Where multiple biomarkers are assessed, patient selection can be made based on a score that takes into account the protein levels of the multiple biomarkers and the relative impact of each biomarker on the risk of disease development and progression.

[0124] The biomarker panels described herein can include biomarkers in addition to any of those described herein, including biomarkers that when having a level above or below a predetermined threshold is an indication that the subject has an ocular inflammatory disease, would benefit from CFH treatment, an inflammatory ocular disease has progressed, and/or a higher dose of CFH is needed for treating, reducing progression, or preventing progression of an ocular inflammatory disease.

[0125] The sample of the subject can be obtained from various tissues. In certain embodiments, the sample of the subject is an ocular sample. In certain embodiments, the ocular sample comprises aqueous humor. In certain embodiments, the sample comprises vitreous humor. In other embodiments, the sample of the subject is a serum sample. In certain embodiments, both an ocular sample and serum sample are obtained.

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[0126] Aqueous humor can be collected via a technique called anterior chamber paracentesis (ACP). This involves the use of a 27- or 30-gauge needle attached to a 1ml syringe that is inserted at the paralimbal clear cornea in a plane above and parallel to the iris with the bevel of the needle facing forward until the whole bevel has penetrated the cornea. Under direct vision, the retina physician then pulls the plunger of the syringe to aspirate a small quantity of aqueous humor. In certain embodiments, 10-300 μL of aqueous humor is obtained from subject. In certain embodiments, no more than 100 μL of aqueous humor is acquired from subjects. In certain embodiments, approximately 10 μL, 20 μL, 30 μL, 40 μL, 50 μL, 60 μL, 70 μL, 80 μL, 90 μL, or 100 μL of aqueous humor is acquired from a human subject.

Detection and Quantification Assays

[0127] Any assay known in the art that has sufficient sensitivity to detect and quantify the biomarkers described herein in either an ocular sample (*e.g.*, aqueous humor or vitreous humor) or serum sample can be used, including, but not limited to, mass spectrometry (MS), multiplex bead immunoassay (*e.g.*, Luminex™ assay, Quanterix™ SIMOA bead technology), enzyme-linked immunoabsorbant assay (ELISA), electrochemiluminescence immunoassay (ECLIA), chemiluminescent microparticle immunoassay (CMIA), radioimmunoassay (RIA), immunodiffusion, immunoelectrophoresis, immunohistochemistry (IHC), flow cytometry, immunoprecipitation assays, complement fixation assays, binding with indexed antibodies in solutions and suspensions, protein arrays using antibodies, or any combinations thereof. In certain embodiments, the assay used for determination of the protein level of a biomarker described herein has a detection limit lower than or equal to 10 μg/mL, lower than or equal to 1 μg/mL, lower than or equal to 0.1 μg/mL, lower than or equal to 10 ng/mL, lower than or equal to 1 ng/mL, lower than or equal to 0.1 ng/mL, lower than or equal to 10 pg/mL, or lower than or equal to 1 pg/mL.

[0128] In certain embodiments, the protein level of a biomarker is obtained using a assay that requires a binding agent (*e.g.*, a detection reagent, or a capture reagent in a sandwich-type immunoassay) that specifically binds the biomarker. In certain embodiments, the binding agent is an antibody. In certain embodiments, where the biomarker of interest is a cleavage fragment (*e.g.*, C3a, Ba, Bb, C5a, and sC5b-9), the binding agent (*e.g.*, antibody) specifically binds the cleavage fragment and not the precursor protein, or binds the cleavage fragment at a higher affinity than that to the corresponding precursor protein (*e.g.*, the dissociation constant of the

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complex with the precursor protein is greater than that with the fragment by at least 5 fold, 10 fold, 20 fold, 50 fold, or 100 fold). For example, for detection of C3a, an antibody that has a higher affinity to C3a than to full-length C3 can be used.

[0129] In certain embodiments, a sandwich-type immunoassay method such as ELISA, ECLIA, CMIA, or RIA is used to quantify the protein level of a biomarker. For example, the method may comprise adding a biological sample to a first antibody combined with a bead, a film, a slide, or a microtiter plate made with a solid substrate, *e.g.*, glass, plastic such as polystyrene, polysaccharide, nylon, or nitrocellulose and then qualitatively or quantitatively detecting a protein (*e.g.*, a protein that directly or indirectly binds the analyte) labeled with a directly or indirectly detectable moiety. Such moieties include, without limitation, radioactive substances such as ^{3}H or ^{125}I, fluorescent substances, chemiluminescent substances, electrochemiluminescent substances, hapten, biotin, digoxigenin, enzymes such as horseradish peroxidase (HRP), alkaline phosphatase (ALP), and malate dehydrogenase capable of colorimetry or luminescence through an reaction with a substrate. Exemplary ELISA methods are described in Gaastra, W., Methods in Molecular Biology, Vol. 1, Walker, J.M. ed., Humana Press, NJ, 1984. Exemplary ECLIA methods are described in Prieto *et al.*, (2010) Clin. Chem. Lab. Med. 48:835–38 and have been developed for commercial use by companies such as Meso Scale Discovery (MSD).

[0130] In certain embodiments, a dipstick assay is used to quantify the protein level of a biomarker. The dipstick assay is a technology widely used in the field of point of care test (POCT). It is a chromatic method for detecting a biomarker, showing color when a biomarker of interest in a sample binds an antibody in the substrate (*e.g.*, nitrocellulose) and combines with a detection antibody due to capillarity when the sample contacts the substrate, *e.g.*, when one end of a dipstick is dipped into the sample.

[0131] In certain embodiments, concentration levels of C3a in human aqueous humor samples are quantified using a MicroVue C3a Plus Enzyme Immunoassay Kit (Quidel, cat#A031). This is a 96 well, direct capture immunoassay where a microassay plate is coated with a murine monoclonal antibody specific for human C3a. When standards, controls, and samples are added to this plate, the antibody on the plate binds to C3a. After the incubation period, a wash cycle removes any unbound material. Next a horseradish peroxidase (HRP)-conjugated anti-C3a is added to each assay well where it binds to the immobilized C3a captured

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in the first step. When a 3,3',5,5' tetramethylbenzidine (TMB), a ready-to-use, chromogenic substrate solution, is added to the assay wells, the bound HRP reacts with the substrate forming a blue color. After the incubation period, the reaction is stopped and the color intensity is measured spectrophotometrically at A450. The color intensity of the reaction mixture is proportional to the concentration of C3a present in the standards, controls, and samples. Results are calculated from the generated standard curve using 5-parameter analysis.

[0132] In certain embodiments, concentration levels of Ba in human aqueous humor samples are quantified using a MicroVue Ba fragment EIA Kit (Quidel, cat# A033). Based on quantitative ELISA format, a microtiter plate is coated with a mouse mAb that specifically binds to human Ba. When standards, controls, and samples are added to the wells, the Ba fragments present in them bind to the immobilized Ab. A horse radish peroxidase (HRP) conjugated polyclonal anti-Ba Ab is used as the detection reagent. This is detected by TMB substrate which will generate color which is directly proportional to the amount of Ba in the samples.

[0133] In certain embodiments, CFH, Factor B, and C3 concentration levels in human aqueous humor samples are quantified using a Luminex -Human Complement Magnetic Bead Panel 2 Kit (Millipore cat# HCMP2MAG-19K). This is a double capture multiplex bead-based Immunoassay which uses spectrally encoded magnetic beads conjugated to specific antibodies, *e.g.*, for CFH, anti-factor H capture antibodies are used as the solid support. FH in samples and QC will bind to these antibodies conjugated to color-coded beads. The bound FH is detected with biotinylated anti-FH antibody producing a Magnetic bead antibody-antigen(Ag)-antibody double capture complex. When a reporter molecule, streptavidin-conjugated to Phycoerythrin (Streptavidin-PE) is added to the mixture, a fluorescence is generated which can be read on a BioPlex Suspension Array instrument. By monitoring the Spectral properties of the magnetic beads and the PE Fluorescence intensity (FI), the concentration of Factor H can be determined.

[0134] In certain embodiments, CFH concentration levels in human plasma are quantified using a MicroVue Factor H EIA (Quidel, cat# A039). This is a quantitative Elisa method where the microtiter plate is coated with a mouse monoclonal antibody that binds specifically to human Factor H. When standards, controls, or specimens are added to the plate, FH will bind to the immobilized anti-Factor H monoclonal antibody. Next, a horseradish peroxidase (HRP)-conjugated murine anti-Factor H antibody is added to each well as the detection reagent which will bind to the FH captured on the plate. When a chromogenic enzyme substrate (TMB) is

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added, the bound HRP-conjugate reacts with the substrate, forming a color which is measured spectrophotometrically at A450. The color intensity of the reaction mixture is proportional to the concentration of Factor H present in the samples.

[0135] In certain embodiments, immunoelectrophoresis such as Ouchterlony plate, western blot, crossed IE, rocket IE, fused rocket IE, or affinity IE, wherein a biomarker may easily detect through antigen-antibody binding, is used to quantify the protein level of a biomarker described herein. Exemplary immunoassays are described in Enzyme Immunoassay, E. T. Maggio, ed., CRC Press, Boca Raton, Florida, 1980.

[0136] In certain embodiments, microparticles or nanoparticles are used in immunoassays to quantify the protein level of a biomarker. Microparticles are often in suspension and may be synthesized in large batches, which reduces labor and allows for higher standardization between assays. Suspension assays also allow for easier mixing, leading to faster reaction times on microspheres. For multiplexed analyses, microspheres can be added or removed to individually choose each protein for the assay. Fluorescence microbeads can be analyzed by various methods, including flow cytometry, laser scanning cytometer or confocal laser scanning microspectrometer. For example, Luminex™ polystyrene beads can be applied to immunoassays. Because each bead serves as a separate replicate and many beads can be fabricated simultaneously in batch, bead-based immunoassays may be more reliable, more reproducible, and cheaper than other immunoassays. Another advantage of microparticles is the ability to trap and concentrate analyte molecules for improved sensitivity. This improved sensitivity is particularly important in microfluidic and point of care devices where detector sensitivity may be compromised because of size limitations.

[0137] In certain embodiments, an array including a microarray or a chip method is used to detect the protein level of a biomarkers described herein. Array techniques are known in the art and can be found in, for example, Schena et al., 1996, Proc Natl Acad Sci USA. 93 (20) :10614-9; Schena et al., 1995, Science 270(5235):467-70; and U.S. Patent. Nos. 5,599,695, 5,556,752, and 5,631,734. A detection reagent may be attached to the surface of a substrate such as glass or nitrocellulose. The detection reagent that may be attached to an array may include an antibody, an antibody fragment, an aptamer, an avidity multimer, or peptidomimetics capable of being specifically binding with one protein.

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[0138] In certain embodiments, the protein level of a biomarker is assessed using mass spectrometry (MS) (see, *e.g.*, Kim, et al. 2010 J Proteome Res. 9: 689-99; Anderson, L et al. 2006. Mol Cell Proteomics 5: 573-88). Mass spectrometry can detect a wide range of proteins in a single measurement without labeling and can be performed from a biological sample, such as an ocular sample or serum. In an embodiment, for example, multiple reaction monitoring (MRM) by utilizing a triple quadrupole LC-MS/MS, QTRAP, etc. is used. The MRM is a method for quantitative measuring of a substance, such as a small amount of biomarker present in biological samples, in multiple ways, in which precursor ions or parent ions among ion fragments are created from an ionization source selectively and sent to a collision cell by using a first mass filter Q1. After that, the precursor ions in the collision cell collide with internal collision gas and splits into product ions or daughter ions, which are sent to a second mass filter Q2. Only characteristic ions are sent to a detecting unit. This is an analytic method with high selectivity and high sensitivity that may detect information on required ingredients only (see Gillette et al., 2013, Nature Methods 10:28-34).

[0139] When mass spectrometry techniques are used to detect and quantify one or more biomarkers in a test sample, the test sample must first be prepared for mass spectrometry analysis. Sample preparation can take place in several ways, but the one that is most commonly used involves contacting the sample with one or more adsorbents attached to a solid phase. The adsorbents may be anionic or cationic groups, hydrophobic groups, metal chelating groups with or without a metal ligand, antibodies, whether polyclonal or monoclonal, or suitable antigens to bind their related antibodies. The solid phase can be a flat surface made of metal, glass, or plastic. The solid phase can also be microparticulate in nature, be they microbeads, amorphous particulates, or insoluble polymers to increase the surface area. In addition, microparticulate materials can be magnetic for handling. In certain embodiments, the biomarker of interest is absorbed in the solid phase and the remaining molecules are washed away. By mass analysis, the biomarker of interest is eluted from the solid phase with a solvent that reduces the affinity of the biomarker for the adsorbent. The biomarkers are then introduced into the mass spectrometer for analysis. Preferably, the peripheral spectra are identified and omitted in the spectrum evaluation. Additionally, immunoassays such as those described above can also be used. Upon termination of an immunoassay, the analyte can be eluted from the immune surface and introduced into the spectrophotometer for analysis.

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[0140] Once the test sample has been prepared, it is introduced into the mass analyzer. Laser ionization desorption (for example, MALDI or SELDI) is a common technique for samples that are presented in solid form. In this technique, the sample is co-crystallized on a target plate with an effective matrix to absorb and transfer the laser energy to the sample. The ions are separated, counted, and calibrated against the ions of known mass and charge. The mass data collected by any sample is an ionic count with a specific mass / charge ratio (m / z). It is anticipated that different sample preparation methods and different ionization techniques will result in different spectra.

[0141] In certain embodiments, the detection of the biomarker can be performed through detection of a peptide derived from a biomarker protein. For example,

[0142] Certain MS methods are suitable for detecting shorter peptides or polypeptides. To analyze a protein of interest having a large size, the sample can be digested with one or more proteases to generate fragments of the protein, and signature fragments can be detected by MS. Where the event to be detected is cleavage of a protein (*e.g.*, C3, CFB, or C5), this technology is particularly suitable for detecting the depletion of a signature fragment of the uncleaved protein. Accordingly, in specific embodiments, the protein level of C3, CFB, or C5 is measured by MS.

[0143] It is understood that the volume of an ocular sample is typically small, for example, no more than 100 μL. As a result, an assay suitable for measurement of the levels of one or more proteins in an ocular sample is generally highly sensitive and/or compatible with small sample volumes. Given the potential benefit of assessing more than one biomarker, in certain embodiments, the assay is a multiplex assay. In certain embodiments, the protein level of the biomarker is measured by a multiplex bead immunoassay (*e.g.*, Luminex™ assay, Quanterix™ SIMOA bead technology). In certain embodiments, the protein level of the biomarker is measured by electrochemiluminescence immunoassay (ECLIA).

Genetic Patient Selection Criteria

[0144] In lieu of or in addition to the biomarker levels disclosed above, one or more of the genetic criteria below can be used for patient selection.

[0145] In some embodiments, the method disclosed herein is used in treatment of a patient having one or more CFH mutations. The presence or absence of any of the CFH mutations disclosed herein can be determined by genetic testing. In some embodiments, the patient has one or more CFH mutations that causes macular degeneration (AMD) or that increases the likelihood

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that a patient develops AMD. In some embodiments, the patient has one or more mutations that causes atypical hemolytic uremic syndrome (aHUS) or that increases the likelihood that a patient develops aHUS. In some embodiments, the one or more mutations are in the patient's CFH gene. In some embodiments, the subject has a loss-of-function mutation in the subject's CFH gene.

[0146] In particular embodiments, the patient expresses a mutant CFH protein, wherein the mutant CFH protein has reduced CFH activity as compared to a wildtype CFH protein (*e.g.*, a CFH protein having the amino acid sequence of SEQ ID NO: 1, 2 or 3). In some embodiments, the CFH activity is the ability to bind to C3b. In some embodiments, the CFH activity is the ability to act as a cofactor with CFI and facilitate C3b cleavage. In some embodiments, the CFH activity is the ability to bind to a cell surface (*e.g.*, an erythrocyte and/or endothelial cell). In some embodiments, the CFH activity is the ability to bind to heparin. In some embodiments, the CFH activity is the ability to inhibit C5b9 levels as a result of complement activation, *e.g.*, as measured in a Wieslab AP assay (*see, e.g.*, Example 1). In some embodiments, the CFH activity is the ability to inhibit hemolysis. In some embodiments, if the mutant CFH protein were tested in a functional assay, the mutant CFH protein would display reduced CFH activity as compared to a wildtype CFH protein (*e.g.*, a CFH protein having the amino acid sequence of SEQ ID NO: 1, 2, or 3). Examples of CFH mutants associated with reduced CFH activity include R2T, R53C, R53H, S58A, D130N, R175Q, R175P, I221V, R303W, R303Q, Y402H, P503A, R567G, G650V, G1194D, or R1210C CFH mutants. As used herein, the amino acid positions in a CFH protein is numbered according a full-length CFH protein including the signal peptide, for example, the amino acid sequence set forth NCBI Reference Sequence NP_000177.2. In some embodiments, if a cell expresses less of a variant CFH polypeptide than the same cell would express a wildtype control CFH polypeptide, then the variant CFH is determined to be functionally impaired. In some embodiments, the patient is homozygous for any of the mutations disclosed herein. In some embodiments, the patient is heterozygous for any of the mutations disclosed herein.

[0147] In some embodiments, the patient is homozygous for the Y402H mutation of the CFH gene. In some embodiments, the patient homozygous for the Y402H mutation of the CFH gene is negative for (a) the ARMS2/HTRA1 risk allele of ARMS2: c298-858T>C in the rs3750846 SNP; (b) homozygous CFH 62V, homozygous C3 102G, and homozygous CFB 32R; and/or (c) rare

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missense CFH mutations selected from the group consisting of R2T, L3V, R53C, R53H, S58A, D90G, D130N, R175Q, R175P, I221V, R303W, R303Q, Q400K, P503A, R567G, G650V, S890I, T956M, G1194D, and R1210C. In some embodiments, the patient homozygous for the Y402H mutation of the CFH gene is negative for any of the mutations in (a), (b), and (c) above.

[0148] In some embodiments, the patient is homozygous for CFH 62V, C3 102G, and CFB 32R.

[0149] In some embodiments, the patient is positive for at least one of the rare missense CFH mutations selected from the group consisting of R2T, L3V, R53C, R53H, S58A, D90G, D130N, R175Q, R175P, I221V, R303W, R303Q, Q400K, P503A, R567G, G650V, S890I, T956M, G1194D, and R1210C.

[0150] In some embodiments, the patient has one or more mutations in the patient's CFH gene. In some embodiments, the treatment and/or prophylactic method is for use in treating a patient in whom it has been determined has one or more of any of the CFH mutations disclosed herein. In some embodiments, the patient has a mutation in one or more of CCP domains 1-20, or any combination thereof. In some embodiments, the patient has a mutation in one or more of CCP domains 1-2 or 18-20. In some embodiments, the patient has a mutation in CCP1. In some embodiments, the patient has a mutation in CCP2. In some embodiments, the patient has a mutation in CCP3. In some embodiments, the patient has a mutation in CCP4. In some embodiments, the patient has a mutation in CCP5. In some embodiments, the patient has a mutation in CCP6. In some embodiments, the patient has a mutation in CCP7. In some embodiments, the patient has a mutation in CCP8. In some embodiments, the patient has a mutation in CCP9. In some embodiments, the patient has a mutation in CCP10. In some embodiments, the patient has a mutation in CCP11. In some embodiments, the patient has a mutation in CCP12. In some embodiments, the patient has a mutation in CCP13. In some embodiments, the patient has a mutation in CCP14. In some embodiments, the patient has a mutation in CCP15. In some embodiments, the patient has a mutation in CCP16. In some embodiments, the patient has a mutation in CCP17. In some embodiments, the patient has a mutation in CCP18. In some embodiments, the patient has a mutation in CCP19. In some embodiments, the patient has a mutation in CCP20. In some embodiments, the patient has one or more mutations in the disulphide bond sites in the CFH protein. In some embodiments, the mutation is one or more of the mutations selected from the group consisting of: H402Y, G69E,

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D194N, W314C, A806T, Q950H, p.Ile184fsX, p.Lys204fsX, c.1697-17_-8del, A161S, A173G, R175Q, V62I, V1007L, S890I, S193L, I216T, A301Nfs*25, W379R, Q400K, Q950H, T956M, R1210C, N1050Y, E936D, Q408X, R1078S, c.350+6T->G, R567G, R53C, R53H, R2T, A892V, R567G, I221V, S159N, P562H, F960S, R303W, R303Q, K666N, G1194D, P258L, G650V, D130N, S58A, R166W, R232Q, R127H, K1202N, G397Stop, Stop450R, R830W, I622L, T732M, S884Y, L24V, Y235H, K527N, R582H, C973Y, V1089M, E123G, T291S, R567K, E625Stop, N802S, N1056K, R1203W, Q1076E, P26S, T46A, T91S, C129Y, R166Q, E167Q, R175P, C192F, W198*, V206M, G218*, M239T, Y277*, C325Y, R341H, R364L, P384R, C431S, D454A, A473V, P503A, N516K, I551T, H699R, F717L, W978R, P981S, A1010V, W1037*, P1051L, I1059T, Q1143E, R1206H, T1227I, L24V, H169R, R257H, K410E, V609I, D619N, A892V, G1002R, G278S, T30*, I32Stop, R78G, Q81P, V111E, W134R, P139S, M162V, E189Stop, K224Del, K224Del, A307A, H332Y, S411T, C448Y, L479Stop, R518T, T519A, C536R, C564P, C569Stop, L578Stop, P621T, C623S, C630W, E635D, K670T, Q672Q, C673Y, C673S, S714Stop, S722*, C733Y, V737V, E762Stop, N774Stop, R780I, G786*, M823T, V835L, E847V, E850K, C853R, C853T, C864S, C870R, H878H, I881L, E889Stop, H893R, Y899Stop, Y899D, C915S, C915Stop, W920R, Q925Stop, C926F, Y951H, C959Y, P968*, I970V, T987A, N997T, G1011*, T1017I, Y1021F, C1043R, T1046T, V1054I, V1060A, V1060L, C1077W, T1097W, T1097T, D1119G, D1119N, P1130L, V1134G, E1135R, E1137L, E1139Stop, Y1142D, Y1142C, C1152S, W1157R, P1161T, C1163T, P1166L, V1168E, V1168Stop, I1169L, E1172Stop, Y1177C, R1182S, W1183L, W1183R, W1183L, W1183Stop, W1183C, T1184R, T1184A, K1186H, K1188Del, L1189R, L1189F, S1191L, S1191W, E1195Stop, V1197A, E1198A, E1198Stop, F1199S, V1200L, G1204E, L1207R, S1211P, R1215Q, R1215G, T1216Del, C1218R, Y1225*, P1226S, L3V, H821Y, E954del, G255E, T1038R, V383A, V641A, P213A, I221V, E229K, R2T, R1072G, G967E, N819S, V579F, G19K, A18S, K834E, T504M, R662I, P668L, G133R, I184T, L697F, H1165Y, G1110A, pIle808_Gln809del, I760L, T447R, I808M, I868M, L765F, N767S, R567G, K768N, S209L, Q628K, D214Y, N401D, I216K, Q464R, I777V, E229D, M823I, R232Ter, S266L, P260S, E23G, C80Y, R78T, R582H, N638D, N638S, P258L, L3F, R257H, G240R, G69R, D855N, M11I, K472N, Q840H, E850K, Y899H, T645M, M805V, K919T, E201G, V407A, I907L, T914K, H332R, V144M, S652G, D195N, C146S, P661R, E677Q, V482I, T34R, A421T, R281G, C509Y, K666N, P440S, C442G, N607D, A425V, G667E, P440L, I49V, R387G,

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E625K, E625Ter, T135S, P43S, K283E, I124V, T36V, I563T, G350E, D619G, T321I, T286A, P384L, T739N, M515L, V158A, G727R, T724K, F717L, M162V, C178R, G700R, A161T, F176L, R295S, F298Y, G297S, P300L, R1040K, V552L, T310I, T531A, G928D, Ter386RextTer 69â€,Q1143K, Y534C, P981L, K308N, D538E, R1215Ter, E105V, T1017I, N1050I, P935S, Y951H, T1097M, D947H, E961D, G962S, G964E, I970V, R1072T, P1114L, S1122T, F960C, R1074C, R1182T, R1074L, S884Y, S890T, V837I, V941F, V158I, D748V, I216T, H371N, L750F, P418T, M432V, D693N, A746E, V111E, c.2237-2A>G, P982S, V579A, E591D, V579I, V65I, P418S, Y1067C, D772N, V72L, E189K, A1027P, D798N, N61D, P384S, N521S, P1068S, E395K, N774S, H577R, E833K, K6E, H337R, R444C, L741F, Y42F, D288E, S705F, R1040G, D214H, N757D, I861M, G848E, P923S, E201K, E902A, R303Q, G366E, D538H, K82R, E721K, Y1008H, R1074P, A806S, Q807R, C389Y, H764Y, K867N, P392T, L394M, E456K, F459L, Y398C, E570K, D214N, I574V, I574T, G631C, T880I, V865F, V576A, N776S, P633S, N22D, P634A, N822I, R885S, R232L, E635D, R778K, L827V, C267R, Y779C, R582C, L77S, R257C, Y327H, N75K, L74F, S836T, Y243H, c.1519+5_1519+8delGT…, K507Q, A892S, I15T, P924L, A14V, N842K, G894R, G894E, Y271C, C9W, T504R, V683M, L385Pheâ€, S898R, Q408H, G409S, T34K, E648G, I412V, E338D, P799S, G480E, D798E, D195Y, R341C, D485H, D485G, K598Q, Y420H, P599T, N434H, R441T, C431G, V149A, V349I, T679A, P43T, G45D, R662G, T519I, L121P, P364L, P621A, H373Y, D538MfsTer14, H371P, T544A, T131A, R166G, V177I, V177A, R729S, F717V, N718S, S991G, L98I, Y1016Ter, T1217del, M1001T, K1004E, A1010T, G1011D, T1017A, T1031A, L1125F, R1203G, L1214M, W1096DfsTer20, H939N F960L, D966H, M1064I, E1071K, N1095K, T1106A, G1107E, C1109W, P1111S, V1197I, Y1075F, S1079N, P1080S, E1082G, or Sto1232. In particular embodiments, the mutation is one or more of the mutations selected from the group consisting of: R2T, L3V, R53C, R53H, S58A, G69E, D90G, R175Q, S193L, I216T, I221V, R303W, H402Y, Q408X, P503A, G650V, R1078S, and R1210C. As used herein, any of the CFH mutant amino acid positions described herein correspond to the amino acid CFH sequence of full-length human CFH including the signal peptide (UniProt Accession No. P08603-1).

[0151] In some embodiments, the patient has any one of the following CFH mutations: R2T, L3V, R53C, R53H, S58A, D90G, D130N, R175Q, R175P, I221V, R303W, R303Q, Q400K, Y402H, P503A, R567G, G650V, S890I, T956M, G1194D, or R1210C. In some embodiments, the patient has any one or more of the following CFH mutations: R2T, R53C, R53H, S58A,

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D130N, R175Q, R175P, I221V, R303W, R303Q, P503A, R567G, G650V, G1194D, or R1210C. In some embodiments, the patient has an R2T mutation. In some embodiments, the patient has an L3V mutation. In some embodiments, the patient has an R53C mutation. In some embodiments, the patient has an R53H mutation. In some embodiments, the patient has an S58A mutation. In some embodiments, the patient has a D90G mutation. In some embodiments, the patient has a D130N mutation. In some embodiments, the patient has an R175Q mutation. In some embodiments, the patient has an R175P mutation. In some embodiments, the patient has an I221V mutation. In some embodiments, the patient has an R303W mutation. In some embodiments, the patient has an R303Q mutation. In some embodiments, the patient has a Q400K mutation. In some embodiments, the patient has a Y402H mutation. In some embodiments, the patient has a P503A mutation. In some embodiments, the patient has an R567G mutation. In some embodiments, the patient has a G650V mutation. In some embodiments, the patient has an S890I mutation. In some embodiments, the patient has a T956M mutation. In some embodiments, the patient has a G1194D mutation. In some embodiments, the patient has an R1210C mutation.

[0152] In certain embodiments, the CFH mutation is a change from the Tyr at amino acid position 402 of human CFH protein to a Histidine and is present in at least one of the two alleles of the CFH gene in the genome of the patient. In certain embodiments, the CFH mutation is a mutation of the Tyr at amino acid position 402 of human CFH protein and is present in both alleles of the CFH gene. In certain embodiments. the genetic variant comprises SNP rs1061170 in at least one allele of the human genome. In certain embodiments. the genetic variant comprises rs1061170 in both alleles of the human genome.

[0153] In some embodiments, the patient expresses less CFH, or less functional CFH, than expressed in a cell or tissue (*e.g.*, eye) than a control patient or population thereof. In some embodiments, the control patient does not have any of the CFH mutations disclosed herein. In some embodiments, the control patient does not have a genetic mutation that impairs CFH function. In some embodiments, the control patient is of the same or similar age and/or of the same sex as the patient selected. In some embodiments, the control patient is a healthy subject, *e.g.*, the subject does not have a disease, disorder, or condition of the eye. In some embodiments, the control patient does not have a disease or disorder of the eye associated with activation of the complement cascade. In some embodiments, the control patient does not have macular

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degeneration. In some embodiments, the eye or a specific cell type of the eye (*e.g.*, cells in the foveal region) in the selected patient expresses at least 95%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, or 1% less CFH or functional CFH as compared to the levels in the control patient. In some embodiments, the eye or a specific cell type of the eye (*e.g.*, cells in the foveal region) in the selected patient express CFH protein having any one or more of the CFH mutations disclosed herein. In some embodiments, the eye or a specific cell type of the eye (*e.g.*, cells in the foveal region) in the control patient do not express a CFH protein having any of the CFH mutations disclosed herein.

Complement Factor H Protein

[0154] The present disclosure provides a complement factor H (CFH) protein, including a biologically active fragment and/or variant of a full-length, wild-type CFH protein, for use in any of the methods disclosed herein. In certain embodiments, the CFH protein is a recombinant protein.

[0155] Mature human wild-type CFH is a 1213 amino acid soluble protein which comprises 20 complement-control protein modules (CCPs 1–20), which are each approximately 60 amino acid residues in length. Alignment of the 20 CCPs demonstrates four invariant cysteine residues arranged in two conserved disulfide bonds, and a near-invariant tryptophan residue. Short three to eight amino acid residue "linkers" are found between the last residue of one CCP and the first residue of the next CCP. Each of the CCPs fold into a distinct three-dimensional β-sheet rich structure (Schmidt C.Q. Clin Exp Immunol. 2008;151(1):14-24). CCP module 1 corresponds to amino acids 1-64 of SEQ ID NO: 1 or a variant thereof; CCP module 2 corresponds to amino acids 65-125 of SEQ ID NO: 1 or a variant thereof; CCP module 3 corresponds to amino acids 126-189 of SEQ ID NO: 1 or a variant thereof; CCP module 4 corresponds to amino acids 190-246 of SEQ ID NO: 1 or a variant thereof; CCP module 5 corresponds to amino acids 247-304 of SEQ ID NO: 1 or a variant thereof; CCP module 6 corresponds to amino acids 306-368 of SEQ ID NO: 1 or a variant thereof; CCP module 7 corresponds to amino acids 369-426 of SEQ ID NO: 1 or a variant thereof; CCP module 8 corresponds to amino acids 428-489 of SEQ ID NO: 1 or a variant thereof; CCP module 9 corresponds to amino acids 497-548 of SEQ ID NO: 1 or a variant thereof; CCP module 10 corresponds to amino acids 549-607 of SEQ ID NO: 1 or a variant thereof; CCP module 11 corresponds to amino acids 610-668 of SEQ ID NO: 1 or a variant thereof; CCP module 12 corresponds to amino acids 671-728 of SEQ ID NO: 1 or a

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variant thereof; CCP module 13 corresponds to amino acids 733-787 of SEQ ID NO: 1 or a variant thereof; CCP module 14 corresponds to amino acids 791-848 of SEQ ID NO: 1 or a variant thereof; CCP module 15 corresponds to amino acids 850-910 of SEQ ID NO: 1 or a variant thereof; CCP module 16 corresponds to amino acids 911-968 of SEQ ID NO: 1 or a variant thereof; CCP module 17 corresponds to amino acids 969-1,027 of SEQ ID NO: 1 or a variant thereof; CCP module 18 corresponds to amino acids 1,028-1,086 of SEQ ID NO: 1 or a variant thereof; CCP module 19 corresponds to amino acids 1,089-1,147 of SEQ ID NO: 1 or a variant thereof; and CCP module 20 corresponds to amino acids 1,152-1,212 of SEQ ID NO: 1 or a variant thereof.

[0156] In some embodiments, the CFH protein comprises at least four CCP modules or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises at least five CCP modules or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises at least six CCP modules or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises at least seven CCP modules or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises at least eight CCP modules or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises at least nine CCP modules or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises at least ten CCP modules or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises at least eleven CCP modules or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises at least twelve CCP modules or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises at least thirteen CCP modules or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises at least fourteen CCP modules or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises at least fifteen CCP modules or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises at least sixteen CCP modules or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises at least seventeen CCP modules or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises at least eighteen CCP modules or a biologically active fragment and/or variant thereof. In some

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embodiments, the CFH protein comprises at least nineteen CCP modules or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises twenty CCP modules or a biologically active fragment and/or variant thereof.

[0157] In some embodiments, the CFH protein comprises any one of or any combination of CCP modules 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and/or 20 or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises at least four of CCP modules 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and/or 20 or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises CCP modules 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-13, 1-14, 1-15, 1-16, 1-17, 1-18, 1-19, or 1-20 or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises CCP modules 2-20, 3-20, 4-20, 5-20, 6-20, 7-20, 8-20, 9-20, 10-20, 11-20, 12-20, 13-20, 14-20, 15-20, 16-20, 17-20, 18-20, or 19-20 or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises CCP modules 1-4 and 19-20, or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises CCP modules 1-4 and 18-20, or a biologically active fragment and/or variant thereof. In some embodiments, the CFH protein comprises CCP modules 1-5 and 18-20, or a biologically active fragment and/or variant thereof.

[0158] A consensus sequence of a full-length CFH protein is shown in SEQ ID NO: 1. Exemplary full-length CFH proteins covered by this consensus sequence are shown in **Table 1**.

Table 1. Amino Acid Substitutions in the CFH Sequence.

	Amino Acid Position		
SEQ ID NO	**62**	**402**	**936**
1	X_1	X_2	X_3
2	V	Y	E
3	V	H	E
4	V	Y	D
5	V	H	D
6	I	Y	E
7	I	H	E
8	I	Y	D
9	I	H	D

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[0159] In some embodiments, the CFH protein comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 1, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 1, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 1, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises the amino acid sequence of SEQ ID NO: 1 or a biologically active fragment thereof.

[0160] In some embodiments, the CFH protein comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 2 (mature CFH amino acid sequence (62V, 402Y, 936E)), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 2, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 2, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises the amino acid sequence of SEQ ID NO: 2 or a biologically active fragment thereof.

[0161] In some embodiments, the CFH protein comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 3 (mature CFH amino acid sequence (62V, 402H, 936E)), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 3, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 3, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises the amino acid sequence of SEQ ID NO: 3 or a biologically active fragment thereof.

[0162] In some embodiments, the CFH protein comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%

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identical to the amino acid sequence of SEQ ID NO: 4 (mature CFH amino acid sequence (62V, 402Y, 936D)), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 4, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 4, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises the amino acid sequence of SEQ ID NO: 4 or a biologically active fragment thereof.

[0163] In some embodiments, the CFH protein comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 5 (mature CFH amino acid sequence (62V, 402H, 936D)), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 5, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 5, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises the amino acid sequence of SEQ ID NO: 5 or a biologically active fragment thereof.

[0164] In some embodiments, the CFH protein comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 6 (mature CFH amino acid sequence (62I, 402Y, 936E)), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 6, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 6, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises the amino acid sequence of SEQ ID NO: 6 or a biologically active fragment thereof.

[0165] In some embodiments, the CFH protein comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 7 (mature CFH amino acid sequence (62I,

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402H, 936E)), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 7, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 7, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises the amino acid sequence of SEQ ID NO: 7 or a biologically active fragment thereof.

[0166] In some embodiments, the CFH protein comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 8 (mature CFH amino acid sequence (62I, 402Y, 936D)), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 8, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 8, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises the amino acid sequence of SEQ ID NO: 8 or a biologically active fragment thereof.

[0167] In some embodiments, the CFH protein comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 9 (mature CFH amino acid sequence (62I, 402H, 936D)), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 9, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 9, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises the amino acid sequence of SEQ ID NO: 9 or a biologically active fragment thereof.

[0168] In certain embodiments, the rCFH protein is characterized by an electrophoretogram comprising peak isoelectric point (pI) values from above 5.9 to below 6.8, wherein the pI values are measured by a capillary isoelectric focusing (cIEF) assay. In certain embodiments, the peak pI values are: about 6.02 ± 0.03, about 6.06 ± 0.03, about 6.11 ± 0.02, about 6.16 ± 0.02, about

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6.22 ± 0.02, about 6.28 ± 0.02, about 6.33 ± 0.01, about 6.40 ± 0.01, about 6.46 ± 0.01, about 6.53 ± 0.01, about 6.60 ± 0.00, about 6.67 ± 0.00, and about 6.73 ± 0.01. In certain embodiments, the rCFH protein is characterized by an electrophoretogram comprising percent area under the curve (AUC) values ordered from most acidic to most basic of: from 3.0% to 3.1%, from 3.6% to 4.4%, from 4.5% to 5.2%, from 5.6% to 6.5%, from 5.9% to 7.2%, from 7.1% to 8.1%, from 7.6% to 8.7%, from 8.0% to 9.1%, from 9.1% to 9.6%, from 9.4% to 10.1%, from 9.3% to 10.7%, from 7.6% to 9.5%, and from 3.3% to 6.5%, wherein the AUC values are measured by a cIEF assay. In certain embodiments, the percent AUC values ordered from most acidic to most basic are: about 3.0% ± 0.0%, about 3.9% ± 0.2%, about 4.8% ± 0.2%, about 5.9% ± 0.2%, about 6.4% ± 0.3%, about 7.4% ± 0.2%, about 8.0% ± 0.2%, about 8.6% ± 0.3%, about 9.4% ± 0.1%, about 9.7% ± 0.1%, about 10.1% ± 0.3%, about 8.9% ± 0.4%, and about 5.5% ± 0.8%.

[0169] In certain embodiments, the CFH protein is characterized by a free cysteine profile of trypsin-digested rCFH protein peptides of: about 0.9% of a Cys160-containing peptide fragment, about 3.2% of a Cys187-containing peptide fragment, about 3% of a Cys476-containing peptide fragment, about 1.2% of a Cys291-containing peptide fragment, about 1.2% of a Cys487-containing peptide fragment, about 0.5% of a Cys774-containing peptide fragment, about 0.6% of a Cys846-containing peptide fragment, about 0.5% of a Cys966-containing peptide fragment, about 0.5% of a Cys965-containing peptide fragment, about 0.8% of a Cys1000-containing peptide fragment, about 1% of a Cys1183-containing peptide fragment, about 1.9% of a Cys1145-containing and Cys1149-containing peptide fragment, and about 0.6% of a Cys1210-containing peptide fragment.

[0170] In certain embodiments, the CFH protein comprises less than 100 pmol of *N*-glycolylneuraminic (Neu5Gc) acid per mol protein, less than 10 pmol of Neu5Gc acid per mol protein, less than 1 pmol of Neu5Gc acid per mol protein, less than 0.1 pmol of Neu5Gc acid per mol protein, or less than 0.003 pmol of Neu5Gc acid per mol protein, as assayed by Ultra Performance Liquid Chromatography (UPLC).

[0171] In some embodiments, the CFH protein comprises at least CCP modules 1-4 or a biologically active fragment and/or variant thereof. As noted above, CCP modules 1-4 corresponds to amino acids 1-246 of SEQ ID NO: 1 or a variant thereof. Accordingly, in some embodiments, the CFH protein comprises an amino acid sequence that is at least 70%, 75%,

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80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to amino acids 1-246 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 90% identical to amino acids 1-246 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 95% identical to amino acids 1-246 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises amino acids 1-246 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof.

[0172] In some embodiments, the CFH protein comprises at least CCP modules 1-5 or a biologically active fragment and/or variant thereof. As noted above, CCP modules 1-5 corresponds to amino acids 1-304 of SEQ ID NO: 1 or a variant thereof. Accordingly, in some embodiments, the CFH protein comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to amino acids 1-304 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 90% identical to amino acids 1-304 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 95% identical to amino acids 1-304 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises amino acids 1-304 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof.

[0173] In some embodiments, the CFH protein comprises at least CCP modules 1-7 or a biologically active fragment and/or variant thereof. As noted above, CCP modules 1-7 corresponds to amino acids 1-426 of SEQ ID NO: 1 or a variant thereof. Accordingly, in some embodiments, the CFH protein comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to amino acids 1-426 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 90% identical to amino acids 1-426 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof. In some embodiments, the CFH protein

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comprises an amino acid sequence that is at least 95% identical to amino acids 1-426 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises amino acids 1-426 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof.

[0174] In some embodiments, the CFH protein comprises at least CCP modules 19-20 or a biologically active fragment and/or variant thereof. As noted above, CCP modules 19-20 corresponds to amino acids 1,089-1,212 of SEQ ID NO: 1 or a variant thereof. Accordingly, in some embodiments, the CFH protein comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to amino acids 1,089-1,212 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 90% identical to amino acids 1,089-1,212 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 95% identical to amino acids 1,089-1,212 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises amino acids 1,089-1,212 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof.

[0175] In some embodiments, the CFH protein comprises at least CCP modules 18-20 or a biologically active fragment and/or variant thereof. As noted above, CCP modules 18-20 corresponds to amino acids 1,028-1,212 of SEQ ID NO: 1 or a variant thereof. Accordingly, in some embodiments, the CFH protein comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to amino acids 1,028-1,212 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 90% identical to amino acids 1,028-1,212 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 95% identical to amino acids 1,028-1,212 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises amino

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acids 1,028-1,212 of SEQ ID NO: 1 (*e.g.*, SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, or 9), or a biologically active fragment thereof.

[0176] In some embodiments, the CFH protein is an FHL1 protein. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 10 (mature FHL1 amino acid sequence), or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 10, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 10, or a biologically active fragment thereof. In some embodiments, the CFH protein comprises the amino acid sequence of SEQ ID NO: 10 or a biologically active fragment thereof.

[0177] The biological activities of full-length, wild-type CFH are known in the art. It is understood that biologically active fragments and/or variants of a reference CFH sequence can be identified by functional characteristics. For example, in some embodiments, the CFH protein or biologically active fragment and/or variant thereof is capable of acting as a cofactor with CFI to facilitate C3b cleavage. In some embodiments, the CFH protein or biologically active fragment and/or variant thereof is capable of binding C3b. In some embodiments, the CFH protein or biologically active fragment and/or variant thereof is capable of facilitating the breakdown of C3b by acting in concert with complement factor I (CFI). In some embodiments, the CFH protein or biologically active fragment and/or variant thereof is capable of destabilizing C3bBb. In some embodiments, the CFH protein or biologically active fragment and/or variant thereof competes with factor B for binding to C3b. In some embodiments, the CFH protein or biologically active fragment and/or variant thereof prevents the formation of a C3 convertase (*e.g.* C3bBb). In some embodiments, the CFH protein or biologically active fragment and/or variant thereof accelerates the decay of convertase complexes (*e.g.* C3bBb). In some embodiments, the CFH protein or biologically active fragment and/or variant thereof accelerates the decay of the alternative pathway C5 convertase (C3bBbC3b). In some embodiments, the CFH protein or biologically active fragment and/or variant thereof is capable of suppressing C3b amplification. In some embodiments, the CFH protein or biologically active fragment and/or variant thereof is capable of binding to a eukaryotic cell surface (*e.g.*, an erythrocyte and/or

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endothelial cell). In some embodiments, the CFH polypeptide or biologically active fragment and/or variant thereof is capable of binding to heparin. In some embodiments, the CFH protein or biologically active fragment and/or variant thereof is capable of reducing C5b9 levels generated as a result of complement activation (*e.g.*, as measured in a Wieslab AP assay. In some embodiments, the CFH protein or biologically active and/or variant fragment thereof is capable of inhibiting hemolysis.

[0178] In some embodiments, the biologically active fragment and/or variant is at least 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 1100, 1200, or 1213 amino acids in length. In some embodiments, the biologically active fragment and/or variant is between 100-1213, 200-1213, 300-1213, 400-1213, 500-1213, 600-1213, 700-1213, 800-1213, 900-1213, 1000-1213, 1100-1213, 500-1213, 100-200, 100-300, 100-400, 100-500, 100-600, 100-700, 100-800, 100-900, 200-300, 200-400, 200-500, 200-600, 200-700, 200-800, 200-900, 300-400, 300-500, 300-600, 300-700, 300-800, 300-900, 400-500, 400-600, 400-700, 400-800, 400-900, 500-600, 500-700, 500-800, 500-900, 600-700, 600-800, 600-900, 700-800, 700-900, or 800-900 amino acids in length. In some embodiments, the biologically active fragment comprises at least 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, or 1200 consecutive amino acids from a sequence that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 1. In some embodiments, the biologically active fragment and/or variant comprises at least 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, or 1200 consecutive amino acids of SEQ ID NO: 1.

[0179] In some embodiments, any of the CFH polypeptides or biologically active fragments and/or variants disclosed herein is a modified CFH polypeptide or biologically active fragment thereof as compared to a reference CFH sequence (*e.g.*, a protein comprising the amino acid sequence of any one of SEQ ID NOs: 1-10). In some embodiments, a modified CFH polypeptide may comprise 1, 2, 3, 4, 5, up to 10, or more amino acid substitutions and/or deletions and/or insertions. A "deletion" may comprise the deletion of individual amino acids, deletion of small groups of amino acids such as 2, 3, 4 or 5 amino acids, or deletion of larger amino acid regions, such as the deletion of specific amino acid domains (*e.g.*, one or more CCP domains) or other features. An "insertion" may comprise the insertion of individual amino acids, insertion of small

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groups of amino acids such as 2, 3, 4 or 5 amino acids, or insertion of larger amino acid regions, such as the insertion of specific amino acid domains or other features (*e.g.*, insertion of a linker). A "substitution" comprises replacing a wild type amino acid with another (*e.g.*, a non-wild type amino acid). In some embodiments, the another (*e.g.*, non-wild type) or inserted amino acid is Ala (A), His (H), Lys (K), Phe (F), Met (M), Thr (T), Gln (Q), Asp (D), or Glu (E). In some embodiments, the another (*e.g.*, non-wild type) or inserted amino acid is A. In some embodiments, the another (*e.g.*, non-wild type) amino acid is Arg (R), Asn (N), Cys (C), Gly (G), Ile (I), Leu (L), Pro (P), Ser (S), Trp (W), Tyr (Y), or Val (V). Conventional or naturally occurring amino acids are divided into the following basic groups based on common side-chain properties: (1) non-polar: Norleucine, Met, Ala, Val, Leu, He; (2) polar without charge: Cys, Ser, Thr, Asn, Gin; (3) acidic (negatively charged): Asp, Glu; (4) basic (positively charged): Lys, Arg; and (5) residues that influence chain orientation: Gly, Pro; and (6) aromatic: Trp, Tyr, Phe, His. Conventional amino acids include L or D stereochemistry. In some embodiments, the another (*e.g.*, non-wild type) amino acid is a member of a different group (*e.g.*, an aromatic amino acid is substituted for a non-polar amino acid). Substantial modifications in the biological properties of the polypeptide are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a β-sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties: (1) Non-polar: Norleucine, Met, Ala, Val, Leu, Ile; (2) Polar without charge: Cys, Ser, Thr, Asn, Gln; (3) Acidic (negatively charged): Asp, Glu; (4) Basic (positively charged): Lys, Arg; (5) Residues that influence chain orientation: Gly, Pro; and (6) Aromatic: Trp, Tyr, Phe, His. In some embodiments, the another (*e.g.*, non-wild type) amino acid is a member of a different group (*e.g.*, a hydrophobic amino acid for a hydrophilic amino acid, a charged amino acid for a neutral amino acid, an acidic amino acid for a basic amino acid, etc.). In some embodiments, the another (*e.g.*, non-wild type) amino acid is a member of the same group (*e.g.*, another basic amino acid, another acidic amino acid, another neutral amino acid, another charged amino acid, another hydrophilic amino acid, another hydrophobic amino acid, another polar amino acid, another aromatic amino acid or another aliphatic amino acid). In some embodiments, the another (*e.g.*, non-wild type) amino acid is an unconventional amino acid. Unconventional amino acids are non-naturally occurring amino

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acids. Examples of an unconventional amino acid include, but are not limited to, aminoadipic acid, beta-alanine, beta-aminopropionic acid, aminobutyric acid, piperidinic acid, aminocaprioic acid, aminoheptanoic acid, aminoisobutyric acid, aminopimelic acid, citrulline, diaminobutyric acid, desmosine, diaminopimelic acid, diaminopropionic acid, N-ethylglycine, N-ethylaspargine, hyroxylysine, allo-hydroxylysine, hydroxyproline, isodesmosine, allo-isoleucine, N-methylglycine, sarcosine, N-methylisoleucine, N-methylvaline, norvaline, norleucine, orithine, 4-hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, σ-N-methylarginine, and other similar amino acids and amino acids (*e.g.*, 4-hydroxyproline). In one aspect, a modified CFH protein or biologically active fragment thereof comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 conservative or non-conservative substitutions relative to the wild-type CFH polypeptide or biologically active fragment thereof (*e.g.*, a polypeptide having the amino acid sequence of any one of SEQ ID NOs: 1-10).

[0180] In certain embodiments, any of the CFH polypeptides or biologically active fragments and/or variants disclosed herein may further comprise post-translational modifications in addition to any that are naturally present in the native polypeptides. Such modifications include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, pegylation (polyethylene glycol) and acylation. As a result, the modified polypeptides may contain non-amino acid elements, such as polyethylene glycols, lipids, mono- or poly-saccharides, and phosphates. Effects of such non-amino acid elements on the functionality of a polypeptide may be tested as described herein for other polypeptide variants. When a polypeptide is produced in cells by cleaving a nascent form of the polypeptide, post-translational processing may also be important for correct folding and/or function of the protein. Different cells have specific cellular machinery and characteristic mechanisms for such post-translational activities and may be chosen to ensure the correct modification and processing of the polypeptides.

CFH-encoding Vectors

[0181] The present disclosure also provides a nucleic acid encoding CFH protein, including a biologically active fragment and/or variant of a full-length, wild-type CFH protein, for use in any of the methods disclosed herein. In certain embodiments, the nucleic acid is present in a vector

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(*e.g.*, a viral vector), which can be formulated in a pharmaceutical composition and administered to a subject in need thereof.

[0182] In certain embodiments, the vector comprises a nucleotide sequence encoding a CFH protein disclosed herein operably linked to an expression control sequence. As used herein, the term "expression control sequence" refers to a nucleotide sequence that directs transcription and/or translation of a nucleic acid. Expression control sequences include but are not limited to transcription initiation, termination, promoter and enhancer sequences; RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*e.g.*, Kozak consensus sequence); and sequences that enhance protein stability. In certain embodiments, the vector comprises a promoter and/or an enhancer operably linked to the CFH-coding sequence.

[0183] In certain embodiments, the vector comprises a constitutive promoter operably linked to the CFH-coding sequence. In certain embodiments, the constitutive promoter is a CMV promoter (optionally with the CMV enhancer), RSV promoter (optionally with the RSV enhancer), SV40 promoter, MoMLV promoter, CB promoter, the dihydrofolate reductase promoter, the chicken β-actin (CBA) promoter, CBA/CAG promoter, and the immediate early CMV enhancer coupled with the CBA promoter, or a EF1a promoter.

[0184] In other embodiments, the vector comprises a cell- or tissue-specific expression control sequence, for example, a promoter and/or an enhancer that can direct expression of CFH preferentially in one or more ocular cell types, such as a rod cell, cone cell, bipolar cell (*e.g.*, on-bipolar cell, off-bipolar cell), horizontal cell, amacrine cell, retinal pigment epithelium (RPE) cell, retinal glial cell (*e.g.*, Muller cell), and/or retinal ganglion cell. In certain embodiments, the expression control sequence comprises a metabotropic glutamate receptor 6 (mGluR6) promoter (see, Vardi et al, mGluR6 Transcripts in Non-neuronal Tissues, J Histochem Cytochem. 2011 December; 59(12): 1076-1086). In certain embodiment, the expression control sequence further comprises an mGluR6 enhancer. In certain embodiment, the expression control sequence comprises a human G-protein-coupled receptor protein kinase 1 (GRK1) promoter (Genbank Accession number AY327580). In certain embodiment, the expression control sequence comprises a human interphotoreceptor retinoid-binding protein proximal (IRBP) promoter. Other useful promoters include, without limitation, the rod opsin promoter, the red-green opsin promoter, the blue opsin promoter, the cGMP-β-phosphodiesterase promoter, the mouse opsin

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promoter (Beltran et al 2010 cited above), the rhodopsin promoter (Mussolino et al, Gene Ther, July 2011, 18(7):637-45); the alpha-subunit of cone transducin (Morrissey et al, BMC Dev, Biol, Jan 2011, 11 :3); beta phosphodiesterase (PDE) promoter; the retinitis pigmentosa (RP1) promoter (Nicoud et al, J. Gene Med, Dec 2007, 9(12): 1015-23); the NXNL2/NXNL1 promoter (Lambard et al, PLoS One, Oct. 2010, 5(10):el3025), the RPE65 promoter; the retinal degeneration slow/peripherin 2 (Rds/perph2) promoter (Cai et al, Exp Eye Res. 2010 Aug;91(2): 186-94); and the VMD2 promoter (Kachi et al, Human Gene Therapy, 2009 (20:31-9)).

[0185] In certain embodiments, the expression control sequence is the native promoter for the gene to be expressed (*e.g.*, the CFH gene or the FHL-1 gene).

[0186] In certain embodiments, the expression control sequence comprises an enhancer. Enhancer sequences useful herein include but are not limited to the IRBP enhancer (Nicoud et al, J. Gene Med, Dec 2007, 9(12): 1015-23), immediate early CMV enhancer, immunoglobulin enhancer, and SV40 enhancer.

[0187] Other expression control sequences useful herein include an intron, optionally positioned between the promoter/enhancer sequence and the gene. In some embodiments, the intron sequence is derived from SV-40, and is a 100 bp mini-intron splice donor/splice acceptor referred to as SD-SA. Another suitable sequence includes the woodchuck hepatitis virus post-transcriptional element (*see, e.g.*, L. Wang and I. Verma, 1999 Proc. Natl. Acad. Sci., USA, 96:3906-3910). PolyA signals may be derived from many suitable species, including, without limitation SV-40, human and bovine.

[0188] Another expression control sequence useful herein is an internal ribosome entry site (IRES). An IRES sequence may be used to produce more than one polypeptide from a single gene transcript (for example, to produce more than one complement system polypeptides). An IRES (or other suitable sequence) is used to produce a protein that contains more than one polypeptide chain or to express two different proteins from or within the same cell. An exemplary IRES is the poliovirus internal ribosome entry sequence, which supports transgene expression in photoreceptors, RPE and ganglion cells. Preferably, the IRES is located 3' to the CFH-coding sequence in the vector.

[0189] Expression control sequences that are small in size are desirable in vectors that have a size limitation, for example, adeno-associated virus (AAV) vectors. In some embodiments, the expression control sequence is less than 1000, 900, 800, 700, 600, 500, 400 or 300 nucleotides in

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size. In some embodiments, the expression control sequence comprises, consists of, or consists essentially of a small promoter, *e.g.*, a CRALBP (RLBP), EF1a, HSP70, AAT1, ALB, PCK1, CAG, RPE65, MECP, sCBA, or CB promoter.

[0190] In certain embodiments, the vector is an AAV vector, for example, a recombinant AAV (rAAV) vector. A rAAV vector comprises one or more heterologous sequences (*i.e.*, nucleic acid sequence not of AAV origin) that are flanked by at least one AAV inverted terminal repeat sequence (ITR). Such rAAV vectors can be replicated and packaged into infectious viral particles in a host cell that has been infected with a suitable helper virus or that is expressing suitable helper genes such as AAV rep and cap. When a rAAV vector is incorporated into a larger polynucleotide (*e.g.*, in a chromosome or in another vector such as a plasmid used for cloning or transfection), the vector is referred to as a "pro-vector" which can be used to produce a mature rAAV vector by replication and encapsidation in the presence of AAV packaging functions and suitable helper functions. An rAAV vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and encapsidated in a viral particle, e.g., an AAV particle. A rAAV vector can be packaged into an AAV virus capsid to generate a recombinant adeno-associated viral particle (rAAV particle).

[0191] AAV particles are present in various serotypes. Naturally occurring AAV serotypes include, without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV10, AAV11, AAV12, AAV-DJ, AAV-DJ8, AAV-DJ9, AAVrh8, AAVrh8R and AAVrh10. Each serotype has its unique capsid proteins and ITR sequences that is related to its tropism. For example, AAV1, AAV2, AAV4, AAV5, AAV7 and AAV8 are suitable for gene transfer to photoreceptor cells or the retinal pigment epithelium (RPE) cells. Non-naturally occurring serotypes can also be created by engineering the ITR and/or capsid. A recombinant AAV can be chimeric, comprising the ITR sequence from one serotype and the capsid from a different serotype.

[0192] Generally, an "inverted terminal repeat" or "ITR" sequence is an approximately 145-nucleotide sequence present at both termini of the native single-stranded AAV genome. The outermost 125 nucleotides of the ITR can be present in either of two alternative orientations, leading to heterogeneity between different AAV genomes and between the two ends of a single AAV genome. The outermost 125 nucleotides also contains several shorter regions of self-

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complementarity (designated A, A', B, B', C, C and D regions), allowing intrastrand base-pairing to occur within this portion of the ITR. In certain embodiments, the AAV vector disclosed herein comprises the ITR sequences of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV10, AAV11, AAV12, AAV-DJ, AAV-DJ8, AAV-DJ9, AAVrh8, AAVrh8R or AAVrh10. In certain embodiments, the AAV vector comprises the ITR sequences of AAV2.

[0193] The capsid of AAV particles includes Cap proteins VP1, VP2, and VP3. In certain embodiments, the AAV vector disclosed herein is present in an AAV particle comprising VP1, VP2, and VP3 of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV10, AAV11, AAV12, AAV-DJ, AAV-DJ8, AAV-DJ9, AAVrh8, AAVrh8R or AAVrh10, or a variant or derivative thereof. In certain embodiments, the AAV particle comprises VP1, VP2, and VP3 of AAV2 or a variant or derivative thereof. In certain embodiments, the AAV capsid serotype is AAV2. In certain embodiments, the AAV capsid serotype is AAV.7m8 (see, *e.g.*, International Patent Application Publication No. WO 2012/145601). In certain embodiments, the capsid is modified by amino acid deletion, insertion, and/or substitution to reduce immunogenicity, improve stability, reduce degradation, and/or improve the efficiency of infecting an ocular cell. It is understood that in a pseudotyped AAV particle the Cap proteins can be encoded by a nucleic acid in the producer cell but not in the AAV genome. Accordingly, in certain embodiments, the AAV particle does not comprise a nucleotide sequence encoding a Cap protein.

[0194] AAV particles contain a single-stranded genome when packaged into the capsid. However, self-complementary AAV having an inverted repeat genome can be produced (see, US 2012/0141422). Such AAV genome can fold into double-stranded DNA without the requirement for DNA synthesis or base-pairing between multiple vector genomes. Because scAAV have no need to convert the single-stranded DNA genome into double-stranded DNA prior to expression, they are more efficient vectors. However, self-complementary AAV genomes has only half of the capacity of the vector relative to single-stranded AAV genomes and are thus useful for small transgenes (*e.g.*, small protein-coding sequences and RNA-based therapies). In some embodiments, the rAAV vector disclosed herein comprises a single-stranded genome. In some embodiments, the rAAV vector disclosed herein comprises a self-complementary genome.

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[0195] rAAV vectors useful in the methods of the disclosure are further described in PCT publication No. WO2015168666 and PCT publication no. WO2014011210, the contents of which are incorporated by reference herein.

[0196] Recombinant AAV particles can be produced by delivering the recombinant AAV vector, rep sequences, cap sequences, and helper functions to a packaging host cell. Methods of generating rAAV virions are well known in the art (*see, e.g.*, K. Fisher et al, J. Virol., 70:520-532 (1993) and U.S. Pat. No. 5,478,745). In some embodiments, recombinant AAVs may be produced using the triple transfection method (described in detail in U.S. Pat. No. 6,001,650). Typically, the recombinant AAVs are produced by transfecting a host cell with an recombinant AAV vector (comprising a transgene) to be packaged into AAV particles, an AAV helper function vector, and an accessory function vector. An AAV helper function vector encodes the "AAV helper function" sequences (e.g., rep and cap), which function in trans for productive AAV replication and encapsidation. In some embodiments, a single nucleic acid encoding all three capsid proteins (e.g., VP1, VP2 and VP3) is delivered into the packaging host cell in a single vector. The accessory functions are conferred by nucleotide sequences for non-AAV derived viral and/or cellular functions upon which AAV is dependent for replication. In certain embodiments, the accessory function vector comprises viral genetic elements, for example, elements derived from any of the known helper viruses such as adenovirus, herpesvirus (other than herpes simplex virus type-1), and vaccinia virus. In certain embodiments, the accessory function vector comprises genetic elements from an adenovirus, *e.g.*, E1a, E1b, E2a, and/or E4ORF6. One or more of the required components for producing a rAAV may be provided by a host cell which has been engineered to contain the components in its genome.

Pharmaceutical Compositions

[0197] Also provided herein are pharmaceutical compositions comprising a CFH protein disclosed herein and a pharmaceutically acceptable carrier or excipient for use in the method disclosed herein. The pharmaceutical compositions may be suitable for any mode of administration described herein; for example, by ocular (*e.g.*, intravitreal) administration. In some embodiments, the pharmaceutical compositions described herein is suitable for ocular injection. In some embodiments, the pharmaceutical composition is suitable for intravitreal injection. In some embodiments, the pharmaceutical composition is suitable for subretinal delivery.

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[0198] Pharmaceutically acceptable carriers and excipients suitable for administration to a subject (*e.g.*, human subject) are well known in the art (see, *e.g.*, Remington's Pharmaceutical Sciences, 15th Edition, pp. 1035-1038 and 1570-1580). Such pharmaceutically acceptable carriers can be sterile liquids, such as oil, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, and the like. Saline solutions and aqueous dextrose, polyethylene glycol (PEG) and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. The pharmaceutical composition may further comprise additional ingredients, for example preservatives, buffers, tonicity agents, antioxidants and stabilizers, nonionic wetting or clarifying agents, viscosity-increasing agents, and the like. The pharmaceutical compositions described herein can be packaged in single unit dosages or in multi-dosage forms. The compositions are generally formulated as sterile and substantially isotonic solution.

[0199] The pharmaceutical composition may further comprise additional ingredients, for example preservatives, buffers, tonicity agents, antioxidants and stabilizers, nonionic wetting or clarifying agents, viscosity increasing agents (viscosity inducing agents), and the like. Examples of useful viscosity-inducing components include, but are not limited to, hyaluronic acid (*e.g.*, polymeric hyaluronic acid), carbomer, polyacrylic acid, cellulose derivatives, polycarbophil, polyvinyl pyrrolidone, gelatin, dextrin, polysaccharide, polyacrylamide, polyvinyl alcohol, polyvinyl acetate, derivatives thereof and mixtures and copolymers thereof.

[0200] The composition is generally formulated as a sterile and substantially isotonic solution. In some embodiments, the compositions of the present disclosure are formulated into a pharmaceutical composition intended for subretinal or intravitreal injection. Such formulations involve the use of a pharmaceutically and/or physiologically acceptable vehicle or carrier, particularly one suitable for administration to the eye, *e.g.*, by subretinal or intravitreal injection, such as buffered saline or other buffers, *e.g.*, HEPES, to maintain pH at appropriate physiological levels, and, optionally, other medicinal agents, pharmaceutical agents, stabilizing agents, buffers, carriers, adjuvants, and/or diluents. For injection, the carrier will typically be a liquid. Exemplary physiologically acceptable carriers include pyrogen-free, phosphate buffered saline. A variety of such known carriers are provided in US Patent No. 7,629,322. In some embodiments, the carrier is an isotonic sodium chloride solution. In some embodiments, the isotonic sodium chloride solution comprises about 0.15 M sodium chloride. In another

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embodiment, the carrier is a balanced salt solution. In one embodiment, the carrier includes a surfactant such as TWEEN®20 or perfluorooctane (Perfluoron liquid). If the CFH polypeptide or biologically active fragment and/or variant thereof is to be stored long-term, it may be frozen in the presence of glycerol or TWEEN®20.

[0201] In some embodiments, the pharmaceutical composition is substantially non-toxic, for example, to intraocular tissues. When the compositions include a liquid carrier and are formulated for ocular administration, it is advantageous that the carrier component has a refractive index substantially similar to that of aqueous humor or vitreous humor (depending on the chamber into which the formulation is introduced). As a result, there is no substantial adverse effect on the patient's vision, for example, by changing the focus after administration. Formulations with a refractive index of water (approximately 1.33 depending on the wavelength of light) can adversely affect the patient's vision at the time after administration, for example, at the boundary between the injected formulation and the vitreous humor after injection.

[0202] The pharmaceutical composition can be formulated at pH levels that are suitable for direct administration to the eye (*e.g.*, intravitreal injection). Generally, pH values that are suitable for safe intravitreal injection range from 3-8 (see, for example, Marra *et al.*, *AAPS PharmSciTech*, 2011) or 4.0-7.4 (see, for example, Lorget *et al.*, *Investigative Ophthalmology & Visual Science*, 2018). In some embodiments, the pharmaceutical composition disclosed herein formulated to maintain a physiological pH (*e.g.*, a pH of about 7). In some embodiments, the pH of the pharmaceutical composition is in the range of about 5.0 to 8.0, about 5.0 to 7.0, about 5.0 to 6.0, about 6.0 to 8.0, about 6.1 to 8.0, about 6.2 to 8.0, about 6.3 to 8.0, about 6.4 to 8.0, about 6.5 to 8.0, about 6.6 to 8.0, about 6.7 to 8.0, about 6.8 to 8.0, about 6.9 to 8.0, about 7.0 to 8.0, about 7.1 to 8.0, about 7.2 to 8.0, about 7.3 to 8.0, about 7.4 to 8.0, about 7.5 to 8.0, about 7.6 to 8.0, about 7.7 to 8.0, about 7.8 to 8.0, about 7.9 to 8.0, about 6.60 to 7.40, about 6.0 to 7.5, about 6.1 to 7.5, about 6.2 to 7.5, about 6.3 to 7.5, about 6.4 to 7.5, about 6.5 to 7.5, about 6.6 to 7.5, about 6.7 to 7.5, about 6.8 to 7.5, about 6.9 to 7.5, about 7.0 to 7.5, about 7.1 to 7.5, about 7.2 to 7.5, about 7.3 to 7.5, about 7.4 to 7.5, about 6 to 7.0, about 6.1 to 7.0, about 6.2 to 7.0, about 6.3 to 7.0, about 6.4 to 7.0, about 6.5 to 7.0, about 6.6 to 7.0, about 6.7 to 7.0, about 6.8 to 7.0, about 6.9 to 7.0. In some embodiments, the pH of the pharmaceutical composition is about 5.0, about 5.5, 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

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6.8, about 6.9, about 7.0, about 7.1, about 7.2, about 7.3, about 7.4, about 7.5, about 7.6, about 7.7, about 7.8, about 7.9, or about 8.0.

[0203] In some embodiments, the pharmaceutical composition disclosed herein comprise buffering agents such as sodium phosphate monobasic monohydrate and/or sodium phosphate dibasic heptahydrate. These buffering agents are used to maintain the pH of the compositions. Other buffering agents are contemplated in the compositions of the present disclosure. Alternative buffer components may be selected from those known in the art. Non-limiting examples of buffers include acetate buffer, citrate buffer, phosphate buffer, borate buffer, and the like, and mixtures thereof.

[0204] In some embodiments, the pharmaceutical composition disclosed herein comprise a stabilizer such as sodium chloride and/or polysorbate 20. In addition to stabilizing the compositions, polysorbate 20 prevents the formation of protein aggregates (*e.g.*, due to agitation) in the composition. Polysorbate 20 protects against agitation-induced stress on the CFH protein in solution. Other stabilizers are contemplated in the compositions of the present disclosure.

[0205] In some embodiments, the pharmaceutical composition disclosed herein is formulated to be isotonic. This can be achieved using a tonicity agent. In some embodiments, the compositions of the present disclosure comprise a tonicity agent, such as sodium chloride. Other tonicity agents are contemplated in the compositions of the present disclosure. Non-limiting examples of tonicity agents include glycerin, lactose, mannitol, dextrose, sodium chloride, sodium sulfate, sorbitol, physiological saline-sodium citrate (SSC), and the like.

[0206] The concentration of the active ingredient (*e.g.*, a CFH protein) in the pharmaceutical composition can be determined based on the desired dose and the volume clinically suitable for intravitreal administration. In certain embodiments, the pharmaceutical composition comprises CFH protein in range of 1-50 mg/mL (*e.g.*, 1-20 mg/mL, 1-10 mg/mL, 5-50 mg/mL, 5-20 mg/mL, 5-10 mg/mL, or 10-20 mg/mL). In certain embodiments, the pharmaceutical composition comprises CFH protein in range of 9.0-11.0 mg/mL. In some embodiments, the concentration of CFH protein in the pharmaceutical composition is about 9.0 mg/mL, about 9.1 mg/mL, about 9.2 mg/mL, about 9.3 mg/mL, about 9.4 mg/mL, about 9.5 mg/mL, about 9.6 mg/mL, about 9.7 mg/mL, about 9.8 mg/mL, about 9.9 mg/mL, about 10 mg/mL, about 10.1 mg/mL, about 10.2 mg/mL, about 10.3 mg/mL, about 10.4 mg/mL, about 10.5 mg/mL, about 10.6 mg/mL, about 10.7 mg/mL, about 10.8 mg/mL, about 10.9 mg/mL, or about 11 mg/mL. In

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some embodiments, the concentration of CFH protein in the pharmaceutical composition is in the range of 9.0-11.0 mg/mL, about 9.0-10.5 mg/mL, about 9.0-10.0 mg/mL, about 9.0-9.5 mg/mL, about 9.5-11.0 mg/mL, about 9.-5-10.5 mg/mL, about 9.5-10.0 mg/mL, about 10.0-11.0 mg/mL, about 10.0-10.5 mg/mL, or about 10.5-11.0 mg/mL.

[0207] In certain embodiments, the pharmaceutical composition used in the method of treatment disclosed herein comprises phosphate-buffer saline (PBS). In certain embodiments, the pharmaceutical composition comprises a surfactant. In certain embodiments, the pharmaceutical composition comprises PBS and a surfactant. In certain embodiments, the CFH protein is administered by intravitreal injection in a solution that comprises 0.02 M sodium phosphate and 0.15 M NaCl. In certain embodiments, the solution further comprises from 0.01% to 0.05% by weight of a surfactant. In certain embodiments, the solution further comprises from 0.01% to 0.05% by weight polysorbate 20.

[0208] In some embodiments, the pharmaceutical composition comprises 5-20 mg/mL (*e.g.*, about 10 mg/mL) CFH protein and one or more excipients selected from sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, sodium chloride, and/or polysorbate 20. In some embodiments, the pharmaceutical compositions include water. In some embodiments, the pharmaceutical composition comprises about 0.02 M sodium phosphate, about 0.15 M sodium chloride, and about 0.01% (w/v) polysorbate 20, at pH 7.0. In some embodiments, the pharmaceutical composition comprises 0.02 M sodium phosphate, 0.15 M sodium chloride, and 0.01% (w/v) polysorbate 20, at pH 7.0.

[0209] In some embodiments, the pharmaceutical composition consists essentially of CFH protein and one or more excipients selected from sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, sodium chloride, and polysorbate 20. In some embodiments, the pharmaceutical composition consists essentially of CFH protein and about 0.02 M sodium phosphate, about 0.15 M sodium chloride, and about 0.01% (w/v) polysorbate 20, at pH 7.0. In some embodiments, the pharmaceutical composition consists essentially of 0.02 M sodium phosphate, 0.15 M sodium chloride, and 0.01% (w/v) polysorbate 20, at pH 7.0.

Route of Administration

[0210] As used herein, "administering" or "administration" of a substance, a compound or an agent to a subject can be carried out using one of a variety of methods known to those skilled in the art. For example, a compound or an agent can be administered intravitreally or subretinally.

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In particular embodiments, the compound or agent is administered intravitreally. In some embodiments, administration may be local. In other embodiments, administration may be systemic. Administering can also be performed, for example, once, a plurality of times, and/or over one or more extended periods. In some aspects, the administration includes both direct administration, including self-administration, and indirect administration, including the act of prescribing a drug. For example, as used herein, a physician who instructs a patient to self-administer a drug, or to have the drug administered by another and/or who provides a patient with a prescription for a drug is administering the drug to the patient.

[0211] In some embodiments, the CFH protein or pharmaceutical composition disclosed herein is administered locally to the cells in the retina for treating diseases such as LCA, retinitis pigmentosa, and age-related macular degeneration. The cells that will be the treatment target in these diseases are either the photoreceptor cells in the retina or the cells of the RPE underlying the neurosensory retina. Delivering the CFH protein or pharmaceutical composition to these cells may be by injection into the subretinal space between the retina and the RPE. In some embodiments, the disclosure provides methods to deliver the CFH protein or pharmaceutical composition to cells of the retina.

[0212] In some embodiments, any of the CFH protein or pharmaceutical composition disclosed herein are administered to a patient such that they target cells of any one or more layers or regions of the retina or macula. For example, the compositions disclosed herein target cells of any one or more layers of the retina, including the inner limiting membrane, the nerve fiber layer, the ganglion cell layer (GCL), the inner plexiform layer, the inner nuclear layer, the outer plexiform layer, the outer nuclear layer, the external limiting membrane, the layer of rods and cones, or the retinal pigment epithelium (RPE). In some embodiments, the compositions disclosed herein target glial cells of the GCL, Muller cells, and/or retinal pigment epithelial cells. In some embodiments, the compositions disclosed herein targets cells of any one or more regions of the macula including, for example, the umbo, the foveolar, the foveal avascular zone, the fovea, the parafovea, or the perifovea. In some embodiments, the route of administration does not specifically target neurons. In some embodiments, the route of administration is chosen such that it reduces the risk of retinal detachment in the patient (*e.g.*, intravitreal rather than subretinal administration).

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[0213] In certain embodiments of the methods described herein, the CFH or pharmaceutical composition disclosed herein is administered to the subject by subretinal injection. In other embodiments, the pharmaceutical composition is administered by intravitreal injection. Other forms of administration that may be useful in the methods described herein include, but are not limited to, direct delivery to a desired organ (*e.g.*, the eye), oral, inhalation, intranasal, intratracheal, intravenous, intramuscular, subcutaneous, intradermal, and other parenteral routes of administration. Routes of administration may be combined, if desired. In certain embodiments, the pharmaceutical compositions of the disclosure are administered after administration of an initial loading dose of the CFH polypeptide or biologically active fragment and/or variant thereof.

[0214] In specific embodiments, the CFH or pharmaceutical composition disclosed herein is administered to the subject by intravitreal (IVT) injection. Procedures for intravitreal injection are known in the art (see, *e.g.*, Peyman, G.A., et al. (2009) Retina 29(7):875-912 and Fagan, X.J. and Al-Qureshi, S. (2013) Clin. Experiment. Ophthalmol. 41(5):500-7). Briefly, a subject for intravitreal injection may be prepared for the procedure by pupillary dilation, sterilization of the eye, and administration of anesthetic. Any suitable mydriatic agent known in the art may be used for pupillary dilation. Adequate pupillary dilation may be confirmed before treatment. Sterilization may be achieved by applying a sterilizing eye treatment, *e.g.*, an iodide-containing solution such as Povidone-Iodine (BETADINE®). A similar solution may also be used to clean the eyelid, eyelashes, and any other nearby tissues (*e.g.*, skin). Any suitable anesthetic may be used, such as lidocaine or proparacaine, at any suitable concentration. Anesthetic may be administered by any method known in the art, including without limitation topical drops, gels or jellies, and subconjuctival application of anesthetic. Prior to injection, a sterilized eyelid speculum may be used to clear the eyelashes from the area. The site of the injection may be marked with a syringe. The site of the injection may be chosen based on the lens of the patient. For example, the injection site may be 3-3.5 mm from the limbus in pseudophakic or aphakic patients, and 3.5-4 mm from the limbus in phakic patients. The patient may look in a direction opposite the injection site. During injection, the needle may be inserted perpendicular to the sclera and pointed to the center of the eye. The needle may be inserted such that the tip ends in the vitreous, rather than the subretinal space. Any suitable volume known in the art for injection may be used. After injection, the eye may be treated with a sterilizing agent such as an

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antibiotic. The eye may also be rinsed to remove excess sterilizing agent. Ophthalmologists are accustomed to providing frequent (*e.g.*, monthly) IVT injections for wet macular degeneration. It is not uncommon for patients to be treated on a regular basis for long periods of time (*e.g.*, years). The risk of severe complications following IVT injection such as uveitis, retinal detachment, extensive IVT hemorrhage, raised intraocular pressure (IOP), or endophthalmitis.

[0215] Furthermore, in certain embodiments, it is desirable to perform non-invasive retinal imaging and functional studies to identify areas of specific ocular cells to be targeted for therapy. In these embodiments, clinical diagnostic tests are employed to determine the precise location(s) for one or more subretinal injection(s). These tests may include ophthalmoscopy, electroretinography (ERG) (particularly the b-wave measurement), perimetry, topographical mapping of the layers of the retina and measurement of the thickness of its layers by means of confocal scanning laser ophthalmoscopy (cSLO) and optical coherence tomography (OCT), topographical mapping of cone density via adaptive optics (AO), functional eye exam, etc.

[0216] These, and other desirable tests, are described in International Patent Application No. PCT/US2013/022628. In view of the imaging and functional studies, in some embodiments, one or more injections are performed in the same eye in order to target different areas of retained bipolar cells. The volume and concentration of the CFH polypeptide or biologically active fragment and/or variant thereof for each injection is determined individually, as further described below, and may be the same or different from other injections performed in the same, or contralateral, eye. In another embodiment, a single, larger volume injection is made in order to treat the entire eye. In one embodiment, the volume and concentration of the CFH polypeptide or biologically active fragment and/or variant thereof composition is selected so that only a specific region of ocular cells is impacted. In another embodiment, the volume and/or concentration of the CFH polypeptide or biologically active fragment and/or variant thereof composition is a greater amount, in order reach larger portions of the eye, including non-damaged ocular cells.

[0217] The composition may be delivered in a volume of from about 0.1 μL to about 1 mL, including all numbers within the range, depending on the size of the area to be treated, the route of administration, and the desired effect of the method. In certain embodiments, the volume is between 10-100 μL. In some embodiments, the volume is between 25-100 μL. In some embodiments, the volume is between 40-60 μL. In one embodiment, the volume is about 50 μL.

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In one embodiment, the volume is 50 μL. In another embodiment, the volume is about 70 μL. In a preferred embodiment, the volume is about 100 μL. In another embodiment, the volume is about 125 μL. In another embodiment, the volume is about 150 μL. In another embodiment, the volume is about 175 μL. In yet another embodiment, the volume is about 200 μL. In another embodiment, the volume is about 250 μL. In another embodiment, the volume is about 300 μL. In another embodiment, the volume is about 450 μL. In another embodiment, the volume is about 500 μL. In another embodiment, the volume is about 600 μL. In another embodiment, the volume is about 750 μL. In another embodiment, the volume is about 850 μL. In another embodiment, the volume is about 1000 μL.

[0218] It is desirable that the lowest effective concentration of CFH protein be utilized in order to reduce the risk of undesirable effects, such as toxicity, retinal dysplasia and detachment. Still other dosages and administration volumes in these ranges may be selected by the attending physician, taking into account the physical state of the subject, preferably human, being treated, the age of the subject, the particular ocular disorder and the degree to which the disorder, if progressive, has developed. For extra-ocular delivery, the dosage will be increased according to the scale-up from the retina.

Combination Therapy

[0219] In certain embodiment, the method disclosed herein is performed in combination with another, or secondary, therapy. The therapy may be any now known, or as yet unknown, therapy which helps prevent, arrest or ameliorate any of the described retinal changes and/or vision loss. In one embodiment, the secondary therapy is encapsulated cell therapy (such as that delivering Ciliary Neurotrophic Factor (CNTF)). *See, e.g.*, Sieving, P.A. et al, 2006. Proc Natl Acad Sci USA, 103(10):3896-3901, which is hereby incorporated by reference. In another embodiment, the secondary therapy is a neurotrophic factor therapy (such as pigment epithelium-derived factor, PEDF; ciliary neurotrophic factor 3; rod-derived cone viability factor (RdCVF) or glial-derived neurotrophic factor). In another embodiment, the secondary therapy is anti-apoptosis therapy (such as that delivering X-linked inhibitor of apoptosis, XIAP). In yet another embodiment, the secondary therapy is rod-derived cone viability factor 2. The secondary therapy can be administered before, concurrent with, or after administration of any of the CFH polypeptides or biologically active fragments and/or variants thereof described above.

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[0220] In some embodiments, the CFH or pharmaceutical composition disclosed herein is administered to a subject in combination with another therapeutic agent or therapeutic procedure. In some embodiments, the additional therapeutic agent is an antagonist of VEGF (*e.g.*, VEGF-A), such as an anti-VEGF antibody or fragment thereof (*e.g.*, ranibizumab or bevacizumab) or a decoy protein (*e.g.*, aflibercept). VEGF antagonists and their uses for treating ocular diseases were reviewed by Fogli *et al.*, Eye (2018) 32, 1010–20. In some embodiments, the additional therapeutic agent is a vitamin or mineral (*e.g.*, vitamin C, vitamin E, lutein, zeaxanthin, zinc or copper), omega-3 fatty acids, and/or Visudyne™. In some embodiments, the inflammatory ocular disease, disorder, or condition is wet AMD or diabetic macular edema, and the CFH or pharmaceutical composition disclosed herein is administered to the subject in combination with an anti-VEGF therapeutic agent. In some embodiments, the other therapeutic procedure is a diet having reduced omega-6 fatty acids, laser surgery, laser photocoagulation, submacular surgery, retinal translocation, and/or photodynamic therapy. In some embodiments, the additional therapeutic agent is a vector (*e.g.*, an AAV vector) encoding a CFH protein or biologically active fragment/variant thereof and/or a CFI protein or a biologically active fragment/variant thereof.

[0221] In some embodiments, the CFH or pharmaceutical composition disclosed herein is administered to a subject in combination with aflibercept. In some embodiments, aflibercept is administered at the dose of 2 mg, 4 mg, or 8 mg. In some embodiments, aflibercept is administered at the dose of 2 mg once every month, once every 4 weeks, once every two months, or once every 8 weeks. In some embodiments, aflibercept is administered at the dose of 8 mg once every two months, once every 8 weeks, once every three months, once every 12 weeks, once every four months, or once every 16 weeks.

[0222] In some embodiments, the CFH or pharmaceutical composition disclosed herein is administered to a subject in combination with an additional agent needed for processing and/or improving the function of CFH. For example, the CFH or pharmaceutical composition disclosed herein may be administered with an antibody (or a vector encoding that antibody) that potentiates the activity of the administered CFH and an endogenous CFH protein. Examples of such antibodies are found in WO2016/028150 or WO2019/139481.

[0223] It is understood that the CFH or pharmaceutical composition disclosed herein can be administered separately, optionally by a different route and/or at a different frequency, from the therapy used in combination.

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Therapeutic Efficacy

[0224] The efficacy of the treatment can be determined by performing functional and imaging studies to the subject. These studies include ERG and *in vivo* retinal imaging, as described in the examples below. In addition, visual field studies, perimetry and microperimetry, pupillometry, mobility testing, visual acuity, contrast sensitivity, color vision testing may be performed.

[0225] In certain embodiments, the progression or severity of the inflammatory ocular disease, disorder, or condition can be assessed by any suitable method known in the art, including, but not limited to best corrected visual acuity (BCVA) score, low luminance visual acuity (LLVA) score, AREDS 9-step severity scale score, area of geographic atrophy, drusen volume, or a retinal architecture parameter. In certain embodiments, the BCVA score is improved by at least 8-letter, at least 9-letter, at least 10-letter, at least 11-letter, or at least 12-letter. In certain embodiments, the LLVA score is improved by at least 4-letter, at least 5-letter, at least 6-letter, or at least 7-letter. The area of geographic atrophy can be assessed by any suitable method, including, but not limited to, color fundus photography, fundus autofluorescence optical coherence tomography-angiography, near infrared imaging, and /or fluorescein angiography. In certain embodiments, the rate of expansion in the area of geographic atrophy is reduced by at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% relative to the rate of expansion in the same subject prior to the treatment. Retinal architecture parameters can be assessed by any suitable method including, but not limited to, optical coherence tomography. Exemplary retinal architecture parameters include total retinal and choroidal thickness, photoreceptor layer thickness, features of nascent geographic atrophy, retinal pigment epithelium thickening, and integrity of retinal pigment epithelium layer. In certain embodiments, one or more retinal architecture parameters are improved based on clinical standards known in the art.

[0226] The retinal diseases described above are associated with various retinal changes. These may include a loss of photoreceptor structure or function; thinning or thickening of the outer nuclear layer (ONL); thinning or thickening of the outer plexiform layer (OPL); disorganization followed by loss of rod and cone outer segments; shortening of the rod and cone inner segments; retraction of bipolar cell dendrites; thinning or thickening of the inner retinal layers including inner nuclear layer, inner plexiform layer, ganglion cell layer and nerve fiber

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layer; opsin mislocalization; overexpression of neurofilaments; thinning of specific portions of the retina (such as the fovea or macula); loss of ERG function; loss of visual acuity and contrast sensitivity; loss of optokinetic reflexes; loss of the pupillary light reflex; and loss of visually guided behavior. In one embodiment, a method of preventing, arresting progression of or ameliorating any of the retinal changes associated with these retinal diseases is provided. In certain embodiment, the method of treatment disclosed herein improves the subject's vision or arrests and/or ameliorates vision loss.

[0227] In particular embodiments, a method of preventing, arresting progression of or ameliorating vision loss associated with an ocular disorder in the subject is provided. Vision loss associated with an ocular disorder refers to any decrease in peripheral vision, central (reading) vision, night vision, day vision, loss of color perception, loss of contrast sensitivity, or reduction in visual acuity.

[0228] In certain embodiments, the method of treatment disclosed herein increases the viability and/or homeostasis of ocular cells in the subject. In one embodiment, the ocular cell is a glial cell. In one embodiment, the ocular cell is an RPE cell. In another embodiment, the ocular cell is a photoreceptor. In another embodiment, the photoreceptor is a cone cell. In another embodiment, the ocular cell is a Muller cell. In another embodiment, the ocular cell is a bipolar cell. In yet another embodiment, the ocular cell is a horizontal cell. In another embodiment, the ocular cell is an amacrine cell. In still another embodiment, the ocular cell is a ganglion cell.

[0229] As used herein "photoreceptor function loss" means a decrease in photoreceptor function as compared to a normal, non-diseased eye or the same eye at an earlier time point. In some embodiments, the method disclosed herein may be used to increase photoreceptor function in a subject in need thereof, i.e., to improve the function of the photoreceptors or increase the number or percentage of functional photoreceptors as compared to a diseased eye (having the same ocular disease), the same eye at an earlier time point, a non-treated portion of the same eye, or the contralateral eye of the same patient. Photoreceptor function may be assessed using the functional studies described above and in the examples below, *e.g.*, ERG, perimetry, or microperimetry, which are conventional in the art.

[0230] In some embodiments, administration of a CFH protein or pharmaceutical composition disclosed herein of the test subject results in an increase in levels of functional CFH

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protein. In some embodiments, ocular administration of a CFH protein or pharmaceutical composition disclosed herein to a subject results in an increase in levels of functional CFH protein in the aqueous humor such that the increased levels are 5-fold to 100-fold, 5-fold to 90-fold, 5-fold to 80-fold, 5-fold to 70-fold, 5-fold to 60-fold, 5-fold to 50-fold, 10-fold to 100-fold, 10-fold to 90-fold, 10-fold to 80-fold, 10-fold to 70-fold, 10-fold to 60-fold, or 10-fold to 50-fold of the levels functional CFH protein in the aqueous humor of a control subject or population thereof (*e.g.*, a control patient described in the "patient selection" subsection). In some embodiments, ocular administration of a CFH protein or pharmaceutical composition disclosed herein to a subject results in an increase in levels of functional CFH protein in the vitreous humor such that the increased levels are 5-fold to 1,000-fold, 5-fold to 500-fold, 5-fold to 200-fold, 5-fold to 100-fold, 5-fold to 90-fold, 5-fold to 80-fold, 5-fold to 70-fold, 5-fold to 60-fold, 5-fold to 50-fold, 10-fold to 1,000-fold, 10-fold to 500-fold, 10-fold to 200-fold, 10-fold to 100-fold, 10-fold to 90-fold, 10-fold to 80-fold, 10-fold to 70-fold, 10-fold to 60-fold, or 10-fold to 50-fold of the levels functional CFH protein in the vitreous humor of a control subject or population thereof (*e.g.*, a control patient described in the "patient selection" subsection).

[0231] In certain embodiments, administration of a CFH protein or pharmaceutical composition disclosed herein to the subject results in a decrease of the protein level of a biomarker positively correlated with activation of the complement pathway. For example, in certain embodiments, the administration results in a decrease of the protein level of Ba by at least 1 ng/mL, 2 ng/mL, 3 ng/mL, or 4 ng/mL 7 days post-treatment. In certain embodiments, the administration results in a decrease of the protein level of Ba by at least 1 ng/mL, 2 ng/mL, 3 ng/mL, 4 ng/mL, 5 ng/mL, 6 ng/mL, 7 ng/mL, or 8 ng/mL 28 days post-treatment. In certain embodiments, the administration results in a decrease of the protein level of Ba by at least 1 ng/mL, 2 ng/mL, 3 ng/mL, 4 ng/mL, or 5 ng/mL 56 days post-treatment. In certain embodiments, the administration results in a decrease of the protein level of C3a by at least 0.5 ng/mL, 0.6 ng/mL, 0.7 ng/mL, 0.8 ng/mL, 0.9 ng/mL, or 1 ng/mL 7 days post-treatment. In certain embodiments, the administration results in a decrease of the protein level of C3a by at least 0.5 ng/mL, 0.6 ng/mL, 0.7 ng/mL, 0.8 ng/mL, 0.9 ng/mL, or 1 ng/mL 56 days post-treatment.

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Kits

[0232] The CFH or pharmaceutical composition disclosed herein can be assembled into a pharmaceutical or diagnostic or research kit to facilitate their use in therapeutic, diagnostic or research applications. Accordingly, the present disclosure provides a kit comprising one or more containers containing a CFH or pharmaceutical composition disclosed herein and instructions for use, wherein the instructions sets a biomarker-based standard disclosed herein. In some embodiments, the kit comprises instructions for administering the CFH or pharmaceutical composition disclosed herein to a subject who has meets one of more of the biomarker tests disclosed herein and optionally has a CFH mutation disclosed herein.

[0233] Reagents useful in an assay to assess the level of a biomarker disclosed herein can also be assembled into a kit. Accordingly, in another aspect, the present disclosure provides a kit comprising one or more containers containing such reagents. For example, where the level of the biomarker is measured by an immunoassay, the kit comprises an antibody or variant thereof useful in the immunoassay. The kit can further comprise other reagents useful in such assay, such as a solid surface (*e.g.*, microplate) and a buffer. In certain embodiments, the kit is suitable for assessing the level of a biomarker in an ocular sample. Compared to blood samples, the volume of ocular sample is typically small. Accordingly, in certain embodiments, the kit is designed to measure the level of a biomarker in a sample that has a volume smaller than or equal to 100 μL, 50 μL, 40 μL, 30 μL, 20 μL, 10 μL, 9 μL, 8 μL, 7 μL, 6 μL, 5 μL, 4 μL, 3 μL, 2 μL, or 1 μL of sample (*e.g.*, aqueous humor sample). In certain embodiments, the kit comprises instructions for measuring the level of the protein marker, optionally further instructions to treat a patient if the level is (i) greater than or equal to a predetermined threshold if the biomarker is positively correlated with activation of the complement pathway or with inflammation or (ii) lower than or equal to a predetermined threshold if the biomarker is negatively correlated with activation of the complement pathway or with inflammation.

[0234] The instructions can define a component of instruction and/or promotion, and typically involves written instructions on or associated with packaging of the disclosure. Instructions also can include any oral or electronic instructions provided in any manner such that a user will clearly recognize that the instructions are to be associated with the kit, for example, audiovisual (*e.g.*, videotape, DVD, etc.), internet, and/or web-based communications, etc. The written instructions may be in a form prescribed by a governmental agency regulating the

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manufacture, use or sale of pharmaceuticals or biological products, which instructions can also reflects approval by the agency of manufacture, use or sale for animal administration.

[0235] The kit may be designed to facilitate use of the methods described herein by researchers and can take many forms. Each of the compositions of the kit, where applicable, may be provided in liquid form (*e.g.*, in solution), or in solid form, (*e.g.*, a dry powder). In certain cases, some of the compositions may be reconstitutable or otherwise processable (*e.g.*, to an active form), for example, by the addition of a suitable solvent or other species (*e.g.*, water or a cell culture medium), which may or may not be provided with the kit.

[0236] The kits disclosed above can be combined. For example, the present disclosure also provides a kit comprising (a) one or more containers containing reagents for assessing the protein level of one or more biomarkers and (b) one or more containers containing a CFH or pharmaceutical composition disclosed herein. The kit can further comprise instructions for administering to a patient the CFH or pharmaceutical composition if the level of the biomarker is (i) greater than or equal to a predetermined threshold if the biomarker is positively correlated with activation of the complement pathway or with inflammation or (ii) lower than or equal to a predetermined threshold if the biomarker is negatively correlated with activation of the complement pathway or with inflammation.

EXAMPLES

[0237] The disclosure is further illustrated by the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present disclosure, and which are not intended to limit the disclosure.

Example 1. Inhibition of the Alternative Pathway of the Complement System by Recombinant CFH in RPE Cell Culture

[0238] This example was designed to assess the impact of exogenous recombinant CFH on the activity of the alternative pathway of the complement system in a culture of RPE cells that lack sufficient complement regulation.

[0239] Briefly, RPE cells were subjected to a CFH level lower than the normal endogenous level by incubating RPE in CFH insufficient human serum (CFH depleted human serum with 50 ug/ml plasma-purified CFH added). These cells showed deposition of C3bBb the C3-convertase of the complement alternative pathway, as observed by immunologic detection of RPE cell-

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bound C3bBb by first incubating cells with a specific mouse anti-human Bb antibody followed by incubation with an horse radish peroxidase (HRP) conjugated anti-mouse antibody and finally quantitation with an HRP-sensitive colorimetric substrate. In addition, the generation of C3a and Ba, cleavage products of active C3-convertase, increased in the culture medium of these cells, as measured by commercially available ELISAs for human C3a and Ba. Recombinant, wild-type CFH protein (rCFH), having the amino acid sequence of SEQ ID NO: 1, was added in the cell culture at various concentrations. As shown in **FIGs. 1A-1C**, the supplementation of the rCFH reduced C3bBb deposition (**FIG. 1A**), generation of C3a (**FIG. 1B**), and generation of Ba (**FIG. 1C**) in a dose-dependent manner.

[0240] These results indicate that rCFH addition is able to modulate dysregulated alternative pathway of the complement system, in particular, the C3 amplification loop, on the surface of RPE cells which are of relevance to AMD pathology. It is understood that the initial levels of C3a and Ba represent the levels of these protein in the human serum added in the RPE cell culture, which were higher than the level of these proteins in an ocular sample. As a result, the doses of rCFH used in this assay also represent the levels required for reducing the levels of complement proteins in the human serum added in the RPE cell culture, which are higher than the ocular levels of these proteins.

Example 2. Phase 1, Single-Ascending Dose Study of Recombinant CFH in Patients with Dry AMD

[0241] The safety, tolerability, pharmacodynamics, immunogenicity, and biomarker information of intravitreal (IVT) injection of rCFH to patients having geographic atrophy (GA) secondary to dry AMD are assessed in a Phase 1, multicenter, open-label, single-dose, dose-escalation study. In this study, rCFH is developed to restore appropriate regulation of the complement system in dry AMD patients with functionally adverse variants in the CFH gene by blocking the detrimental effects (*e.g.*, inappropriate cell lysis, immune response) and retaining the beneficial effects (*e.g.*, clearance of extracellular debris, repair of oxidative damage) of complement activation. The rCFH is intended to be used as a CFH replacement therapy for the treatment of dry AMD and is administered to patients by IVT administration. A diagram of a clinical study design is shown in **FIG. 2**.

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Objectives and End Points

[0242] The primary objectives of the study are (1) to evaluate the safety and tolerability of rCFH; and (2) to establish the single IVT maximum tolerated dose of rCFH. The endpoints for the primary objectives are: intraocular and systemic adverse events, including seriousness, severity and relationship to study drug; results of ocular examinations, clinical laboratory assessments and vital signs.

[0243] The secondary objectives are (1) to evaluate the concentration of rCFH in aqueous humor; (2) to evaluate the endogenous CFH concentration pre and post rCFH administration; and (3) to evaluate the immunogenicity of rCFH in serum and aqueous humor. The endpoints for the secondary objectives include: aqueous humor concentrations of rCFH, systemic (plasma) endogenous CFH concentrations, occurrence and severity of anti-drug antibodies in the serum and in aqueous humor.

[0244] The exploration objectives include (1) to describe the impact of rCFH on biomarkers in aqueous humor samples and (2) to describe any changes from baseline in clinical parameters such as best corrected visual acuity, retinal architecture, and pathology. The endpoints for the exploratory objectives are: aqueous humor biomarker values; plasma biomarker values, Age Related Eye Disease Study (AREDS) 9-Step Scale Score; best corrected visual acuity (BCVA) and low luminance visual acuity (LLVA)scores as assessed by Early Treatment Diabetic Retinopathy Study (ETDRS); area of geographic atrophy (GA) in mm^2 as assessed by color fundus photography (CFP), fundus autofluorescence (FAF), optical coherence tomography (OCT), near infrared imaging (NI), and fluorescein angiography (FA); drusen volume, total retinal and choroidal thickness, photoreceptor layer thickness, features of nascent geographic atrophy, retinal pigment epithelium (RPE) thickening and integrity of RPE layer as assessed by OCT.

Methodology and Study Design

[0245] The rCFH is administered to 1 eye only, which is designated at Baseline (Visit 1), based on eligibility, and prior to any baseline measurements. Detailed assessments of intraocular pressure (IOP), visual acuity (VA) and function, and ophthalmic imaging is performed as described in **Table 2**.

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Table 2. Schedule of Assessments for Single-Ascending Dose

Study Period	Screening		Single Dose and Safety Follow-up						End of Study
Visit ID	V01		V02	V03	V04	V05	V06		V07
Visit Description	Genetic testing (a)	IC/EC Screen (a)	Baseline & Dosing	24 hr Post-Dose	1 Week Safety Visit	2 Week Safety Visit	4 Week Safety Visit	8 Week Safety Visit	
Study Day (First Dose = Day 1)	Prior to Dosing	-28 to -3	1	2	8 (±2 days)	15 (±2 days)	29 (±2 days)	57 (±2 days)	
Informed Consent (b)	X								
Oral DNA Biosampling – Genetic Testing (a)	X								
Medical and Ocular History		X	X*						
Inclusion/Exclusion Criteria (c)		X	X*						
Demographics		X							
Physical Examination Vital Signs		X	X*	X	X	X	X	X	
Concomitant Medications		X	X*		X	X	X	X	
rCFH Administration			X						
Adverse Events			X	X	X	X	X	X	
Ocular Assessments									
Ophthalmic Biomicroscopy Examination		OU	OU*	OU	OU	OU	OU	OU	
Intraocular Pressure Assessment (d)		X	Pre/30 min	X	X	X	X	X	
ETDRS BCVA		OU	OU*	OU	OU	OU	OU	OU	
ETDRS BCVA LEVA			OU*			OU	OU	OU	
Imaging and Visual Function Assessments									
Color Fundus Photography (3-Field)		OU	OU**				OU	OU	
Fundus AutoFluorescence		OU	OU*			OU	OU	OU	
Fluorescein Angiography		OU						OU	
Near Infrared Imaging		OU	OU**			OU	OU	OU	
Optical Coherence Tomography (SD-OCT [required] and OCT-A [optional])		OU	OU*		OU	OU	OU	OU	

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Other Assessments									
Identification of Study Eye (c)		X							
Pregnancy Testing		X	X*						
Clinical Safety Laboratories		X	X*			X	X	X	X
Routine Urinalysis			X*						
Blood Sampling – Exploratory Genetics	X								
Blood Sampling – ADA			X*			X	X	X	X
Blood Sampling – Endogenous CFH (e)		X	Pre/2	Post 24		X	X	X	X
Blood Sampling – Exploratory Biomarkers			X*			X	X	X	X
Aqueous Humor Sampling (f)			SE*			SE		SE	SE

Abbreviations: ADA=anti-drug antibodies; BCVA=Best Corrected Visual Acuity; ETDRS=Early Treatment Diabetic Retinopathy Study; LLVA=Low Luminance Visual Acuity; MFD=maximum feasible dose; MTD=maximum tolerated dose; OCT=optical coherence tomography; OCT-A=OCT-angiography-; OU=oculus uterque (both eyes; eyes are tested separately); SD-OCT=spectral domain-OCT; SE=study eye.
* Assessment during a dosing visit that is obtained prior to dosing.
**Color Fundus Photography and Near Infrared Imaging are performed (prior to dosing) at the Baseline visit only if a) the investigator believes that there has been clinically significant change in vision or in ocular health, or b) the screening assessments ≥29 days prior to the baseline assessment.

(a) Saliva and blood samples for genetic testing may occur before or after assessment of eligibility criteria, but must occur prior to dosing with rCFH. Sites should allow at least 10 days for analysis.
(b) Informed consent must be obtained before any study assessment is performed.
(c) Some entry criteria apply only to the study eye. Therefore, the study eye must be identified during the screening process in order to assess entry criteria.
(d) Intraocular pressure assessments are obtained on dosing days prior to the dose and 30 minutes after the dose, with an additional sample at 60 minutes if the IOP has not resolved to ≤30 mmHg at the 30-minute assessment.
(e) Blood samples for endogenous CFH assessments are timed relative to the prior rCFH dose. Pre/2 = pre-dose and 2 hours (±5 min) post-dose.
This assessment is called Biomarker Plasma B in the laboratory manual.
(f) Based on sample volume, the priorities for analysis of aqueous humor are, in order, aqueous humor biomarkers, ADA, and rCFH concentration.

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[0246] A comprehensive ophthalmic examination of both the study and fellow eye (if possible) is performed by the Investigator or qualified designee at the timepoints specified in the schedule of assessments shown in **Table 2**. These examinations include an assessment of the visual acuity and visual function using validated assessment tools, and ocular imaging tests to visualize the appearance and pathology of the retina and associated ocular tissues. All findings are recorded in the eCRF. On dosing days, IOP is assessed pre-dose (prior to any aqueous humor sampling) and then 30 minutes after the dose. If the IOP 30 minutes post-dose exceeds 30 mmHg then the IOP is reassessed 60 minutes after the dose. If the IOP continues to exceed 30 mmHg, then standard IOP-lowering interventions (per institutional guidelines) should be followed. If IOP is $\leq$5 mmHg, the subject should be treated per institutional guidelines.

[0247] Only 1 eye is selected as the study eye. If both eyes meet all eye-specific entry criteria, then the eye with the poorer visual function as determined by the investigator and the subject are selected as the study eye. If neither the investigator nor the subject can make this determination, the eye with the larger GA lesion (total area) is selected as the study eye.

[0248] Safety and tolerability of a single dose of rCFH is assessed based on the occurrence of dose-limiting toxicities (DLTs). DLTs are assessed only during the DLT window, which begins at the date and time of the first dose (Day 1) and continues through the 14th calendar day after the first dose, i.e., through Study Day 15. A treated subject is considered DLT-evaluable if the subject either (a) experiences a DLT in the DLT window, or (b) experiences no DLTs during the window and continues beyond Study Day 15. If a subject has no DLTs but discontinues from the study during the DLT window, the subject is not DLT-evaluable, and an additional subject is consented and treated to achieve the required number of DLT-evaluable subjects in the same cohort.

[0249] DLTs include reduced visual acuity (VA), defined as: (1) at least 5 line (25 letter) decrease, as assessed on post-injection Day 2, 8, or 15; (2) at least a 4 line (20 letter) decrease on or before Day 8 is still present at the next scheduled visit; or (3) at least a 3 line (15 letter) decrease on or before Day 8 that persists until Day 15, intraocular inflammation defined as increase in ocular inflammation on 2 units on a standard grading scale as assessed through Day 15; elevated intraocular pressure (IOP), defined as sustained elevation of IOP characterized by an increase of 10 mmHg over the baseline value for $\geq$30 min; and "other DLTs" including any serious adverse event (SAE) that in the opinion of the investigator is related to the rCFH.

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[0250] Cohorts of 3 to 6 DLT-evaluable subjects receive a single IVT dose of rCFH. Three escalating dose cohorts (50 μg/eye, 100 μg/eye, and 250 μg/eye) are planned **(FIG. 2)**. Each cohort begins with dosing one sentinel subject on Day 1; the next 2 subjects in the cohort are dosed only after a favorable safety and tolerability assessment of the sentinel subject has been completed on or after Day 15 (Day 14 post dose). Subjects dosed after the sentinel subject may be dosed concurrently (at the same dose level).

[0251] If none of the first 3 DLT-evaluable subjects in a cohort experience a DLT, the dose is considered tolerable, and dose escalation proceeds to the next highest dose cohort. If exactly 1 DLT-evaluable subject experiences a DLT, 3 additional subjects are treated in order to obtain 6 DLT-evaluable subjects. The dose level for the cohort is considered tolerable if none of the 3 additional DLT-evaluable subjects experiences a DLT. If $\geqslant$2 of the first 3 DLT-evaluable subjects experience a DLT, the dose level is considered not tolerable.

[0252] The "3+3" single-ascending dose (SAD) study design is selected to ensure a conservative step-wise approach to investigating the MTD/MFD for rCFH. Cohorts of 3 to 6 DLT-evaluable subjects receive a single IVT dose of rCFH. Three escalating dose cohorts (50 μg/eye, 100 μg/eye, and 250 μg/eye) are planned. After review of all available data from doses $\leq$250 μg/eye, the sponsor can decide to open an additional cohort to evaluate a higher dose or an intermediate dose that is between a dose level found to be not tolerable and the highest previously tested tolerable dose level.

[0253] Cohorts of 3 to 6 DLT-evaluable subjects receive a single IVT dose of rCFH. Three escalating dose cohorts (50 μg/eye, 100 μg/eye, and 250 μg/eye) are planned. After review of all available data from doses $\leq$250 μg/eye, the sponsor may decide to open an additional cohort to evaluate a higher dose or an intermediate dose that is between a dose level found to be not tolerable and the highest previously tested tolerable dose level.

[0254] The minimum planned duration of each subject's participation is approximately 3 months: 1 month for screening, and 2 months for follow-up after the day of dosing.

[0255] A minimum of 9 subjects are planned for inclusion. Up to 18 subjects may be enrolled depending on the need for cohort expansion based on the safety response to dosing with rCFH. If a fourth cohort is opened, this number would increase by 3 to 6 more subjects.

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Eligibility Criteria

Inclusion Criteria

[0256] Subjects must meet all of the following inclusion criteria for consideration for participation in the study: (1) at least 50 years old at the time of signed informed consent; (2) positive for the CFH 402HH variant by targeted sequencing (Table 3); (3) sufficiently clear ocular media, adequate pupillary dilation, fixation to permit quality; fundus imaging, and able to cooperate sufficiently for adequate ophthalmic visual function testing and anatomic assessment; (4) understands the full nature and purpose of the study, including possible risks of study procedures, and provides informed consent prior to initiation of any study procedure; all subjects with a reproductive potential must agree to use effective contraceptive methods for 90 days from their last dose of rCFH; (5) best corrected visual acuity (BCVA) in the study eye using Early Treatment Diabetic Retinopathy Study [ETDRS] Chart VAS of 5 to 45 letters (equivalent to Snellen VA of approximately 20/800 – 20/125); and (6) confirmed diagnosis of central GA in the study eye with the following characteristics:

a. GA can be multifocal, and cumulative GA lesion must reside completely within the fundus autofluorescence (FAF) imaging field (field 2, 30-degree image centered on the fovea), as confirmed by the Image Reading Center (IRC);

b. GA must be central, defined as GA that affects the foveal center point (diagnosis of GA and location relative to the foveal center point is determined by an IRC eligibility read, based on multi-modal imaging with color fundus photography [CFP], fluorescein angiography [FA], optical coherence tomography [OCT], and near infrared reflectance imaging [NIR]); and

c. Total size of all GA lesions in the study eye must be within 0.5 to 15.0 Disk Areas (DAs).

Exclusion Criteria

[0257] A subject who meets any of the following exclusion criteria is ineligible to participate in the study:

(1) carriers of high-genetic-risk AMD variants (Table 3), to include AMD risk locus: homozygous for age-related maculopathy susceptibility protein [ARMS2]/HTRA1 p.A69S) and complete complotype (CFH 62VV + C3 102GG + complement factor B [CFB] 32RR);

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(2) presence of the following ocular conditions – in the study eye:

a. Exudative AMD or choroidal neovascularization (CNV);

b. Any active ocular disease, disorder, or condition that that could confound the assessment of the macula or be a contraindication to IVT injection, *e.g.*, macular hole (stage 3 or 4), GA or maculopathies due to any disease other than AMD, uveitis, uncontrolled glaucoma, ocular infection (diabetes mellitus without retinopathy is not a criterion for exclusion);

c. Any intraocular surgery (with the exception of intraocular lens replacement surgery more than 3 months prior to consent);

d. Aphakia or absence of the posterior capsule;

e. History of laser therapy to the macula or fundus (exception: laser therapy to treat peripheral retinal tears is not exclusionary);

f. Prior corneal transplant;

(3) presence of any of the following ocular conditions – in either eye:

a. History of herpetic infection;

b. Ongoing treatment with antiangiogenic therapies in the fellow eye or completed treatment in the study eye with antiangiogenic therapies within 5 half-lives of first rCFH dose;

c. Concurrent disease that could require medical or surgical intervention during the study period;

d. Active uveitis and/or vitritis (grade: trace or above);

e. History of idiopathic or autoimmune-associated uveitis;

f. Active infectious conjunctivitis, keratitis, scleritis, or endophthalmitis;

g. Any ophthalmologic condition that reduces the clarity of the media and that, in the opinion of the Investigator, interferes with ophthalmologic examination;

(4) in the opinion of the Investigator, the subject has any prior or ongoing medical condition (*e.g.*, ocular other than dry AMD, systemic, psychiatric) or clinically significant screening laboratory value that may present a safety risk, interfere with study compliance, interfere with consistent study follow-up, or confound data interpretation throughout the longitudinal follow-up period; or

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(5) female subjects must not be pregnant or lactating; 6) current use of medications known to be toxic to the lens, retina, or optic nerve (deferoxamine, chloroquine / hydroxychloroquine [Plaquenil®], tamoxifen, phenothiazines, ethambutol, digoxin, and aminoglycosides). (Current use is defined as the administration of first dose of rCFH within 5 half-lives of the prohibited medication).

Biomarkers

[0258] Blood samples for plasma isolation are obtained at the time points specified in the SOAs for analyses to identify and evaluate disease-related biomarkers, where local regulations and blood volume permits (Table 2). A specific sample for biomarker analysis is being collected in this study; however, remaining blood samples collected in this study may also be used to analyze additional biomarkers of potential clinical interest, if there is sufficient sample and where local regulations permit.

[0259] Aqueous humor samples are obtained at the time points specified in the SOAs for biomarker analyses to identify and evaluate disease-related biomarkers according to the schedule set forth in Table 2. Aqueous humor samples collected are obtained for the primary purpose of PD biomarker analysis.

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Table 3. List of Genetic Variants Tested via Targeted Sequencing

Chromosome	Position (hg19)	Reference Allele	Alternate Allele	dbSNPs	AMD Risk Nomenclature	Inclusion Exclusion Criteria	Eligible genotypes
1	196,659,237	C	T	rs1061170	CFH: c.1204T>C (p.His402=)	Inclusion	Positive for CFH p.[His402=;His402=] (CFH 402HH)
1	196,642,233	G	A	rs800292	CFH: c.184A>G (p.Val62=)	Joint Exclusion	Negative for CFH p.[Val62=;Val62=] AND CFB p.[Arg32=;Arg32=] AND C3 p.[Arg102Gly;Arg102Gly]
6	31,914,179	C	T	rs12614	CFB: c.94T>C (p.Arg32=)		
6	31,914,180	G	A	rs641153	CFB: c.95A>G (p.Arg32=)		
19	6,718,387	G	C	rs2230199	C3: C.304C>G (p.Arg102Gly)		
10	124,215,565	T	C	rs3750846	ARMS2: c298-858T>C	Exclusion	Negative for ARMS2: c.[298-858T>C;298-858T>C]

Abbreviations: A=adenine; AMD=age-related macular degeneration; ARMS2=age-related maculopathy susceptibility protein 2; C=cytosine; CFB=complement factor B; CFH=complement factor H; dbSNP=single nucleotide polymorphism database; G=guanine; T=thymine

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Example 3. Results of the Phase 1, Single-Ascending Dose Study of Recombinant CFH in Patients with Dry AMD

[0260] Patients with geographic atrophy secondary to dry AMD were treated in a single ascending dose study as described in Example 2. Specifically, nine patients were equally divided in three cohorts, one for testing each rCFH dose. The sponsor reviewed all available data from doses ≤250 μg/eye, observed no dose-limiting toxicities, serious adverse events, or related adverse events, and opened an additional cohort of three patients to evaluate a higher dose of 500 μg/eye. As a result, in this clinical study, the patients were treated at four different doses, namely, 50 μg, 100 μg, 250 μg, and 500 μg per eye. No dose-limiting toxicities, serious adverse events, or related adverse events were observed during the course of treatment.

[0261] It is understood that measurement of protein biomarkers in ocular samples has been technically challenging, given the limited sample volumes and the low levels of many protein biomarkers in ocular samples. In a clinical setting, where a plurality of biomarkers are assessed from the same sample, it would be desirable to conduct multiplex measurements of the multiple protein biomarkers under a single level of dilution. The inventors successfully measured the levels of IL-6, IL-8, VEGF-A, Eotaxin-2, and CCL2 from undiluted and 1:4 diluted aqueous humor samples from patients having wet AMD or cataract using the ECLIA method of Meso Scale Discovery (MSD). The inventors also successfully measured the levels of CXCL5, IL-10, and IL-18 from undiluted aqueous humor samples from the same patients using the same method. Additional biomarkers (up to ten in total) can be measured in a U-PLEX multiplex assay.

[0262] In aqueous humor, supraphysiological levels of CFH were achieved and maintained, along with a reduction in complement activation biomarkers. The baseline levels of Ba in the treated patients ranged from 8.4 ng/mL to 41.2 ng/mL, whereas the Ba levels in healthy patients generally ranged from 6.2 ng/mL to 11.5 ng/mL and averaged 7.8 ng/mL. The baseline levels of C3a in the treated patients ranged from 1.5 ng/mL to 9.6 ng/mL, whereas the C3a levels in healthy patients generally ranged from 1.6 ng/mL to 2.9 ng/mL and averaged 2.2 ng/mL. Given that all the patients appeared to have received sufficient amounts of rCFH to induce protein level changes of certain biomarkers at least within 7 days after administration, the results of all the patients receiving different doses were pooled. As shown in **FIGs. 3A-3B**, the levels of complement activation fragments Ba and C3a were generally higher in the patients at pre-

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treatment stage, reflecting the state of uncontrolled ocular C3-convertase activity. After treatment, the levels of activation fragments were reduced, indicating the ability of rCFH to regulate excessive C3-convertase activity. This is the first demonstration in a clinical study that in patients with geographic atrophy, intravitreal administration of a complement regulator can restore complement regulation and reduce levels of pathogenically relevant complement products.

[0263] The BCVA and LLVA scores of the patients were also measured. As shown in **FIGs. 4A-4B**, the rCFH treatment stabilized the disease progression. There also appeared to be a trend of improvement of visual outcome, but the difference did not arise to a level of statistical significance. More significant improvement of clinical outcome is expected be observed in a phase 2 clinical trial with more patients enrolled and multiple doses of rCFH administered.

Example 4- Phase 2, Multiple Dose Study of Recombinant CFH (rCFH) in Patients with Dry AMD

[0264] The safety, tolerability, pharmacodynamics, and immunogenicity of repeat intravitreal (IVT) injections of rCFH to patients having geographic atrophy (GA) secondary to dry AMD were assessed in a Phase 2, multicenter, open-label, multiple dose study. An optimal dose of rCFH was developed and safety, tolerability, immunogenicity, PK (measured as concentration of CFH in aqueous humor)/PD, and early clinical effect were assessed. This phase 2 clinical study was designed, in part, on the basis of the phase 1 Single Ascending Dose Study described in Example 3.

[0265] Repeat doses of rCFH were administered by IVT injection to subjects with dry AMD. The initial MD study dose (dose level) of up to 250 μg was based on safety data from single- and repeat-dose (3 monthly doses) IVT toxicology studies in cynomolgus monkeys, PK modeling and simulation of the profiles of rCFH after a single IVT injection in cynomolgus monkeys, and the endogenous level of CFH in human RPE (the target ocular tissue of rCFH replacement therapy in dry AMD patients), as well as data from initial dose cohorts in the Phase 1 SAD Study as described in Example 3. The initial 250 μg dose level was based on the clinical experience with 50 μg, 100 μg, and 250 μg in the Phase 1 SAD Study (as described in Example 2). The inclusion of the 500 μg dose level was supported by nonclinical toxicology studies and the clinical experience with 50 μg, 100 μg, 250 μg, and 500 μg in the Phase 1 SAD Study. A diagram of a clinical study design for Phase 2 is shown in **FIG. 5**.

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Objectives and End Points

[0266] The primary objectives of the study were to establish the safety and tolerability of multiple IVT injections of rCFH. The endpoints for the primary objectives were: intraocular and systemic adverse events, including seriousness, severity and relationship to study drug; results of ocular examinations; and clinical laboratory assessments.

[0267] The secondary objectives of the study were: (1) to describe the clinical effect of rCFH IVT injection; (2) to evaluate the total CFH levels in aqueous humor after rCFH IVT injection; and (3) to evaluate the immunogenicity of rCFH in serum after rCFH IVT injection. The endpoints for the secondary objectives include: change from baseline in best corrected visual acuity (BCVA) scores as assessed by Early Treatment Diabetic Retinopathy Study (ETDRS); change from baseline in area and/or rate of progression of GA as assessed by fundus autofluorescence (FAF) and optical coherence tomography (OCT); change from baseline in low luminance visual acuity (LLVA), and derived low luminance deficit (LLD) value (BCVA – LLVA); total CFH concentration levels in aqueous humor; and generation of anti-drug antibodies in the serum.

[0268] The exploration objectives of the study included (1) to describe any changes from baseline in retinal architecture and pathology after rCFH IVT injection; (2) to describe any changes from baseline in functional parameters (reading speed) after rCFH IVT injection; (3) to describe any changes from baseline in quality of life after rCFH IVT injection; (4) to describe the impact of rCFH IVT injection on biomarkers in aqueous humor sample. The endpoints for the exploratory objectives include: (1) descriptive analyses of changes from baseline in: retinal fluid/hemorrhage or development of choroidal neovascularization (CNV) by color fundus photography (CFP), fluorescein angiography (FA), and OCT; drusen morphology and volume, total retinal and photoreceptor layer thickness, features of nascent GA and rate of progression, RPE thickening, and integrity of RPE layer as assessed by FAF, OCT, optical coherence tomography-angiography (OCT-A) (optional), and near infrared reflectance imaging (NIR); (2) change from baseline in MNRead (Minnesota Low-vision Reading Test) scores; (3) change from baseline in NEI-VFQ-25 (National Eye Institute Visual Functioning Questionnaire-25) scores; and (4) change from baseline in aqueous humor complement proteins, complement split products, and related protein values as potential biomarkers of (pharmacodynamics) PD.

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Methodology

[0269] Subjects were assigned to 1 of 6 cohorts, based on subject genetic profile and location of GA (discussed below in "Inclusion Criteria" section).

- Multiple dose (MD) cohort 1 subjects had genetic profile A and noncentral GA;
- MD cohort 2 subjects had genetic profile A and central GA;
- MD Cohort 3 subjects had genetic profile B and either central or noncentral GA;
- MD cohort 4 included subjects who participated in the Phase 1 SAD Study (described in Example 2) and completed their EOS Visit, who had neither genetic profile A (CFH 402HH) nor B (Rare CFH/complotype), but who otherwise met all eligibility criteria and wish to continue receiving rCFH. Treatment naïve subjects that did not participate in the Phase 1 SAD Study and who meet all eligibility criteria (except the genetic profiles criteria) may also be included in MD cohort 4. Patients were enrolled in MD cohort 4 at the discretion of the medical monitor, in consultation with the Investigator;
- MD cohort 5 subjects had genetic profile A; and
- MD cohort 6 subjects had genetic profile B.

[0270] Subjects in MD cohorts 1-4 (as described in this section) received 250 μg rCFH either monthly or every other month (EOM), based on their initial treatment assignment. For subjects in MD cohorts 1-4, dose escalation from 250 μg to 500 μg monthly was considered when at least 3 subjects had completed at least 2 doses of rCFH at 250 μg and the available safety data had been assessed by the MRC. Subjects in MD cohorts 5 and 6 received 500 μg rCFH monthly. The study had two periods of follow-up: a 6-month initial dosing period and a 12-month extended dosing period. Based on MRC reviews of ongoing data, the dose and/or dosing frequency could be adjusted during the 12-month extended dosing period. An overview of MD cohorts is provided in Table 4.

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Table 4. Overview of MD Cohorts

MD Cohort	GA Location	Genetics	N	Dose Level (µg)
MD Cohort 1	Noncentral	Profile A (CFH 402HH, no ARMS2/HTRA1)	~ 60 Total	250 µg Monthly/EOM - 500 µg Monthly
MD Cohort 2	Central	Profile A (CFH 402HH, no ARMS2/HTRA1)		250 µg Monthly/EOM - 500 µg Monthly
MD Cohort 3	Noncentral/ central	Profile B (Rare CFH/complete complotype)		250 µg Monthly - 500 µg Monthly
MD Cohort 4	Noncentral/ central	Neither Profile A nor Profile B		250 µg Monthly - 500 µg Monthly
MD Cohort 5	Noncentral/ central	Profile A (CFH 402HH)		500 µg Monthly
MD Cohort 6	Noncentral/ central	Profile B (Rare CFH/complete complotype)		500 µg Monthly

Abbreviations: CFH=complement factor H; EOM=every other month; GA=geographic atrophy; ARMS2/HTRA1= age-related maculopathy susceptibility 2/high temperature requirement A serine peptidase 1; MD=multiple dose; N=number of subjects

[0271] The rCFH was administered to 1 eye only, which was designated during the screening process based on eligibility and prior to any baseline measurements. If both eyes met all eye-specific entry criteria, then the study eye was determined by the Investigator and subject together. If neither the Investigator nor the subject could make this determination, the eye with the larger GA lesion (total area) would be selected as the study eye. The fellow eye may be followed for comparative analyses, where appropriate. A schedule of assessments for 6-month initial dosing period and 12-month extended dosing period are described in Table 5 and Table 6, respectively.

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[0272] **Table 5. Schedule of Assessments —6-Month Initial Dosing Period (Primary Endpoint)**

Visit Description	Screening		Baseline [a]	48 hr Post-dose Phone Call	Week 1 Visit	Week 2 Phone Call	Month 1 Visit	Month 2 Visit	Month 3 Visit	Month 4 Visit	Month 5 Visit	Month 6 Visit
	Genetic Testing [a]	IC/EC Screen [a]										
Study Day (First Dose=Day 1)	**Prior to Day 1**	**-35 to -3**	**1**	**3 (±1)**	**8 (±2)**	**15 (±1)**	**31 (±4)**	**61 (±4)**	**91 (±4)**	**121 (±4)**	**151 (±4)**	**181 (±4)**
Informed Consent for Genetic Testing	x											
Informed Consent for 6-Month Initial Dosing Period [b]		x										
Informed Consent for 12-Month Extended Dosing Period												x
Oral DNA Biosample – Genetic Testing *	x											
Historical imaging (including imaging type) and visual acuity	*Obtained between Baseline and Month 3*											
Medical and Ocular History *[c]		x	x									
Inclusion/Exclusion Criteria *[d]		x	x									
Demographics		x										
Complete Physical Examination		x										x
Symptom-Directed Physical Examination *			x				x	x	x	x	x	
ECG		x										
Vital Signs *[c]		x	x				x**	x	x**	x	x**	x**
Concomitant Medications *		x	x	x	x	x	x	x	x	x	x	x
rCFH Administration (monthly)			SE				SE	SE	SE	SE	SE	SE [f]
Adverse Events			x	x	x	x	x	x	x	x	x	x
Ocular Assessments												

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Visit Description	Screening		Baseline [a]	48 hr Post-dose Phone Call	Week 1 Visit	Week 2 Phone Call	Month 1 Visit	Month 2 Visit	Month 3 Visit	Month 4 Visit	Month 5 Visit	Month 6 Visit
	Genetic Testing [a]	IC/EC Screen [a]										
Study Day (First Dose=Day 1)	**Prior to Day 1**	**-35 to -3**	**1**	**3 (±1)**	**8 (±2)**	**15 (±1)**	**31 (±4)**	**61 (±4)**	**91 (±4)**	**121 (±4)**	**151 (±4)**	**181 (±4)**
Complete Ophthalmic Examination *		OU	OU		OU		OU	OU	OU	OU	OU	OU
Intraocular Pressure Assessment [g]		OU	OU [g]		OU		OU [g]	OU [g]	OU [g]	OU [g]	OU [g]	OU [g]
ETDRS BCVA *		OU	OU		OU		OU	OU	OU	OU	OU	OU
ETDRS BCVA LLVA *		OU							OU			OU
Imaging and Visual Function Assessments (Imaging assessments may be optionally performed at any in-clinic visit if deemed medically necessary for subject safety.)												
Color Fundus Photography (7-Field) *		OU	OU [h]						OU			OU
Fundus Autofluorescence *		OU	OU [h]						OU			OU
Fluorescein Angiography *		OU	OU [i]									
Near Infrared Imaging *		OU	OU [h]						OU			OU
Optical Coherence Tomography (SD-OCT [required] and OCT-A [optional]) *		OU	OU [h]						OU			OU
NEI-VFQ-25 *			X									OU
MNRead *			OU									OU
Other Assessments												
Identification of Study Eye [d]		X										
Pregnancy Testing [j]		X										X [f]
Clinical Safety Laboratory Assessments *[k]		X	X					X		X		X
Routine Urinalysis [l]		X										X
Blood Sampling – Exploratory Genetics [a]	X											X
Blood Sampling for serum ADA			X				X	X		X		X

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Visit Description	Screening		Baseline [a]	48 hr Post-dose Phone Call	Week 1 Visit	Week 2 Phone Call	Month 1 Visit	Month 2 Visit	Month 3 Visit	Month 4 Visit	Month 5 Visit	Month 6 Visit
	Genetic Testing [a]	IC/EC Screen [a]										
Study Day (First Dose=Day 1)	Prior to Day 1	-35 to -3	1	3 (±1)	8 (±2)	15 (±1)	31 (±4)	61 (±4)	91 (±4)	121 (±4)	151 (±4)	181 (±4)
*												
Aqueous Humor Sampling *			SE				SE**	SE	SE**	SE	SE**	SE**

Abbreviations: ADA=anti-drug antibodies; BCVA=best corrected visual acuity; EOM=every other month; ECG=electrocardiogram; ETDRS=Early TreatmentDiabetic Retinopathy Study; IOP=intraocular pressure; LLVA=low luminance visual acuity; MNRead=Minnesota Low-vision Reading Test; NEI-VFQ- 25=National Eye Institute Visual Functioning Questionnaire-25; OCT=optical coherence tomography; OCT-A=OCT-angiography; OU=oculus uterque (both eyes; eyes will be tested separately); SD-OCT=spectral domain-OCT; SE=study eye

* Assessment obtained prior to dosing, as applicable.

** Assessment obtained on dosing visits only, prior to dosing and at month 6 if the subject does not agree to continue into the 12-Month Extended Dosing Period.

a Results of saliva or blood samples for genetic testing must be available before dosing. Prior genetic testing results from a Sponsor-qualified assay may be acceptable after consultation with the medical monitor. The Baseline visit may be performed over several days if necessary but must be completed within 1week.

b Informed consent must be obtained before any study assessment is performed.

c Medical and ocular history also includes social history and family ophthalmic history.

d Some entry criteria apply only to the SE. Therefore, the SE must be identified during the screening process in order to assess entry criteria.

e Vital sign measurements include systolic and diastolic blood pressures, heart rate, respiratory rate, and body temperature. When procedures overlap and are scheduled to occur at the same time point, vital sign measurements should precede any blood collection.

f Dose administration and pregnancy testing at this visit only occurs if the subject has consented to the 12-Month Extended Dosing Period.

g On dosing days, IOP assessments are obtained prior to the dose and 30±15 minutes after the dose, with an additional sample at 60±15 minutes if the IOP hasnot resolved to ≤30 mmHg at the 30-minute assessment. If the IOP continues to exceed 30 mmHg, then standard IOP-lowering interventions (per institutional guidelines) should be followed. If IOP is ≤5 mmHg, the subject should be treated per institutional guidelines. Regardless of whether applanation tonometry or a tonopen is used to measure IOP, the same method should be used for the subject for the entirety of their participation in this study.

h These baseline imaging assessments are only required if the screening images were obtained >35 days prior to baseline. They may be redone at baseline if the Investigator deems it clinically relevant, or if there were quality issues with the screening images.

i Baseline assessment of fluorescein angiography is not required, but may be assessed if the Investigator deems it clinically relevant, or if there were quality issues with the screening images.

j Highly sensitive (serum or urine) human chorionic gonadotropin pregnancy test as needed for women of childbearing potential.

k Clinical safety laboratory assessments include the following: hematology (platelet count, red blood cell count, hemoglobin count, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, percent reticulocytes, and white blood cell count with differential) and clinical chemistry (blood urea, nitrogen, creatinine, glucose, potassium, sodium, total calcium, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total

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and direct bilirubin, total protein, standard electrolytes – magnesium, phosphate, and chloride). Baseline clinical safety labs do not need to be drawn if screening labswere drawn within 7 days prior to baseline.

1 Routine urinalysis includes macroscopic and microscopic examination.

Table 6. Schedule of Assessments —12-Month Extended Dosing Period

Visit Description	Month 7 Visit	Month 8 Visit	Month 9 Visit	Month 10 Visit	Month 11 Visit	Month 12 Visit	Month 13 Visit	Month 14 Visit	Month 15 Visit	Month 16 Visit	Month 17 Visit	EOS Visit
Study Day (First Dose=Day 1)	**210 (±4)**	**241 (±4)**	**271 (±4)**	**301 (±4)**	**331 (±4)**	**361 (±4)**	**391 (±4)**	**421 (±4)**	**451 (±4)**	**481 (±4)**	**501 (±4)**	**531 (±4)**
Complete Physical Examination						x						x
Symptom-Directed Physical Examination *	x	x	x	x	x		x	x	x	x	x	
Vital Signs * [a]	x	x	x	x	x	x	x	x	x	x	x	x
Concomitant Medications *	x	x	x	x	x	x	x	x	x	x	x	x
rCFH Administration	SE	SE	SE	SE	SE	SE	SE	SE	SE	SE	SE	
Adverse Events	x	x	x	x	x	x	x	x	x	x	x	x
Ocular Assessments												
Complete Ophthalmic Examination *	OU	OU	OU	OU	OU	OU	OU	OU	OU	OU	OU	OU
Intraocular Pressure Assessment	OU	OU	OU	OU	OU	OU	OU	OU	OU	OU	OU	OU
ETDRS BCVA *	OU	OU	OU	OU	OU	OU	OU	OU	OU	OU	OU	OU
ETDRS BCVA LLVA *			OU			OU			OU			OU
Imaging and Visual Function Assessments (Imaging assessments may be optionally performed at any in-clinic visit if deemed medically necessary for subject safety)												
Color Fundus Photography (7-Field) *			OU			OU			OU			OU
Fundus Autofluorescence *			OU			OU			OU			OU
Fluorescein Angiography *						OU						OU
Near Infrared Imaging *			OU			OU			OU			OU
Optical Coherence Tomography (SD-OCT [required] and OCT-A [optional]) *			OU			OU			OU			OU

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Visit Description	Month 7 Visit	Month 8 Visit	Month 9 Visit	Month 10 Visit	Month 11 Visit	Month 12 Visit	Month 13 Visit	Month 14 Visit	Month 15 Visit	Month 16 Visit	Month 17 Visit	EOS Visit
Study Day (First Dose=Day 1)	**210 (±4)**	**241 (±4)**	**271 (±4)**	**301 (±4)**	**331 (±4)**	**361 (±4)**	**391 (±4)**	**421 (±4)**	**451 (±4)**	**481 (±4)**	**501 (±4)**	**531 (±4)**
NEI-VFQ-25 *						OU						OU
MNRead *						OU						OU
Other Assessments												
Pregnancy Testing [c]												x
Clinical Safety Laboratory Assessments *[d]			x			x			x			x
Routine Urinalysis [e]			x			x			x			x
Blood Sampling for serum ADA *						x						x
Aqueous Humor Sampling *			SE			SE			SE			SE

Abbreviations: ADA=anti-drug antibodies; BCVA=best corrected visual acuity; EOS=End of Study; ECG=electrocardiogram; ETDRS=Early Treatment Diabetic Retinopathy Study; hr=hour; IOP=intraocular pressure; LLVA=low luminance visual acuity; MNRead=Minnesota Low-vision Reading Test; NEI-VFQ-25=National Eye Institute Visual Functioning Questionnaire-25; OCT=optical coherence tomography; OCT-A=OCT-angiography; OU=oculus uterque (both eyes; eyes will be tested separately); SD-OCT=spectral domain-OCT; SE=study eye.

* Assessment obtained prior to dosing, as applicable.

a Vital sign measurements include systolic and diastolic blood pressures, heart rate, respiratory rate, and body temperature. When procedures overlap and are scheduled to occur at the same time point, vital sign measurements should precede any blood collection.

b IOP assessments are obtained prior to the dose and 30±15 minutes after the dose, with an additional sample at 60±15 minutes if the IOP has not resolved to ≤30 mmHg at the 30-minute assessment. If the IOP continues to exceed 30 mmHg, then standard IOP-lowering interventions (per institutional guidelines) should be followed. If IOP is ≤5 mmHg, the subject should be treated per institutional guidelines. Regardless of whether applanation tonometry or a tonopen is used to measure IOP, the same method should be used for the subject for the entirety of their participation in this study.

c Highly sensitive (serum or urine) human chorionic gonadotropin pregnancy test as needed for women of childbearing potential.

d Clinical safety laboratory assessments include the following: hematology (platelet count, red blood cell count, hemoglobin count, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, percent reticulocytes, and white blood cell count with differential) and chinical chemistry (blood urea nitrogen, creatinine, glucose, potassium, sodium, total calcium, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total and directbilirubin, total protein, standard electrolytes – magnesium, phosphate, and chloride).

e Routine urinalysis includes macroscopic and microscopic examination.

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Genetic Profiles

Genetic variants tested via targeted sequencing are listed in Table 7.

Table 7. List of Genetic Variants Tested via Targeted Sequencing

Chromosome	Position (hg19)	Reference Allele	Alternate Allele	dbSNPs	AMD Risk Nomenclature	Genetic Profile A	Genetic Profile B
1	196,659,237	C	T	rs1061170	CFH: c.1204T>C (p.His402=)	**Positive** for CFH p.[His402=; His402=] (CFH 402HH)	Any genotype
10	124,215,565	T	C	rs3750846	ARMS2: c.298-858T>C	Any genotype	Any genotype
1	196,642,233	G	A	rs800292	CFH: c.184A>G (p.Val62=)	**Negative** for complete complement	**Positive** for complete complement
6	31,914,179	C	T	rs12614	CFB: c.94T>C (p.Arg32=)	(CFH p.[Val62=;Val62=] AND CFB p.[Arg32=;Arg32=] AND C3 p.[Arg102Gly;Arg102Gly])	(CFH p.[Val62=;Val62=] AND CFB p.[Arg32=;Arg32=] AND C3 p.[Arg102Gly;Arg102Gly])
6	31,914,180	G	A	rs641153	CFB: c.95A>G (p.Arg32=)		
19	6,718,387	G	C	rs2230199	C3: c.304C>G (p.Arg102Gly)		
1	196,621,252	G	C	rs142266551	CFH: c.5G>C (p.Arg2Thr)	**Negative for all**	OR **Positive for any** one of the 20 rare missense CFH variants
1	196,621,254	C	G	rs139254423	CFH: c.7C>G (p.Leu3Val)		
1	196,642,206	C	T	rs757785149	CFH: c.157C>T (p.Arg53Cys)		
1	196,642,207	G	A	rs757785149	CFH: c.158G>A (p.Arg53His)		
1	196,642,221	T	G	rs141336681	CFH: c.172T>G (p.Ser58Ala)		
1	196,643,011	A	G	rs123969589 9	CFH: c.269A>G (p.Asp90Gly)		
1	196,645,156	G	A	rs147002633	CFH: c.388G>A (p.Asp130Asn)		
1	196,646,702	G	A	rs130049367 1	CFH: c.524G>A (p.Arg175Gln)		
1	196,646,702	G	C	rs139360826	CFH: c.524G>C (p.Arg175Pro)		
1	196,648,794	A	G	rs774239374	CFH: c.661A>G (p.Ile221Val)		
1	196,654,310	C	T	rs142937931	CFH: c.907C>T (p.Arg303Trp)		

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Chromosome	Position (hg19)	Reference Allele	Alternate Allele	dbSNPs	AMD Risk Nomenclature	Genetic Profile A	Genetic Profile B
1	196,654,311	G	A	rs766408580	CFH: c.908G>A (p.Arg303Gln)	20 rare missense CFH variants	
1	196,659,231	C	A	rs201671665	CFH: c.1198C>A (p.Gln400Lys)		
1	196,683,035	C	G	rs570523689	CFH: c.1507C>G (p.Pro503Ala)		
1	196,694,253	A	G	rs757756991	CFH: c.1699A>G (p.Arg567Gly)		
1	196,695,675	G	T	rs143237092	CFH: c.1949G>T (p.Gly650Val)		
1	196,706,677	G	T	rs515299	CFH: c.2669G>T (p.Ser890Ile)		
1	196,709,833	C	T	rs145975787	CFH: c.2867C>T (p.Thr956Met)		
1	196,716,328	G	A	rs761877050	CFH: c.3581G>A (p.Gly1194Asp)		
1	196,716,375	C	T	rs121913059	CFH: c.3628C>T (p.Arg1210Cys)		

Abbreviations: A=adenine; AMD=age-related macular degeneration; ARMS2=age-related maculopathy susceptibility 2; C=cytosine; C3=complement component 3; CFB=complement factor B; CFH=complement factor H; dbSNP=single nucleotide polymorphism database; G=guanine; T=thymine

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[0273] Emerging data, including safety, PK/PD, biomarker, and clinical data, were reviewed on an ongoing basis, and may be reviewed at specific timepoints during the study (*e.g.*, when all ongoing subjects have been treated with rCFH for 12 weeks and again at 6 and 12 months during the treatment period).

[0274] Data was collected on demographics, medical and ocular history, family history, and concomitant medications. Additionally, ophthalmic anatomic assessments and multi-modal imaging, genetic and biomarker analyses, and aqueous humor sampling was done for each enrolled subject.

[0275] For evaluation of post treatment effect, historical data, including visual acuity and anatomical endpoints such as rate of progression of GA (measured from diagnosis to study screen where available), drusen volume, and retinal architecture, can also be used.

Study Duration

[0276] The planned duration of each subject's participation in the initial dosing period was approximately 7 months: 1 month for screening and 6 months for dosing and follow-up. The planned duration of each subject's participation in the extended dosing period was approximately 12 months of dosing and follow-up.

[0277] Approximately 60 subjects are in the study.

Eligibility Criteria

Inclusion Criteria

[0278] Subjects must meet all of the following inclusion criteria for consideration for participation in the study. (1) at least 50 years old at the time of signed informed consent; subjects younger than 50 may be considered for participation after consultation; (2) subjects in MD cohorts 5 and 6 must have one of the following genetic profiles (*see* Table 7):

a. Genetic profile A (CFH 402HH)- positive for the CFH 402HH variant and negative for rare missense CFH variants and complete complotype;
b. Genetic profile B (Rare CFH/complete complotype)- positive for at least one of the rare high-risk missense CFH genetic variants or complete complotype (CFH 62VV + C3 102GG + CFB 32RR);

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(3) BCVA in the study eye of 24 to 83 letters using the ETDRS Chart VA Scale (approximately equivalent to Snellen VA of 20/25 to 20/320); (4) confirmed diagnosis of central GA in the study eye with the following characteristics:

a. total GA area must be $\geq$1.25 and $\leq$17.5 mm^2 (0.5- and 7-disc areas (DA), respectively), determined by screening images of fundus autofluorescence (FAF);

b. if GA is multifocal, at least one focal lesion must be $\geq$1.25 mm^2 (0.5 DA); and

(5) the entire atrophic lesion must be able to be photographed within field 2 on FAF; (6) sufficiently clear ocular media, adequate pupillary dilation, fixation to permit quality fundus imaging, and able to cooperate sufficiently for adequate ophthalmic visual function testing and anatomic assessment in the study eye; (7) understands the full nature and purpose of the study, including possible risks of study procedures, and provides informed consent prior to initiation of any study procedure; all subjects with a reproductive potential must agree to use effective contraceptive methods through the end of study (EOS) Visit.

Exclusion Criteria

[0279] A subject who meets any of the following exclusion criteria was ineligible to participate in the study:

(1) presence of the following ocular conditions – in the study eye:

a. Any history of exudative AMD or choroidal neovascularization (CNV);

b. Any active ocular disease or condition that could confound the assessment of the macula or be a contraindication to IVT injection, *e.g.*, macular hole (any stage, including lamellar hole), epiretinal membrane, GA or maculopathies due to any disease other than AMD, uncontrolled glaucoma (intraocular pressure (IOP) of >24 mmHg when on 2 or more IOP-lowering medications), severe glaucoma (cup to disc ratio of 0.8 or greater), ocular infection of any nature, diabetic retinopathy graded as moderate non-proliferative diabetic retinopathy or worse, with or without diabetic macular edema);

c. Any intraocular surgery, with the exception of stable intraocular lens replacement surgery more than 3 months prior to consent (*e.g.*, any glaucoma surgeries, including microinvasive glaucoma surgery, and/or any intraocular surgery requiring vitrectomy);

d. Aphakia or complete absence of the posterior capsule;

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e. History of laser therapy to the macula or fundus or extensive laser (*e.g.*, pan-retinal photocoagulation) to the retina (exception: laser therapy to treat peripheral retinal tears is not exclusionary); and

f. Prior corneal transplant;

(2) presence of any of the following ocular conditions – in either eye:

a. History of herpetic infection;

b. Concurrent disease that could require medical or surgical intervention during the study period;

c. Active uveitis and/or vitritis (grade: trace or above);

d. History of idiopathic or autoimmune-associated uveitis;

e. Active blepharitis, conjunctivitis, keratitis, episcleritis, scleritis, or endophthalmitis; and

f. Any ophthalmologic condition that reduces the clarity of the media and that, in the opinion of the Investigator, interferes with ophthalmologic examination;

(3) in the opinion of the Investigator, the subject had any prior or ongoing medical condition (*e.g.*, ocular other than dry AMD, systemic, psychiatric) or clinically significant screening laboratory value that may present a safety risk, interfere with study compliance, interfere with consistent study follow-up, or confound data interpretation throughout the longitudinal follow-up period; (4) female subjects must not be pregnant or lactating, nor plan to become pregnant during the study; (5) current use of medications known to be toxic to the lens, retina, or optic nerve (deferoxamine, chloroquine / hydroxychloroquine [Plaquenil®], tamoxifen, phenothiazines, ethambutol, digoxin, and aminoglycosides). (Current use is defined as the administration of first dose of rCFH within 5 half-lives of the prohibited medication). Past use of these medications, without history of ocular side effects, is not considered exclusionary; or (6) use of any investigational new drug or other experimental treatment in the last 6 months, and/or receipt of any prior gene therapy (*e.g.*, AAV) or ocular device implantation (other than Posterior chamber intraocular lens (PCIOL) placement following cataract surgery).

rCFH Dose and Route, Regimen

[0280] rCFH was supplied in sterile, single use vials as a 10 mg/mL concentrated liquid that, if necessary, can be diluted to the appropriate concentration prior to dosing at the site. All dose administration was open label.

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Biomarkers

[0281] Aqueous humor samples were collected prior to and after receiving single IVT injections of rCFH to evaluate complement proteins and possibly inflammatory cytokine profiles to determine their potential as PD biomarkers for usefulness in a pivotal study. rCFH concentrations in aqueous humor were evaluated after completion of PD analyses, if volume permits. Imaging endpoints such as GA lesion size, and clinical endpoints such as BCVA, LLVA, and low luminance deficit (LLD), were collected as described in Tables 5 and 6.

Example 5. Results of the Phase 2, Multiple Dose Study of Recombinant CFH in Patients with Dry AMD

[0282] Patients with geographic atrophy secondary to dry AMD were treated in multiple dose study as described in Example 4. Specifically, patients were assigned to 1 of 6 cohorts, based on subject genetic profile and location of GA. Patients in MD cohorts 1-4 (as described in this Example 4) received 250 µg rCFH either monthly or every other month (EOM), based on their initial treatment assignment. For patients in MD cohorts 1-4, dose escalation from 250 µg to 500 µg monthly was done when at least 3 patients completed at least 2 doses of rCFH at 250 µg. Patients in MD cohorts 5 and 6 received 500 µg rCFH monthly.

PK of rCFH is Dose Proportional and Supports Every Other Month Dosing

[0283] CFH concentration levels in aqueous humor (AH) were quantified. For quantification of CFH levels in AH, Luminex-Human Complement Magnetic Bead Panel 2 Kit (Millipore catalog number HCMP2MAG-19K) was used. The double capture multiplex bead-based immunoassay uses spectrally encoded magnetic beads conjugated to CFH and anti-factor H capture antibodies are used as the solid support. CFH in samples and control samples bound to the antibodies conjugated to color-coded beads. The bound CFH was detected with biotinylated anti-FH antibody producing a magnetic bead antibody-antigen-antibody double capture complex. When a reporter molecule, streptavidin-conjugated to Phycoerythrin (Streptavidin-PE) was added to the mixture, a fluorescence was generated. The fluorescence signal was read using a BioPlex Suspension Array instrument. The concentration of CFH was determined by monitoring the spectral properties of the magnetic beads and the PE Fluorescence intensity (FI). Factor B and C3 were also analyzed in this assay with specific anti-FB and anti-C3 antibody coated beads. The lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) for CFH for

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this assay was 1.10 ng/mL and 67.50 ng/mL, respectively. **FIG. 6** is a graph showing CFH levels in aqueous humor after administration of 250 μg of rCFH monthly or 500 μg of rCFH monthly. By the indicated time points, the patients were administered either 250 μg of rCFH monthly or 500 μg of rCFH monthly. No dose escalation had occurred at the time points indicated in **FIG. 6**. As shown in **FIG. 6**, both doses achieved multiple-fold (7-fold for 250 μg to 500 μg of rCFH monthly and 14-fold for 500 μg of rCFH monthly) increase over baseline CFH levels. In addition, little difference in CFH mean C_{max} produced by administering rCFH at dose level was observed. Incremental and cumulative aqueous humor CFH AUCs were significantly higher at a dose of 500 μg monthly. The graph also shows that the pharmacokinetics of rCFH is dose proportional. Combining the results from Phase 1 clinical study, which showed that the half-life of rCFH in AH is more than 7 days and the phase 2 clinical study, the pharmacokinetics of rCFH supports a dosing frequency of every other month.

Administration of rCFH Restores Regulation to Complement in Dry AMD Patients

Ba Concentration Levels

[0284] Quantitative measurement of Ba fragment in human aqueous humor was done using a MicroVue Ba fragment EIA Kit (Quidel, cat# A033). Based on quantitative ELISA format, a microtiter plate was coated with a mouse mAb that specifically bound to human Ba. Upon addition of standards, controls, and samples to the wells, the Ba fragments present in them bound to the immobilized antibody. A horse radish peroxidase (HRP) conjugated polyclonal anti-Ba antibody was used as the detection reagent. The amount of Ba in the samples was detected by 3,3', 5,5' tetramethylbenzidine dihydrochloride (TMB) substrate, a ready-to-use chromogenic substrate solution. TMB substrate generated color which was directly proportional to the amount of Ba in the samples. The LLOQ and ULOq values of Ba in this assay were 131.69 pg/mL and 2100 pg/mL, respectively.

[0285] Ba levels were examined in quartile range with quartiles 1-4 having pre-treatment Ba levels in the ranges between 5.4 ng/mL and 16.65 ng/mL; between 16.91 ng/mL and 25.87 ng/mL; between 26.25 ng/mL and 44.22 ng/mL; and between 44.46 ng/mL and 165.6 ng/mL, respectively. **FIGs. 7A-C** are a set of graphs showing Ba concentration levels in aqueous humor after administration of either 250 μg of rCFH monthly or 500 μg of rCFH monthly (FIG. 7A); 250 μg of rCFH monthly (FIG. 7B); or 500 μg of rCFH monthly (FIG. 7C). As shown in **FIG. 7A-C**, administration of rCFH has a robust and prolonged impact on the Ba levels, by regulating

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the complement pathway, in the dry AMD patients who had an elevated initial level prior to rCFH treatment. Ba levels did not reduce below healthy levels in patients without elevated Ba levels showing that administration of rCFH did not affect the regulatory mechanism.

C3a Concentration Levels

[0286] Quantitative measurement of C3a fragment in human aqueous humor was done using a MicroVue C3a Plus Enzyme Immunoassay Kit (Quidel, cat#A031). Briefly, a 96 well microassay plate was coated with a murine monoclonal antibody specific for human C3a. Upon addition of standards, controls, and samples to the coated plate, the antibody on the plate bound to C3a. After the incubation period, the plate was washed to remove any unbound material. Next, a horseradish peroxidase (HRP)-conjugated anti-C3a was added to each assay well where it bound to the immobilized C3a captured in the first step. When a 3,3',5,5' tetramethylbenzidine (TMB), a ready-to-use, chromogenic substrate solution, was added to the assay wells, the bound HRP reacted with the substrate forming a blue color. After the incubation period, the reaction was stopped and the color intensity was measured spectrophotometrically at A450. The color intensity of the reaction mixture was proportional to the concentration of C3a present in the standards, controls, and samples. Results were calculated from the generated standard curve using 5-parameter analysis. The LLOQ and ULOq values of C3a in this assay were 31.81 pg/mL and 2052.63 pg/mL, respectively.

[0287] C3a levels were examined in quartile range with quartiles 1-4 having pre-treatment C3a levels in the ranges between 1097 pg/mL and 3442 pg/mL; between 3510 pg/mL and 4950 pg/mL; between 5040 pg/mL and 6968 pg/mL; and between 7022 pg/mL and 17882 pg/mL, respectively. **FIG. 8** is a graph showing C3a concentration levels in aqueous humor after administration of either 250 μg of rCFH monthly or 500 μg of rCFH monthly. As shown in **FIG. 8**, rCFH reduced C3a levels in the patients who had an elevated initial level prior to rCFH treatment. C3a levels did not reduce below healthy levels in patients without elevated C3a levels, which shows that administration of rCFH did not affect the regulatory mechanism. **FIG. 9** is a scatter dot plot showing correlation Ba and C3a concentration levels at baseline and after administration of rCFH. Analysis of the quantification levels of Ba and C3a showed that reduction in concentration levels of both biomarkers (Ba and C3a) was dependent upon baseline level of corresponding biomarker in each patient. As expected, patients with healthy levels of Ba and C3a showed no significant further reduction in Ba and C3a levels.

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Example 6. Phase 2, Multiple Dose Study of Recombinant CFH (rCFH) as an Adjunct to Standard of Care Aflibercept Therapy in Patients with Neovascular AMD (nAMD)

[0288] The safety, tolerability, immunogenicity, PK/PD, complement biomarkers, and clinical efficacy of repeat intravitreal (IVT) injection of rCFH plus standard of care (SoC) defined as aflibercept every other month (EOT), compared with sham plus SoC in subjects with nAMD are assessed in a Phase 2, multicenter and multiple-dose study.

[0289] Data from the Phase I single ascending dose study (described in Example 2) is used to inform the selection of the 500 μg/eye dose for evaluation in this Phase 2 multiple-dose study in subjects with nAMD. In this study, repeat doses of rCFH are administered by IVT to subjects with nAMD. The 500 μg/eye is based on safety data from single- and repeat-dose (up to 3 and up to 6 once monthly) IVT toxicology studies in cynomolgus monkeys, PK modeling and simulation of the profiles of rCFH after a single IVT injection in cynomolgus monkeys, and the endogenous level of CFH in human RPE (the target ocular tissue of rCFH replacement therapy in dry AMD patients), as well as data from the Phase 1 SAD study (described in Example 2).

[0290] All subjects treated with rCFH will receive 500 μg rCFH IVT. One dosing regimen (EOM) will be evaluated in each treatment group. Subjects are randomized to 1 of 2 treatment groups: rCFH plus SoC or sham plus SoC. A diagram of a clinical study design is shown in FIG. 10.

Objectives and End Points

[0291] The primary endpoint of the study is to assess rCFH plus SoC versus sham plus SoC at 6 months, and also through an additional extended 6-month dosing period. Secondary and exploratory endpoints are to assess rCFH plus SoC versus sham plus SoC at the same timepoints and may be evaluate at additional timepoints.

[0292] The primary objective of this study is evaluate the safety and tolerability of rCFH plus SoC versus sham plus SoC. The endpoint for the primary objective is treatment-related intraocular adverse events (AEs).

[0293] The secondary objectives are (1) to evaluate total CFH in aqueous humor after rCFH IVT injection; (2) to describe the effect on best corrected visual acuity (BCVA); (3) to describe the effect on macular atrophy (MA) size in subjects with MA present at baseline. The endpoints for the secondary objectives include: aqueous humor concentrations of total CFH; mean change in BCVA from baseline in Early Treatment Diabetic Retinopathy Study (ETDRS) letters; and

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mean change in size of MA evaluated by fundus autofluorescence (FAF) and optical coherence tomography (OCT) from baseline.

[0294] The exploratory objectives are (1) to evaluate the immunogenicity of rCFH in serum after rCFH IVT injection; (2) to describe the effect of rCFH IVT injection on biomarkers in aqueous humor; (3) to describe the effect on incidence of new MA in subjects without MA at baseline; (4) to describe the effect on choroidal neovascularization (CNV) size; (5) to describe the effect on thickness of retinal layers; (6) to describe the effect on subretinal and intraretinal fluid; (7) to describe the effect on low luminance visual acuity (LLVA); and (8) to describe the effect on National Eye Institute Visual Functioning Questionnaire 25 item Version (NEI-VFQ-25) / Minnesota Low-vision Reading Test (MNRead). The endpoints for the exploratory objectives include: generation of serum rCFH antidrug antibodies; change from baseline in complement protein, complement split products, and related cytokines protein values in aqueous humor; new MA evaluated by FAF and OCT from baseline; mean reduction from baseline in CNV size as evaluated by fluorescein angiography (FA) and OCT; mean change from baseline in center point thickness and central subfield thickness in μm as evaluated by spectral domain optical coherence tomography (SD-OCT); incidence of new fluid or disappearance of existing fluid, both subretinal and intraretinal, as evaluated by SD-OCT; mean change in LLVA from baseline in ETDRS letters; and mean change in score from baseline on NEI-VFQ-25 / MNRead.

Methodology

[0295] rCFH or sham, along with SoC aflibercept, is administered to 1 eye only, which is designed during the screening process, based on eligibility, and prior to any baseline measurements. If both eyes meet all eye-specific entry criteria, then the study eye is determined by the Investigator and the subject together. The fellow eye may be followed for comparative analyses. Detailed assessments of intraocular pressure (IOP), visual acuity (VA) and function, and retinal parameters such as CNV and MA lesion size is performed for each subject enrolled

[0296] A schedule of assessments for 6-month initial dosing period is described in Table 8.

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[0297] **Table 8 . Schedule of Assessments**

	IC/EC Screen [a]	Baseline	48 hr Post-dosePhone Call	Week 1	Week 2	Month 2	Month 4	Month 6	Month 8	Month 10	Month 12 EOS
Clinical Visit (C) or Phone Visit (P)	C	C	P	P	C	C	C	C	C	C	C
Study Day (First Dose = Day 1)	-35 to -3	1	3 (±1)	8 (±2)	15 (±2)	61 (±4)	121 (±4)	181 (±4)	241 (±4)	301 (±4)	361(±4)
Informed consent [b]	X										
Blood sample – exploratory genetics *	X										
Medical and Ocular History *[c]	X	X									
Inclusion/Exclusion Criteria *[d]	X	X									
Demographics	X										
Complete Physical Examination	X										X
ECG	X										
Vital Signs *[e]	X	X			X	X	X	X	X	X	X
Concomitant Medications *	X	X	X	X	X	X	X	X	X	X	X
Treatments and AEs											
Randomization†‡		X									
Aflibercept SoC Administration		SE				SE	SE	SE	SE	SE	
Treatment (Sham or rCFH)Administration		SE				SE	SE	SE	SE	SE	
Adverse Events	X	X	X	X	X	X	X	X	X	X	X [f]
Ocular Assessments											
Complete Ophthalmic Examination *[g]	OU	OU			OU	OU	OU	OU	OU	OU	OU
Intraocular Pressure Assessment	OU	OU			OU	OU	OU	OU	OU	OU	OU
ETDRS BCVA *	OU	OU			OU	OU	OU	OU	OU	OU	OU
ETDRS BCVA LLVA *		OU					OU	OU			OU
Imaging and Visual Function Assessments ** (Imaging assessments may be optionally performed at any in-clinic visit if deemed medically necessary for subject safety)											
Color Fundus Photography (3-Field) *	OU	OU [i]						OU			OU
Fundus Autofluorescence *	OU	OU [i]				OU	OU	OU	OU	OU	OU

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IC/EC Screen [a]	Baseline	48 hr Post-dose Phone Call	Week 1	Week 2	Month 2	Month 4	Month 6	Month 8	Month 10	Month 12 EOS
Clinical Visit (C) or Phone Visit (P)	C	C	P	C	C	C	C	C	C	C
Study Day (First Dose = Day 1)	-35 to -3	1	3 (±1)	8 (±2)	15 (±2)	61 (±4)	121 (±4)	181 (±4)	241 (±4)	301 (±4)	361(±4)
Fluorescein Angiography *[j]	OU							OU			OU
Near Infrared Reflectance Imaging *	OU	OU [i]						OU			OU
Optical Coherence Tomography (SD-OCT)	OU	OU [i]			OU	OU	OU	OU	OU	OU	OU
OCTA [k]		OU [i]						OU			OU
NEI-VFQ-25 *[m]		X						X			X
MNRead *[l]		X						X			X
Other Assessments											
Selection of Study Eye [d]		X									
Pregnancy Testing [n]	X										X
Clinical Safety Laboratory Assessments *[o]	X	X			X	X	X	X	X	X	X
Routine Urinalysis [p]	X										X
Plasma for CFH		X					X				
Blood Sampling – ADA *		X			X	X	X	X	X	X	X
Aqueous Humor Sampling *		SE			SE	SE	SE	SE	SE	SE	SE

Abbreviations: ADA=antidrug antibodies; AE=adverse event; BCVA=best corrected visual acuity; CFH=complement factor H; EC=exclusion criteria; ECG=electrocardiogram; EOS=End of Study; ETDRS=Early Treatment Diabetic Retinopathy Study; hr=hour; IC=inclusion criteria; IOP=intraocular pressure; LLVA=low luminance visual acuity; MNRead=Minnesota Low-vision Reading Test; NEI VFQ-25=National Eye Institute Visual Functioning Questionnaire-25;OCT=optical coherence tomography; OCT-A=OCT angiography; OU=oculus utcrque (both eyes; eyes will be tested separately); SAE=serious adverse event; SD-OCT=spectral domain OCT; SE=study eye; SoC=standard of care (2 mg aflibercept every other month); VA=visual acuity

* Assessment obtained prior to dosing, as applicable.
** Imaging (color fundus photography, fundus autofluorescence, fluorescein angiography, near infrared reflectance imaging, SD-OCT) should be repeated atbaseline if >28 days after screening assessment, or desired by the Investigator.
† Randomization may occur up to 2 days prior to the baseline visit.

a Blood sampling for exploratory genetics will occur prior to first dosing.
b Informed consent must be obtained before any study assessment is performed.
c Medical and ocular history also includes social history and family ophthalmic history.

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d Some entry criteria apply only to the SE. Therefore, the SE must be identified during the screening process in order to assess entry criteria.

e Vital sign measurements include systolic and diastolic blood pressures, heart rate, respiratory rate, and body temperature. The subject will be seated for at least 5 minutes before all measurements are taken. When procedures overlap and are scheduled to occur at the same timepoint, vital sign measurements should precede any blood collection.

f AEs will be reported for a minimum of 30 days after the last administration of study treatment. If a subject experiences an SAE that is considered to berelated to study treatment at any time after the study, it must be reported to the Sponsor.

g Complete ophthalmic examinations of both the study and fellow eye (if possible) will include an assessment of the VA and visual function using validatedassessment tools, a complete ophthalmic biomicroscopy examination including fundus examination, IOP, and ocular imaging tests to visualize the appearance and pathology of the retina and associated ocular tissues.

h On dosing days, IOP assessments are obtained prior to the dose and 30±15 minutes after the dose, with an additional sample at 60±15 minutes if the IOP hasnot resolved to ≤30 mmHg at the 30-minute assessment. If the IOP continues to exceed 30 mmHg, then standard IOP-lowering interventions (per institutional guidelines) should be followed. If IOP is ≤5 mmHg, the subject should be treated per institutional guidelines. Regardless of whether applanation tonometry or a Tono-Pen® is used to measure IOP, the same method must be used for the subject for the entirety of their participation in this study.

i These baseline imaging assessments are only required if the screening images were obtained >28 days prior to baseline. They may be redone at baseline if the Investigator deems it clinically relevant, or if there were quality issues with the screening images.

j Baseline assessment of fluorescein angiography is not required, but may be assessed if the Investigator deems it clinically relevant, or if there were quality issues with the screening images.

k Assessment is optional and will only be conducted if the site has approval from the Sponsor.

l Assessment will be performed as per Minnesota Low-vision Reading Test (MNRead) Charts (*see* Altinbay *et al.* (2016). "The Evaluation of Reading Performance with Minnesota Low Vision Reading Charts in Patients with Age-related Macular Degeneration". Middle East African Journal of Ophthalmology. 23 (4): 302–306).

m Assessment will be performed as per National Eye Institute Visual Functioning Questionnaire 25-item Version (NEI-VFQ-25) with Near and Distance Activity Subscale Scores (developed at RAND (https://www.rand.org) under the sponsorship of the NEI).

n Highly sensitive (serum or urine) human chorionic gonadotropin pregnancy test as needed for women of childbearing potential.

o Clinical safety laboratory assessments include the following: hematology (platelet count, red blood cell count, hemoglobin count, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, percent reticulocytes, and white blood cell count with differential) and clinical chemistry (blood urea nitrogen, creatinine, glucose, potassium, sodium, total calcium, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total and directbilirubin, total protein, standard electrolytes – magnesium, phosphate, and chloride).

p Routine urinalysis includes macroscopic and microscopic examination.

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Study duration

[0298] The minimum planned duration of each subject's participation is approximately 13 months: 1 month for screening and 12 months dosing and follow-up.

[0299] Approximately, 45 subjects (~ 30 treated with rCFH plus SoC and ~ 15 treated with sham plus SoC.

Eligibility Criteria

Inclusion Criteria

[0300] Subjects must meet all of the following inclusion criteria to be considered for participation: (1) at least 50 years old at the time of signed informed consent; subjects younger than 50 may be considered for participation after consultation with the Medical Monitor; (2) CNV related to nAMD with the following features, as determined by the Image Reading Center

a. Maximum CNV lesion size of 12 disc areas;

b. Subretinal hemorrhage $\leqslant$50% of lesion size

[0301] (3) must be on aflibercept treatment prior to Day 1 (with at least one aflibercept dose received within 2 months prior to Day 1) and be able to continue aflibercept at an every-other-month frequency for the duration of the study; (4) BCVA in the study eye of 24 to 75 letters using the ETDRS Chart VA Scale (approximately equivalent to Snellen VA of between 20/32 and 20/320); (5) sufficiently clear ocular media, adequate pupillary dilation, fixation to permit quality fundus imaging, and able to cooperate sufficiently for adequate ophthalmic visual function testing and anatomic assessment in the study eye; (6) understands the full nature and purpose of the study, including possible risks of study procedures, and provides informed consent prior to initiation of any study procedure; and (7) all subjects with reproductive potential must agree to use effective contraceptive methods through the end of study (EOS) visit.

Exclusion Criteria

[0302] A subject who meets any of the following exclusion criteria is ineligible to participate in the study: (1) presence of the following ocular conditions in the study eye:

a. any active ocular disease or condition that could confound the assessment of the macula or be a contraindication to IVT injection, *e.g.*, retinal pigment epithelium tears or rips involving the macula, macular hole (any stage, including lamellar hole), epiretinal membrane, MA or

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maculopathies due to any disease other than AMD, uncontrolled glaucoma (IOP of >24 mmHg when on 2 or more IOP-lowering medications), severe glaucoma (cup to disc ratio of ≥0.8), diabetic retinopathy graded as moderate nonproliferative diabetic retinopathy or worse (with or without diabetic macular edema);

b. any intraocular surgery, (with the exception of intraocular lens replacement surgery less than 3 months prior to consent) e.g., any glaucoma surgeries including microinvasive glaucoma surgery and/or any intraocular surgery requiring vitrectomy;
c. aphakia or complete absence of the posterior capsule;
d. prior corneal transplant;
e. scar or fibrosis ≥50% of CNV lesion or involving center of fovea;

[0303] (2) presence of any of the following ocular conditions – in either eye:

a. history of herpetic infection, idiopathic polypoidal choroidal vasculopathy (PCV), pathologic myopia, central serous chorioretinopathy (CSCR), adult onset foveal pattern dystrophy;
b. concurrent disease that could require medical or surgical intervention during the study period;
c. active/suspected ocular/periocular infection or active intraocular inflammation;
d. history of idiopathic or autoimmune-associated uveitis;

[0304] (3) in the opinion of the Investigator, the subject has any prior or ongoing medical condition (*e.g.*, ocular other than wet AMD, systemic, psychiatric) or clinically significant screening laboratory value that may present a safety risk, interfere with study compliance, interfere with consistent study follow-up, or confound data interpretation throughout the longitudinal follow-up period; (4) subject has experienced a cardiovascular or cerebrovascular event within 12 months of informed consent; (5) female subjects must not be pregnant or lactating, nor plan to become pregnant during the study; (6) current use of medications known to be toxic to the lens, retina, or optic nerve (deferoxamine, chloroquine/hydroxychloroquine [Plaquenil®], tamoxifen, phenothiazines, ethambutol, digoxin, and aminoglycosides). (Current use is defined as the administration of first dose of rCFH within 5 half-lives of the prohibited

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medication). Past use of these medications, without history of ocular side effects, is not considered exclusionary. Use of medications that may potentially exacerbate macular edema or macular degeneration is permitted if, in the opinion of the Investigator, in consultation with the Medical Monitor, such use will not confound the interpretation of study results; or (7) use of any investigational new drug or other experimental treatment in the last 6 months, and/or receipt of any prior gene therapy (*e.g.* Adeno-associated virus) or ocular device implantation (other than posterior chamber intraocular lens placement after cataract surgery).

rCFH Dose and Route, Regimen

[0305] rCFH is supplied in single-use vials with a 0.25 mL fill volume at a concentration of 10 mg/mL rCFH. Aflibercept administered IVT EOM is SoC therapy. Subjects assigned to the rCFH plus SoC group are administered aflibercept (2 mg/50 µL) first, followed by rCFH (500 µg/50 µL) 15 minutes (±5 minutes) later. Study drug is not mixed in the same syringe with other products. Subjects are randomized to 1 of 2 treatment groups: rCFH plus SoC or sham plus SoC. Subjects randomized to the rCFH plus SoC treatment group receive treatment EOM (6 doses). All subjects assigned to the rCFH plus SoC treatment are treated with 500 µg rCFH per eye.

[0306] Subjects randomized to the sham plus SoC treatment group receive SoC with a sham IVT injection instead of rCFH, to keep the subject masked to their treatment assignment.

Biomarkers

[0307] Aqueous humor is collected prior to and after receiving single IVT injections of rCFH to evaluate complement proteins and possibly inflammatory cytokine profiles to determine their potential as PD biomarkers for usefulness in a pivotal study. Total CFH concentrations in aqueous humor is evaluated. Imaging endpoints such as CNV lesion size, MA lesion size, retinal layer thickness or presence of fluid, and clinical endpoints such as BCVA, LLVA, and low luminance deficit (LLD), are collected as described in Table 8.

[0308] CFH concentration levels in human plasma can be quantified using MicroVue Factor H Enzyme Immunoassay (EIA) (Quidel, cat# A039). This is a quantitative Elisa method where the microtiter plate is coated with a mouse monoclonal antibody that binds specifically to human Factor H. When standards, controls, or specimens are added to the plate, FH binds to the immobilized anti-Factor H monoclonal antibody. Next, a horseradish peroxidase (HRP)-conjugated murine anti-Factor H antibody is added to each well as the detection reagent which

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binds to the FH captured on the plate. When a chromogenic enzyme substrate (TMB) is added, the bound HRP-conjugate reacts with the substrate, forming a color which is measured spectrophotometrically at A450. The color intensity of the reaction mixture is proportional to the concentration of Factor H present in the samples. Human ocular samples obtained from patient group that have been monthly administered rCFH plus SoC are expected to show elevated levels of CFH over baseline CFH levels.

INCORPORATION BY REFERENCE

[0309] All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

EQUIVALENTS

[0310] The present application 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 application described herein. Scope of the present application 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.

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CLAIMS

1. A method of treating a subject having an inflammatory ocular disease, disorder, or condition, the method comprising administering to the subject 50 μg, 100 μg, 250 μg, or 500 μg of CFH protein per eye, thereby treating the inflammatory ocular disease, disorder, or condition.

2. The method of claim 1, wherein the inflammatory ocular disease, disorder, or condition is selected from the group consisting of macular degeneration, diabetic retinopathy, diabetic macular edema, retinal vein occlusion, uveitis, sterile conjunctivitis, keratitis, episcleritis, and Stargardt's Disease.

3. The method of claim 2, wherein the macular degeneration is AMD.

4. The method of claim 3, wherein the AMD is dry AMD.

5. The method of claim 4, wherein the inflammatory ocular disease, disorder, or condition comprises geographic atrophy secondary to dry AMD.

6. The method of claim 1, wherein the AMD is neovascular AMD.

7. The method of claim 6, wherein the subject has been treated with a VEGF-A antagonist.

8. The method of claim 2, wherein the uveitis is anterior uveitis.

9. The method of any one of claims 1-8, wherein the CFH is administered by intravitreal injection.

10. The method of any one of claims 1-9, wherein the CFH protein comprises a recombinant CFH protein.

11. The method of any one of claims 1-10, wherein the CFH protein is administered at a dose of 50 μg per eye.

12. The method of any one of claims 1-10, wherein the CFH protein is administered at a dose of 100 μg per eye.

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13. The method of any one of claims 1-10, wherein the CFH protein is administered at a dose of 250 μg per eye.

14. The method of any one of claims 1-10, wherein the CFH protein is administered at a dose of 500 μg per eye.

15. The method of any one of claims 1-14, wherein the CFH protein is administered once every four weeks, once every eight weeks, once every month, once every two months, once every three months, once every four months, once every five months, or once every six months.

16. The method of any one of claims 1-15, wherein the dose of CFH protein is 250 μg per eye, administered once every month.

17. The method of any one of claim 1-15, wherein the dose of CFH protein is 250 μg per eye, administered once every two months.

18. The method of any one of claims 1-15, wherein the dose of CFH protein is 500 μg per eye, administered once every month.

19. The method of any one of claims 1-15, wherein the dose of CFH protein is 500 μg per eye, administered once every two months.

20. The method of any one of claims 1-19, wherein the CFH protein comprises the amino acid sequence of SEQ ID NO: 2.

21. The method of any one of claims 1-20, wherein a lesion associated with the inflammatory ocular disease, disorder, or condition is observed in the subject by fundus imaging.

22. The method of any one of claims 1-21, wherein the subject carries a genetic variant associated with the inflammatory ocular disease, disorder, or condition.

23. The method of claim 22, wherein the inflammatory ocular disease, disorder, or condition is geographic atrophy, and wherein the genetic variant results in a mutation of the Tyr at amino acid position 402 of human CFH protein and is present in both alleles of the genome.

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24. The method of claim 23, wherein the genetic variant results in a mutation of the Tyr at amino acid position 402 of human CFH protein to His residue and is present in both alleles of the genome.

25. The method of claim 23 or 24, wherein the genetic variant comprises rs1061170 in both alleles of the human genome.

26. The method of any one of claims 23-25, wherein the subject is negative for any one of the missense CFH mutations selected from the group consisting of R2T, L3V, R53C, R53H, S58A, D90G, D130N, R175Q, R175P, I221V, R303W, R303Q, Q400K, P503A, R567G, G650V, S890I, T956M, G1194D, R1210C, and any combination(s) thereof.

27. The method of any one of claims 23-26, wherein the subject is negative for a combination of homozygous CFH 62V, homozygous C3 102G, and homozygous CFB 32R.

28. The method of claim 22, wherein the subject is (a) homozygous for the Y402H mutation of CFH; (b) negative for any one of the missense CFH mutations selected from the group consisting of R2T, L3V, R53C, R53H, S58A, D90G, D130N, R175Q, R175P, I221V, R303W, R303Q, Q400K, P503A, R567G, G650V, S890I, T956M, G1194D, R1210C, and any combination(s) thereof; and (c) negative for a combination of homozygous CFH 62V, homozygous C3 102G, and homozygous CFB 32R.

29. The method of claim 22, wherein the subject is positive for a missense CFH mutation selected from the group consisting of R2T, L3V, R53C, R53H, S58A, D90G, D130N, R175Q, R175P, I221V, R303W, R303Q, Q400K, P503A, R567G, G650V, S890I, T956M, G1194D, R1210C, and any combination(s) thereof.

30. The method of claim 22, wherein the subject is positive for a combination of homozygous CFH 62V, homozygous C3 102G, and homozygous CFB 32R.

31. The method of any one of claims 1-30, wherein the subject is at least 50 years old.

32. The method of any one of claims 1-31, further comprising administering to the subject an effective amount of a VEGF antagonist, wherein the subject has neovascular AMD.

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33. The method of claim 32, wherein the VEGF antagonist comprises aflibercept.

34. The method of claim 33, wherein the effective amount of aflibercept is 2 mg per eye.

35. The method of claim 34, wherein the aflibercept is administered once every four weeks, once every eight weeks, once every month, or once every two months.

36. The method of any one of claims 1-35, wherein the treatment slows the progression of the inflammatory ocular disease, disorder, or condition in the subject.

37. The method of any one of claims 1-36, wherein the treatment reduces the severity of the inflammatory ocular disease, disorder, or condition in the subject.

38. The method of claim 36 or 37, wherein the progression or severity of the inflammatory ocular disease, disorder, or condition is assessed by one or more methods selected from:

(a) best corrected visual acuity (BCVA) score;

(b) low luminance visual acuity (LLVA) score;

(c) AREDS 9-step severity scale score;

(d) area of geographic atrophy assessed by color fundus photography, fundus autofluorescence, optical coherence tomography, optical coherence tomography – angiography, near infrared imaging, and/or fluorescein angiography;

(e) drusen volume; and

(f) one or more retinal architecture parameters assessed by optical coherence tomography, selected from total retinal and choroidal thickness, photoreceptor layer thickness, features of nascent geographic atrophy, retinal pigment epithelium thickening, and integrity of retinal pigment epithelium layer.

39. A method for treating a subject having an inflammatory ocular disease, disorder, or condition, the method comprising:

(a) obtaining or having obtained a measurement of the protein level of at least one biomarker in an ocular sample of the subject, wherein the biomarker is selected from the group consisting of (i) complement component C3, complement factor B (CFB), complement component C5, cleavage fragments thereof, and complement factor H (CFH), (ii) proteins associated with ocular inflammation, and (iii) proteins associated with choroidal

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neovascularization;

(b) determining whether the protein level of the biomarker is greater than or lower than a predetermined threshold; and

(c) administering to the subject an effective amount of CFH if the protein level of the biomarker is (i) greater than or equal to the threshold if the biomarker is positively correlated with activation of the complement pathway, ocular inflammation, or choroidal neovascularization; or (ii) lower than or equal to the threshold if the biomarker is negatively correlated with activation of the complement pathway, ocular inflammation, or choroidal neovascularization,

thereby treating the inflammatory ocular disease, disorder, or condition.

40. The method of claim 39, wherein the inflammatory ocular disease, disorder, or condition is selected from the group consisting of macular degeneration, diabetic retinopathy, diabetic macular edema, retinal vein occlusion, uveitis, sterile conjunctivitis, keratitis, episcleritis, and Stargardt's Disease.

41. The method of claim 40, wherein the macular degeneration is age-related macular degeneration (AMD).

42. The method of claim 41, wherein the AMD is dry AMD.

43. The method of claim 42, wherein the inflammatory ocular disease, disorder, or condition comprises geographic atrophy secondary to dry AMD.

44. The method of claim 41, wherein the AMD is neovascular AMD.

45. The method of claim 44, wherein the subject has been treated with a VEGF-A antagonist.

46. The method of claim 40, wherein the uveitis is anterior uveitis.

47. The method of any one of claims 39-46, wherein the ocular sample comprises aqueous humor.

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48. The method of any one of claims 39-47, wherein the at least one biomarker comprises a cleavage fragment of C3, CFB, or C5, and the protein level of the cleavage fragment is positively correlated with activation of the complement pathway.

49. The method of claim 48, wherein the cleavage fragment is selected from the group consisting of C3a, Ba, and C5a.

50. The method of claim 49, wherein the cleavage fragment is C3a.

51. The method of claim 50, wherein the threshold is 2 ng/mL.

52. The method of claim 49, wherein the cleavage fragment is Ba.

53. The method of claim 52, wherein the threshold is 8 ng/mL.

54. The method of any one of claims 39-53, wherein the at least one biomarker comprises CFH, and the protein level of CFH is negatively correlated with activation of the complement pathway.

55. The method of claim 54, wherein the threshold is 60 ng/mL.

56. The method of any one of claims 39-55, wherein the at least one biomarker comprises a protein associated with ocular inflammation.

57. The method of claim 56, wherein the protein associated with ocular inflammation is selected from the group consisting of IL-1β, IL-6, IL-8, IL-10, IL-18, TNF-α, CCL2, CXCL5, and Eotaxin-2, and the protein level of the biomarker is positively correlated with ocular inflammation.

58. The method of any one of claims 39-57, wherein the at least one biomarker comprises a protein associated with choroidal neovascularization.

59. The method of claim 58, wherein the protein associated with choroidal neovascularization is VEGF-A, and the protein level of the biomarker is positively correlated with choroidal neovascularization.

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60. The method of any one of claims 39-59, wherein the CFH is administered by intravitreal injection.

61. The method of any one of claims 39-60, wherein the CFH comprises a CFH protein.

62. The method of claim 61, wherein the CFH protein comprises a recombinant CFH protein.

63. The method of claim 61 or 62, wherein the CFH protein is administered at a dose of 50 μg, 100 μg, 250 μg, or 500 μg per eye.

64. The method of any one of claims 61-63, wherein the CFH protein is administered once every four weeks, once every eight weeks, once every month, once every two months, once every three months, once every four months, once every five months, or once every six months.

65. The method of any one of claims 39-60, wherein the CFH comprises a vector encoding a CFH protein.

66. The method of claim 65, wherein the vector is an adeno-associated virus vector.

67. The method of any one of claims 61-66, wherein the CFH protein comprises the amino acid sequence of SEQ ID NO: 2.

68. The method of any one of claims 39-67, wherein the steps (a)-(c) are conducted after an initial administration of CFH to the subject.

69. The method of any one of claims 39-68, wherein a lesion associated with the inflammatory ocular disease, disorder, or condition is observed in the subject by fundus imaging.

70. The method of any one of claims 39-69, wherein the subject carries a genetic variant associated with the inflammatory ocular disease, disorder, or condition.

71. The method of claim 70, wherein the inflammatory ocular disease, disorder, or condition is geographic atrophy, and wherein the genetic variant results in a mutation of the Tyr at amino acid position 402 of human CFH protein and is present in both alleles of the genome.

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72. The method of claim 71, wherein the genetic variant comprises rs1061170 in both alleles of the CFH gene.

73. The method of any one of claims 39-72, wherein the subject is at least 50 years old.

74. The method of any one of claims 39-73, further comprising administering to the subject an effective amount of a VEGF antagonist.

75. The method of any one of claims 39-74, wherein the treatment slows the progression of the inflammatory ocular disease, disorder, or condition in the subject.

76. The method of any one of claims 39-75, wherein the treatment reduces the severity of the inflammatory ocular disease, disorder, or condition in the subject.

77. The method of claim 75 or 76, wherein the progression or severity of the inflammatory ocular disease, disorder, or condition is assessed by one or more methods selected from:

(a) best corrected visual acuity (BCVA) score;

(b) low luminance visual acuity (LLVA) score;

(c) AREDS 9-step severity scale score;

(d) area of geographic atrophy assessed by color fundus photography, fundus autofluorescence, optical coherence tomography, optical coherence tomography – angiography, near infrared imaging, and/or fluorescein angiography;

(e) drusen volume; and

(f) one or more retinal architecture parameters assessed by optical coherence tomography, selected from total retinal and choroidal thickness, photoreceptor layer thickness, features of nascent geographic atrophy, retinal pigment epithelium thickening, and integrity of retinal pigment epithelium layer.

78. A method for selecting a subject for treatment of an inflammatory ocular disease, disorder, or condition, the method comprising:

(a) obtaining or having obtained a measurement of the protein level of at least one biomarker in an ocular sample of the subject, wherein the biomarker is selected from the group consisting of (i) complement component C3, CFB, complement component C5, cleavage fragments thereof, and CFH, (ii) proteins associated with ocular inflammation, and (iii) proteins

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associated with choroidal neovascularization;

(b) determining whether the protein level of the biomarker is greater than or lower than a predetermined threshold; and

(c) selecting the subject for treatment of the inflammatory ocular disease, disorder, or condition if the protein level of the biomarker is (i) greater than or equal to the threshold if the biomarker is positively correlated with activation of the complement pathway, ocular inflammation, or choroidal neovascularization; or (ii) lower than or equal to the threshold if the biomarker is negatively correlated with activation of the complement pathway, ocular inflammation, or choroidal neovascularization,

wherein the treatment comprises administering to the subject CFH.

79. The method of claim 78, wherein step (a) comprises measuring the protein level of the biomarker in an ocular sample of the subject.

80. The method of claim 78 or 79, wherein the inflammatory ocular disease, disorder, or condition is selected from the group consisting of macular degeneration, diabetic retinopathy, diabetic macular edema, retinal vein occlusion, uveitis, sterile conjunctivitis, keratitis, episcleritis, and Stargardt's Disease.

81. The method of claim 80, wherein the macular degeneration is AMD.

82. The method of claim 81, wherein the AMD is dry AMD.

83. The method of claim 82, wherein the inflammatory ocular disease, disorder, or condition comprises geographic atrophy secondary to dry AMD.

84. The method of claim 80, wherein the AMD is neovascular AMD.

85. The method of claim 84, wherein the subject has been treated with a VEGF-A antagonist.

86. The method of claim 80, wherein the uveitis is anterior uveitis.

87. The method of any one of claims 78-84, wherein the ocular sample comprises aqueous humor.

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88. The method of any one of claims 78-87, wherein the at least one biomarker comprises a cleavage fragment of C3, CFB, or C5, and the protein level of the cleavage fragment is positively correlated with activation of the complement pathway.

89. The method of claim 88, wherein the biomarker is selected from the group consisting of C3a, Ba, and C5a.

90. The method of claim 89, wherein the biomarker is C3a.

91. The method of claim 90, wherein the threshold is 2 ng/mL.

92. The method of claim 89, wherein the biomarker is Ba.

93. The method of claim 92, wherein the threshold is 8 ng/mL.

94. The method of any one of claims 78-93, wherein the at least one biomarker comprises CFH, and the protein level of CFH is negatively correlated with activation of the complement pathway.

95. The method of claim 94, wherein the threshold is 60 ng/mL.

96. The method of any one of claims 78-95, wherein the at least one biomarker comprises a protein associated with ocular inflammation.

97. The method of claim 96, wherein the protein associated with ocular inflammation is selected from the group consisting of IL-1β, IL-6, IL-8, IL-10, IL-18, TNF-α, CCL2, CXCL5, and Eotaxin-2, and the protein level of the biomarker is positively correlated with ocular inflammation.

98. The method of any one of claims 78-97, wherein the at least one biomarker comprises a protein associated with choroidal neovascularization.

99. The method of claim 98, wherein the protein associated with choroidal neovascularization is VEGF-A, and the protein level of the biomarker is positively correlated with choroidal neovascularization.

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100. The method of any one of claims 78-99, wherein the CFH is administered by intravitreal injection.

101. The method of any one of claims 78-100, wherein the CFH comprises a CFH protein.

102. The method of claim 101, wherein the CFH protein comprises a recombinant CFH protein.

103. The method of claim 101 or 102, wherein the CFH protein is administered at a dose of 50 μg, 100 μg, 250 μg, or 500 μg per eye.

104. The method of any one of claims 101-103, wherein the CFH protein is administered once every four weeks, once every eight weeks, once every month, once every two months, once every three months, once every four months, once every five months, or once every six months.

105. The method of any one of claims 78-100, wherein the CFH comprises a vector encoding a CFH protein.

106. The method of claim 105, wherein the vector is an adeno-associated virus vector.

107. The method of any one of claims 101-106, wherein the CFH protein comprises the amino acid sequence of SEQ ID NO: 2.

108. The method of any one of claims 78-107, wherein the steps (a)-(c) are conducted after an initial administration of CFH to the subject.

109. The method of any one of claims 78-108, wherein a lesion associated with the inflammatory ocular disease, disorder, or condition is observed in the subject by fundus imaging.

110. The method of any one of claims 78-109, wherein the subject carries a genetic variant associated with the inflammatory ocular disease, disorder, or condition.

111. The method of claim 110, wherein the inflammatory ocular disease, disorder, or condition is geographic atrophy, and wherein the genetic variant results in a mutation of the Tyr at amino acid position 402 of human CFH protein and is present in both alleles of the genome.

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112. The method of claim 111, wherein the genetic variant comprises rs1061170 in both alleles of the human genome.

113. The method of any one of claims 78-112, wherein the subject is at least 50 years old.

114. The method of any one of claims 78-113, wherein the treatment further comprises administering to the subject an effective amount of a VEGF antagonist.

115. The method of any one of claims 78-114, wherein the treatment slows the progression of the inflammatory ocular disease, disorder, or condition in the subject.

116. The method of any one of claims 78-115, wherein the treatment reduces the severity of the inflammatory ocular disease, disorder, or condition in the subject.

117. The method of claim 115 or 116, wherein the progression or severity of the inflammatory ocular disease, disorder, or condition is assessed by one or more methods selected from:

(a) best corrected visual acuity (BCVA) score;

(b) low luminance visual acuity (LLVA) score;

(c) AREDS 9-step severity scale score;

(d) area of geographic atrophy assessed by color fundus photography, fundus autofluorescence, optical coherence tomography, optical coherence tomography – angiography, near infrared imaging, and/or fluorescein angiography;

(e) drusen volume; and

(f) one or more retinal architecture parameters assessed by optical coherence tomography, selected from total retinal and choroidal thickness, photoreceptor layer thickness, features of nascent geographic atrophy, retinal pigment epithelium thickening, and integrity of retinal pigment epithelium layer.

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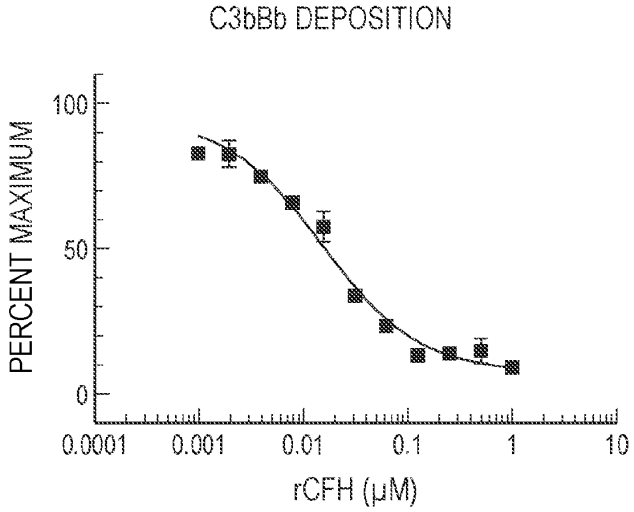

C3bBb DEPOSITION
PERCENT MAXIMUM
100
50
0
0.0001
0.001
0.01
0.1
1
10
rCFH (μM)

FIG. 1A

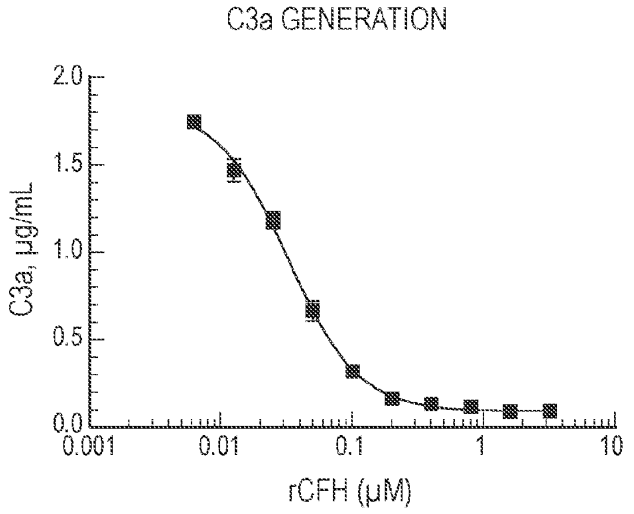

C3a GENERATION
C3a, μg/mL
2.0
1.5
1.0
0.5
0.0
0.001
0.01
0.1
1
10
rCFH (μM)

FIG. 1B

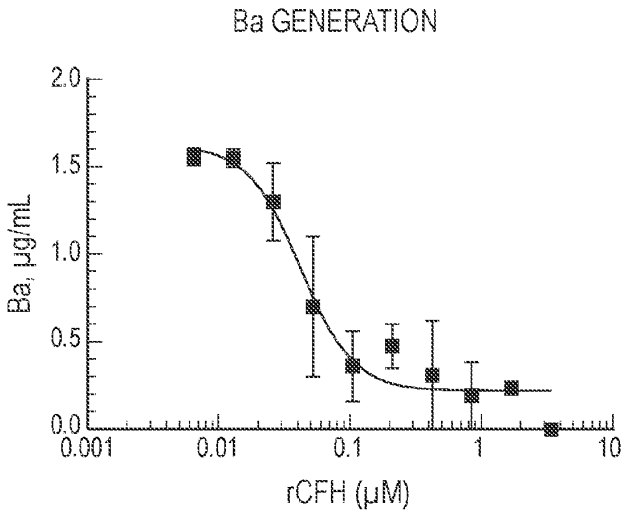


FIG. 1C

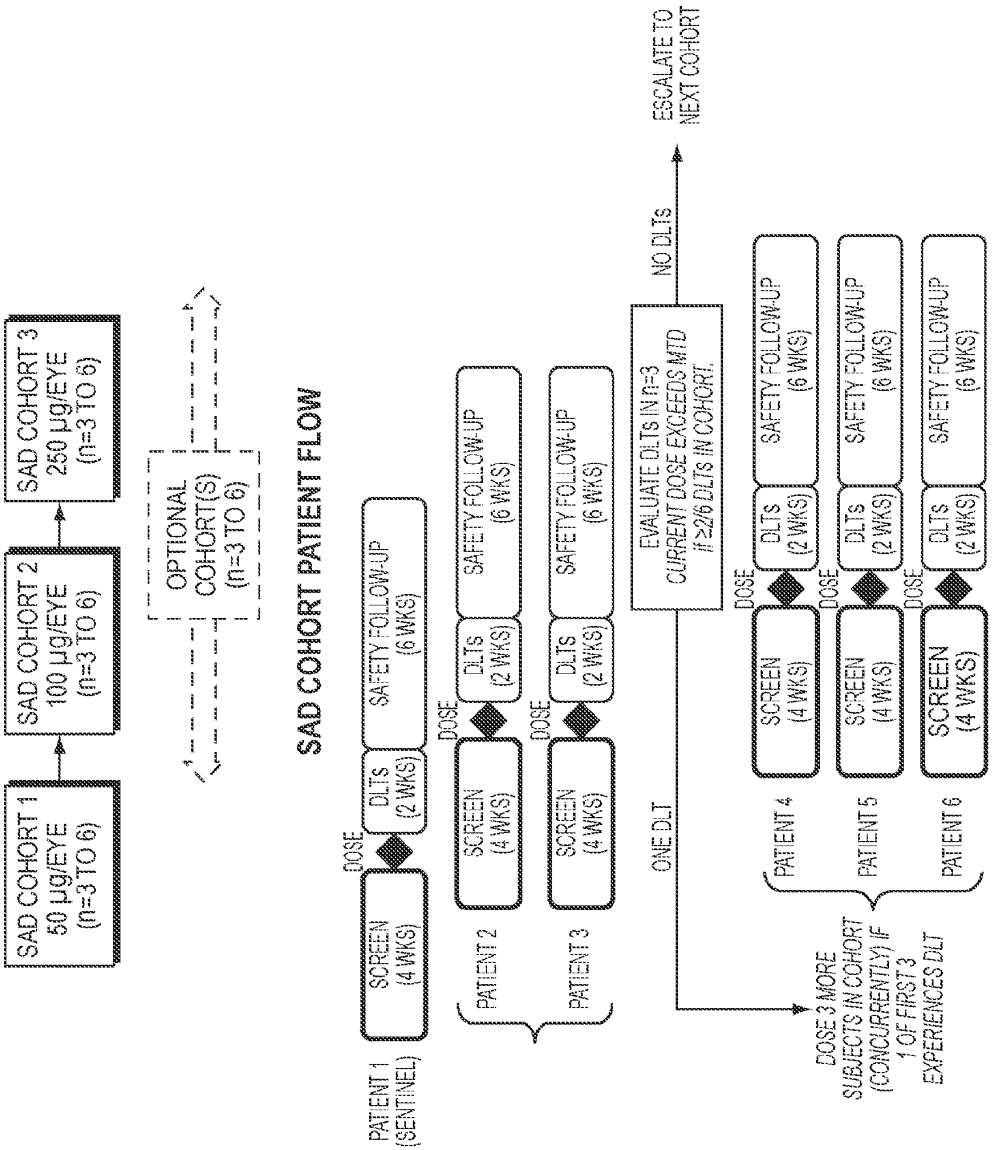

SAD COHORT 1
50 µg/EYE
(n=3 TO 6)
SAD COHORT 2
100 µg/EYE
(n=3 TO 6)
SAD COHORT 3
250 µg/EYE
(n=3 TO 6)
OPTIONAL
COHORT(S)
(n=3 TO 6)
SAD COHORT PATIENT FLOW
PATIENT 1
(SENTINEL)
DOSE
SCREEN
(4 WKS)
DLTs
(2 WKS)
SAFETY FOLLOW-UP
(6 WKS)
PATIENT 2
DOSE
SCREEN
(4 WKS)
DLTs
(2 WKS)
SAFETY FOLLOW-UP
(6 WKS)
PATIENT 3
DOSE
SCREEN
(4 WKS)
DLTs
(2 WKS)
SAFETY FOLLOW-UP
(6 WKS)
EVALUATE DLTS IN n=3
CURRENT DOSE EXCEEDS MTD
IF ≥2/6 DLTs IN COHORT
NO DLTs
ESCALATE TO
NEXT COHORT
ONE DLT
DOSE 3 MORE
SUBJECTS IN COHORT
(CONCURRENTLY) IF
1 OF FIRST 3
EXPERIENCES DLT
PATIENT 4
DOSE
SCREEN
(4 WKS)
DLTs
(2 WKS)
SAFETY FOLLOW-UP
(6 WKS)
PATIENT 5
DOSE
SCREEN
(4 WKS)
DLTs
(2 WKS)
SAFETY FOLLOW-UP
(6 WKS)
PATIENT 6
DOSE
SCREEN
(4 WKS)
DLTs
(2 WKS)
SAFETY FOLLOW-UP
(6 WKS)

FIG. 2

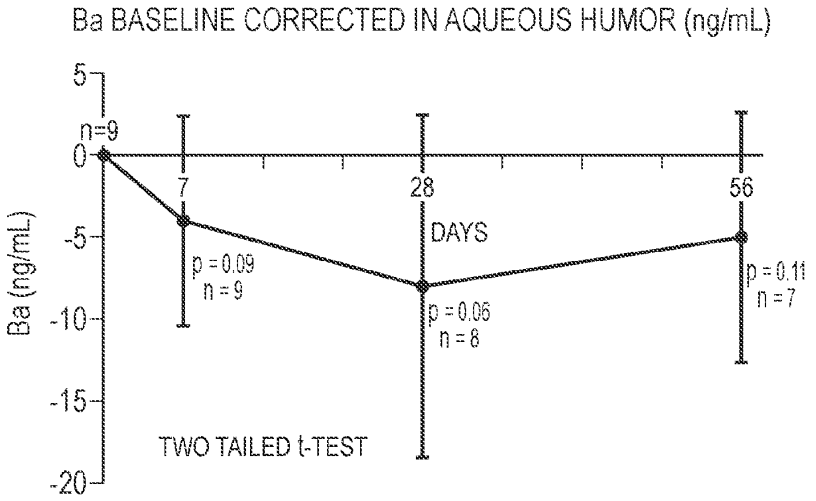

Ba BASELINE CORRECTED IN AQUEOUS HUMOR (ng/mL)
Ba (ng/mL)
5
0
-5
-10
-15
-20
n=9
7
28
56
DAYS
p = 0.09
n = 9
p = 0.06
n = 8
p = 0.11
n = 7
TWO TAILED t-TEST

FIG. 3A

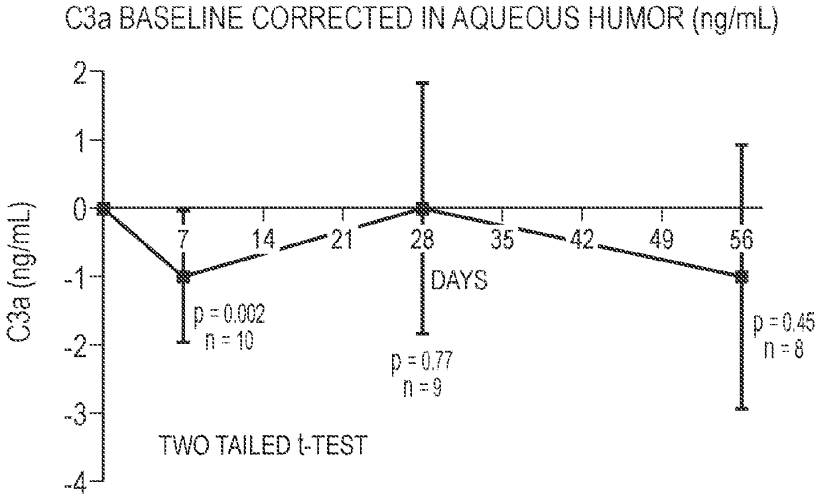

C3a BASELINE CORRECTED IN AQUEOUS HUMOR (ng/mL)
C3a (ng/mL)
2
1
0
-1
-2
-3
-4
7
14
21
28
35
42
49
56
DAYS
p = 0.002
n = 10
p = 0.77
n = 9
p = 0.45
n = 8
TWO TAILED t-TEST

FIG. 3B

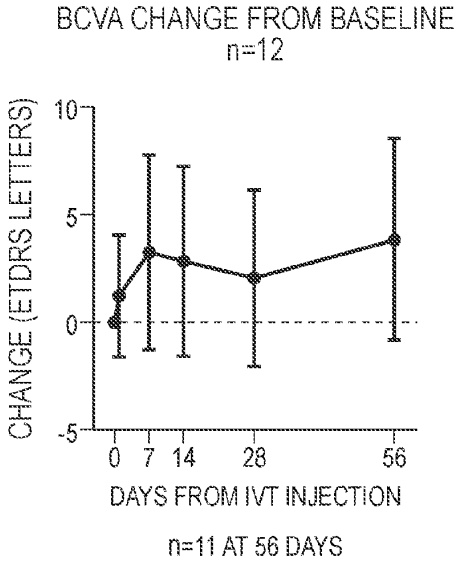

BCVA CHANGE FROM BASELINE
n=12
CHANGE (ETDRS LETTERS)
10
5
0
-5
0 7 14 28 56
DAYS FROM IVT INJECTION
n=11 AT 56 DAYS

FIG. 4A

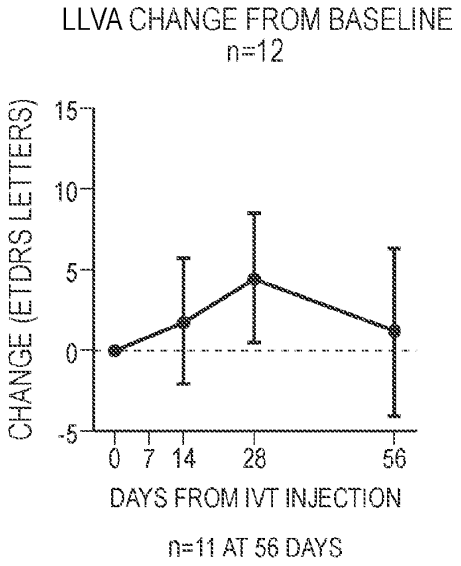

LLVA CHANGE FROM BASELINE
n=12
CHANGE (ETDRS LETTERS)
15
10
5
0
-5
0 7 14 28 56
DAYS FROM IVT INJECTION
n=11 AT 56 DAYS

FIG. 4B

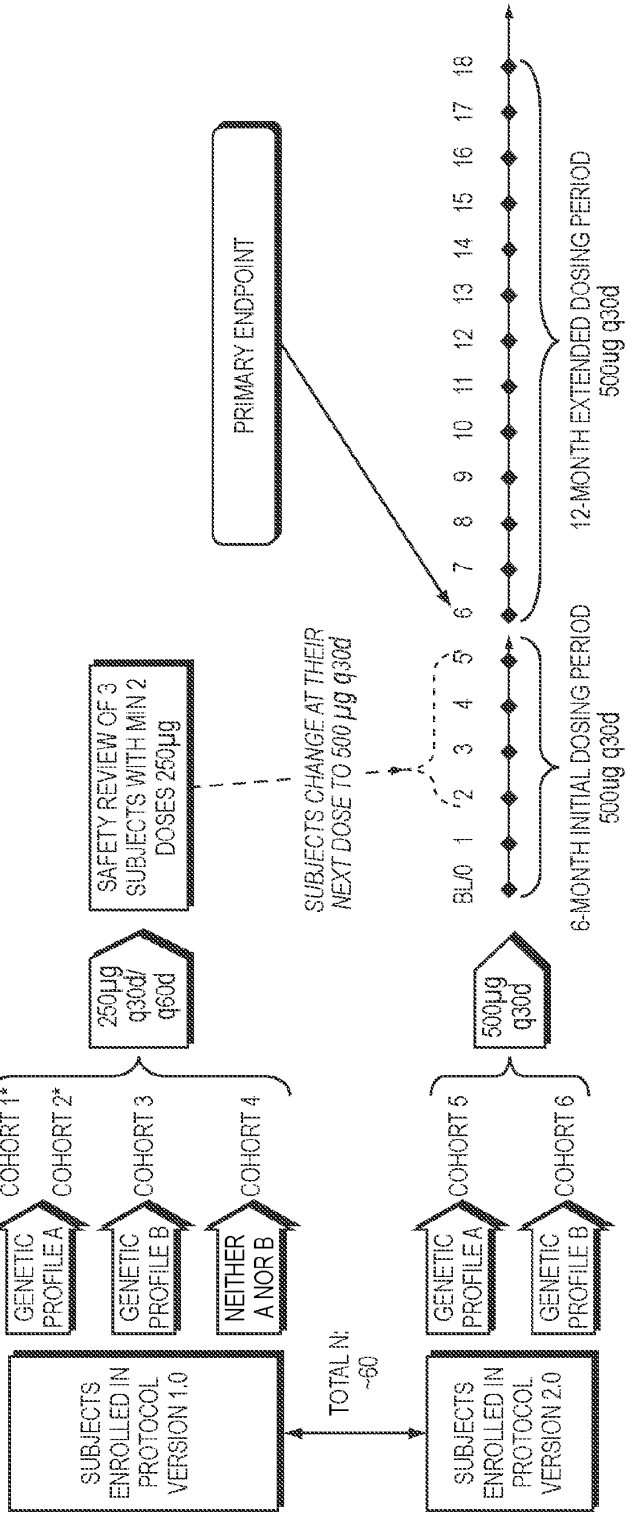

SUBJECTS ENROLLED IN PROTOCOL VERSION 1.0
TOTAL N: ~60
SUBJECTS ENROLLED IN PROTOCOL VERSION 2.0
GENETIC PROFILE A
COHORT 1*
COHORT 2*
GENETIC PROFILE B
COHORT 3
NEITHER A NOR B
COHORT 4
250µg q30d/ q60d
SAFETY REVIEW OF 3 SUBJECTS WITH MIN 2 DOSES 250µg
GENETIC PROFILE A
COHORT 5
GENETIC PROFILE B
COHORT 6
500µg q30d
SUBJECTS CHANGE AT THEIR NEXT DOSE TO 500 µg q30d
PRIMARY ENDPOINT
BL/0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
6-MONTH INITIAL DOSING PERIOD 500ug q30d
12-MONTH EXTENDED DOSING PERIOD 500ug q30d

FIG. 5

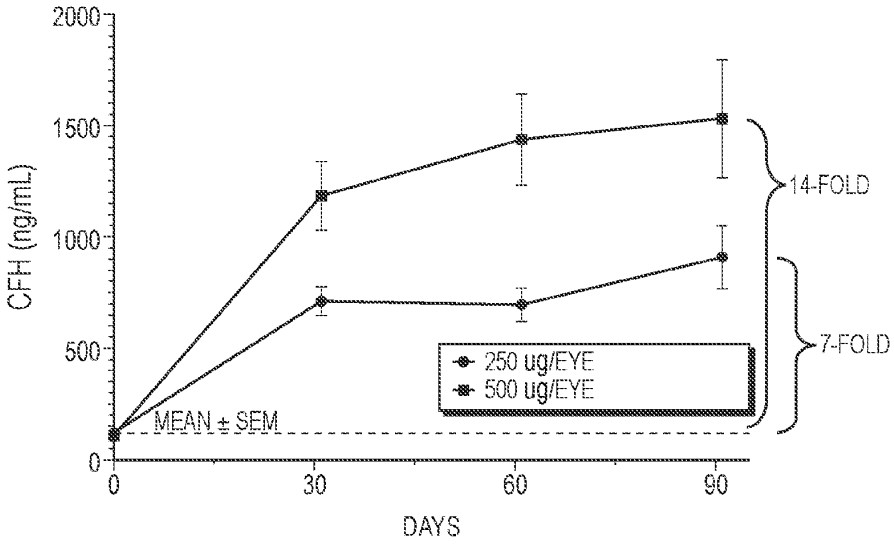

2000
1500
1000
500
0
CFH (ng/mL)
0
30
60
90
DAYS
MEAN ± SEM
250 ug/EYE
500 ug/EYE
14-FOLD
7-FOLD

FIG. 6

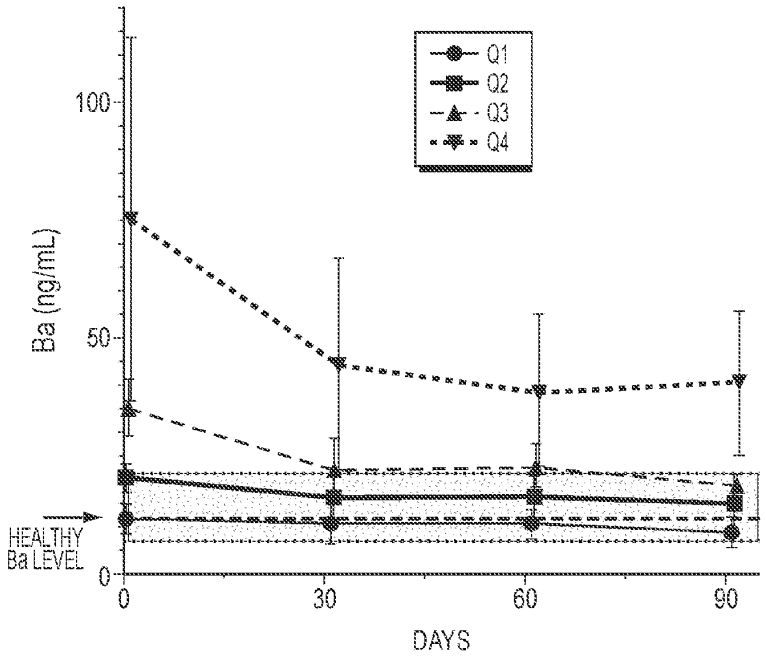


FIG. 7A

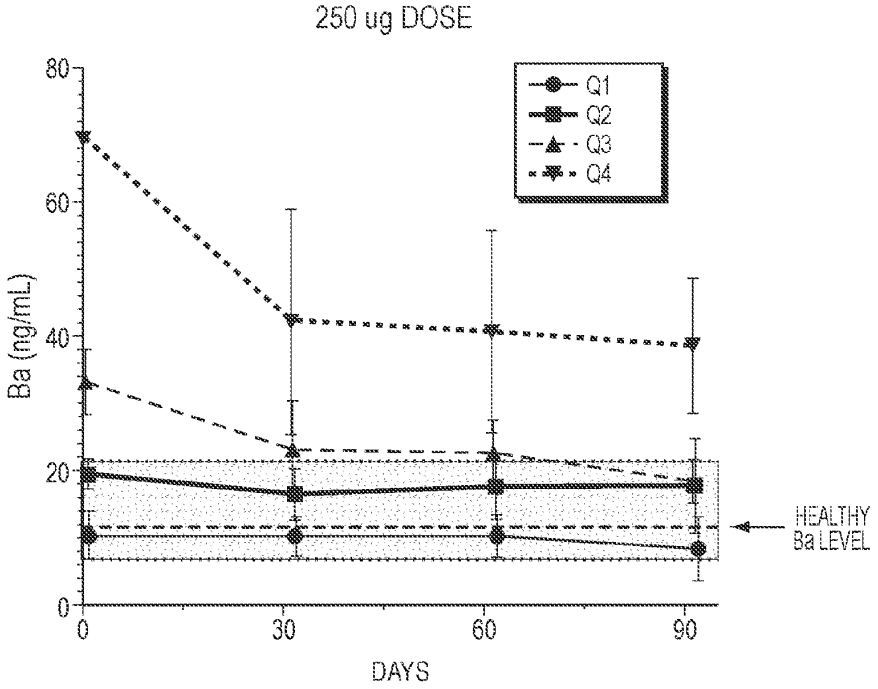

250 ug DOSE
Q1
Q2
Q3
Q4
Ba (ng/mL)
80
60
40
20
0
0
30
60
90
DAYS
HEALTHY
Ba LEVEL

FIG. 7B

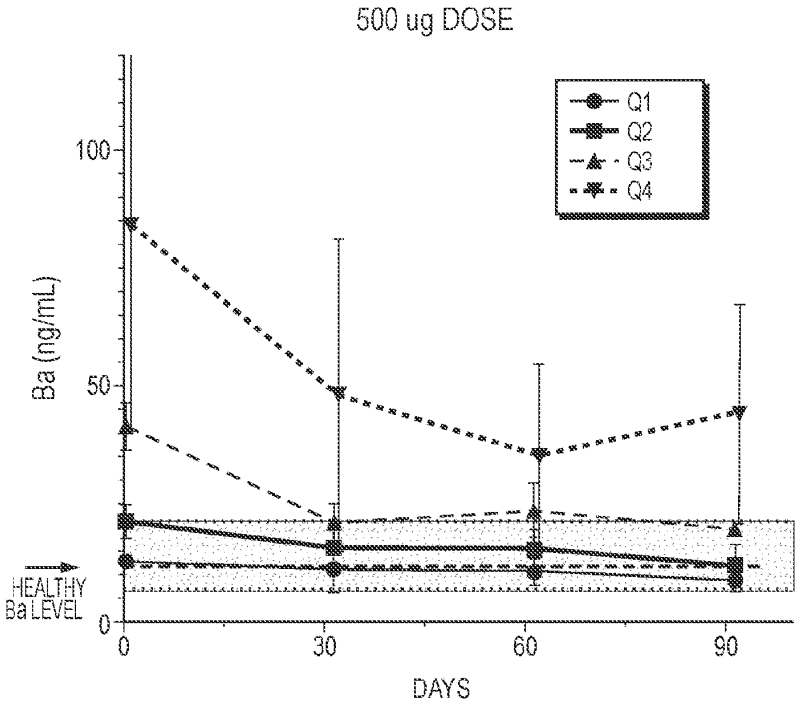

500 ug DOSE
Q1
Q2
Q3
Q4
Ba (ng/mL)
100
50
0
HEALTHY
Ba LEVEL
0
30
60
90
DAYS

FIG. 7C

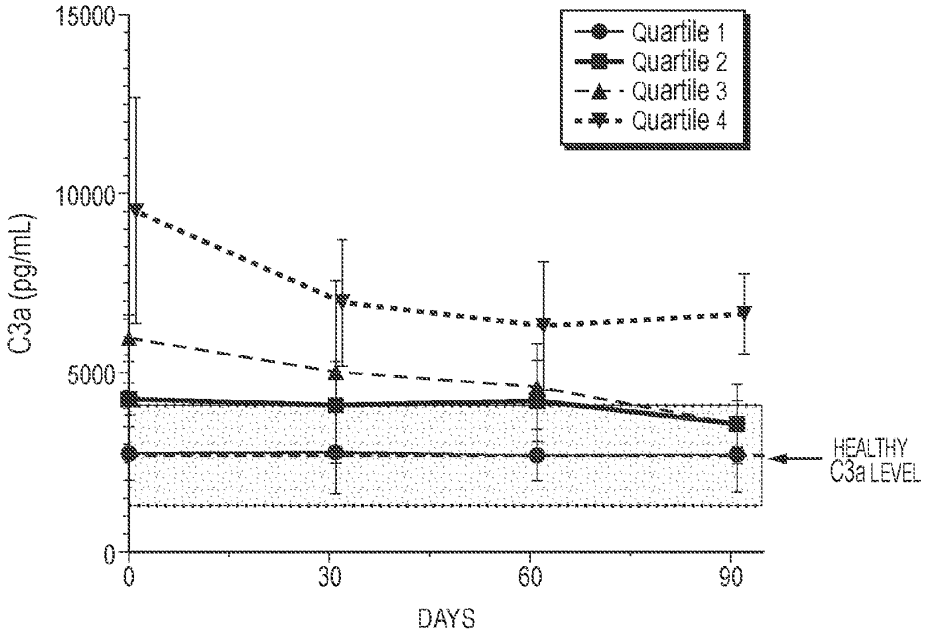

15000
10000
5000
0
C3a (pg/mL)
Quartile 1
Quartile 2
Quartile 3
Quartile 4
HEALTHY
C3a LEVEL
0
30
60
90
DAYS

FIG. 8

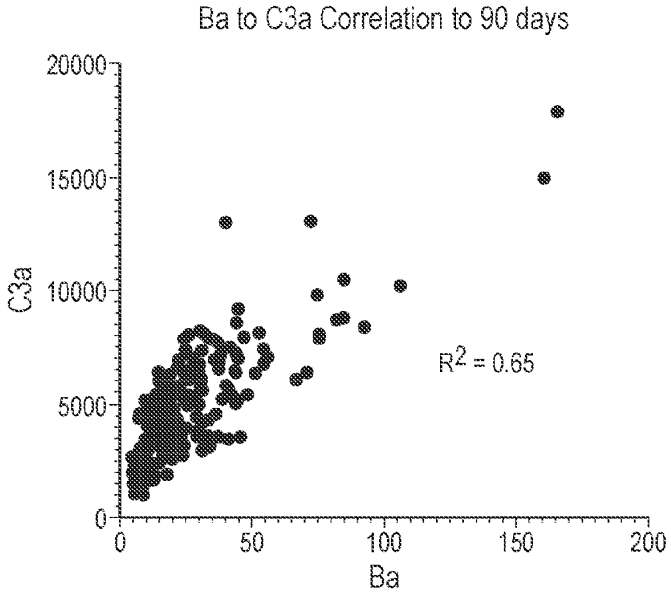

Ba to C3a Correlation to 90 days
20000
15000
10000
5000
0
C3a
R^2 = 0.65
0
50
100
150
200
Ba

FIG. 9

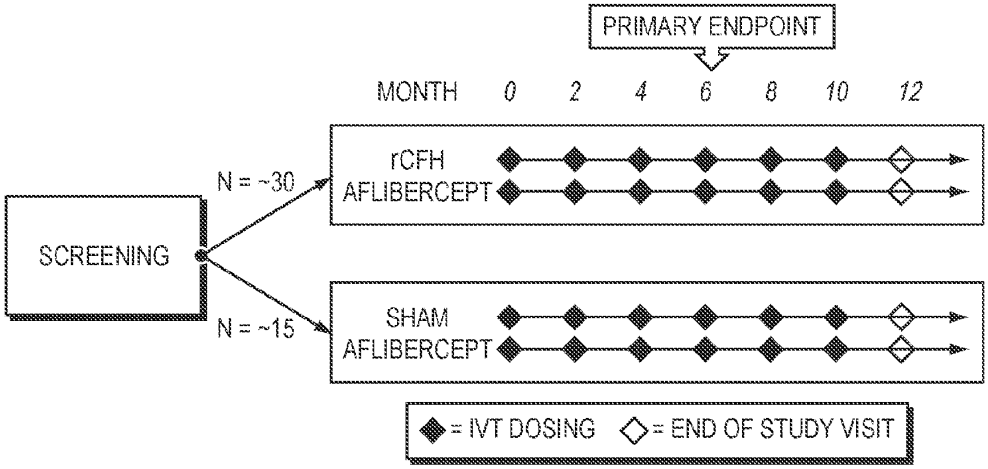

PRIMARY ENDPOINT
MONTH 0 2 4 6 8 10 12
rCFH
AFLIBERCEPT
N = ~30
SCREENING
N = ~15
SHAM
AFLIBERCEPT
= IVT DOSING
= END OF STUDY VISIT

FIG. 10

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(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, 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).

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))*

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(54) Title: PHARMACEUTICAL FORMULATION

(57) Abstract: Described herein are stable pharmaceutical formulations for bioactive agents, including macromolecular bioactive agents such as polynucleotides and polypeptides. Also provided are medicaments and treatments using the pharmaceutical formulations described herein, as well as kits and methods of treating, preventing or managing a disease or disorder using the pharmaceutical formulations described herein.

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PHARMACEUTICAL FORMULATION

RELATED APPLICATIONS

This application claims the benefit of priority under 35 U.S.C. 119(e) to U.S. Provisional Application No. 63/113,300 filed on November 13, 2020. The entire contents of the foregoing application are hereby incorporated by reference.

FIELD OF THE INVENTION

The present disclosure relates to the field of pharmaceutical formulations. Specifically, a stable pharmaceutical formulation for biotherapeutics is provided.

BACKGROUND

Biotherapeutics, which include macromolecule therapeutics, such as peptides, proteins, antibodies, polysaccharides, and nucleic acids; as well as cell-based therapeutics are a rapidly growing portion the therapeutics available to medical practitioners for treatment of a wide range of diseases and disorders. One reason for the success of biotherapeutics is the high specificity towards targets and superior safety profile as compared to small molecule therapeutics.

The structural complexity of biotherapeutics make them susceptible to physical or chemical degradation, which can result in structural and functional instability and reduced safety and efficacy. Although manufacturing and purification processes typically result in a high purity product, many biotherapeutics degrade over time, for example, during storage, transport and administration. Degradation can be due to chemical instability, which includes, but is not limited to, deamidation, isomerization, hydrolysis, racemization, beta-elimination, glycation, oxidation, adduct formation and disulfide scrambling; or physical instability, which includes, but is not limited to, denaturation, aggregation, precipitation, particle formation, and surface adsorption.

The composition of the pharmaceutical formulation for a biotherapeutic can significantly affect stability and, consequently, the safety and efficacy of the therapeutic. The pharmaceutical formulation also can affect the ease and frequency of administration and pain upon injection.

Surfactants are often included in pharmaceutical formulations to help stabilize the biotherapeutic and prevent aggregation or particle formation. Non-ionic surfactants such as

polysorbate 80, polysorbate 20 and poloxamer 188 are commonly used in pharmaceutical formulations due to their proven safety profile.

However, polysorbate 80 and polysorbate 20 are known to be susceptible to degradation during storage by oxidative and hydrolytic pathways. Free fatty acids (FFA), one degradation product, can be sources of particle formation. Degradation can occur during storage of the polysorbate raw material, which can result in particle formation when the polysorbate surfactant is introduced into a pharmaceutical formulation. Degradation of the polysorbate surfactant in the pharmaceutical formulation can also result in aggregation. Additionally, degradation of polysorbates over time can result in an effective reduction in the amount of polysorbate in the formulations, adversely impacting the protective effect of the surfactant.

Despite advances made in the use of biotherapeutics and the knowledge relating to instability processes, there remains a need for pharmaceutical formulations with enhanced long-term stability. A formulation with improved long-term stability could help increase safety and efficacy of biotherapeutics. Thus, there remains a need for formulations that retain long-term stability under a variety of different manufacturing and storage conditions. The present invention satisfies this need and provides additional advantages as well.

SUMMARY

A stable pharmaceutical formulation is provided herein that includes a bioactive agent and D-α-Tocopheryl polyethylene glycol succinate (TPGS) as a surfactant. In one aspect, the stable pharmaceutical formulation includes a bioactive agent and D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS 1000) as a surfactant. In one aspect, the formulation includes a buffer and has a pH from about 3 to about 9, about 4 to about 8, or about 5.5 to about 7.5.

In one aspect, the formulation is a liquid formulation. In one aspect, the formulation is an aqueous formulation. In one aspect, the formulation is lyophilized.

In one aspect, the bioactive agent is a biotherapeutic. In one aspect, the bioactive agent is a therapeutic macromolecule.

In one aspect, the bioactive agent includes a therapeutic polypeptide. In one aspect, the therapeutic polypeptide is selected from an antibody or antigen-binding antibody fragment, an enzyme or enzymatically-active polypeptide, a soluble receptor or receptor ligand, hormone, neurotransmitter, growth factor, integrin, interferon or an antigen. In one aspect, the antibody or

antigen-binding antibody fragment includes a monoclonal antibody. In one aspect, the antibody or antigen-binding antibody fragment is selected from human, humanized, chimeric, multispecific, bispecific, Fab', F(ab')2, Fv, single chain Fv (scFv), diabodies, peptibodies, linear antibodies and single-chain antibodies. In one aspect, the therapeutic polypeptide includes a fusion polypeptide, a proteolysis targeting chimera (protac), or an antibody-drug conjugate (ADC).

In one aspect, the bioactive agent includes a therapeutic polynucleotide. In one aspect, the therapeutic polynucleotide includes single-stranded or double-stranded DNA or RNA. In one aspect, the therapeutic polynucleotide is selected from cDNA, antisense RNA, microRNA (miRNA), shorthairpin RNA (shRNA), RNA interference (RNAi), small interfering RNA (siRNA) and ribozymes. In one aspect, the therapeutic polynucleotide includes a plasmid, phagemid, cosmid or yeast artificial chromosomes (YAC). In one aspect, the therapeutic polynucleotide includes a viral vector, such as a retroviral, adenoviral, poxviral, adeno-associated viral (AAV), Newcastle disease viral (NDV) or herpes simplex viral vector.

In one aspect, the bioactive agent includes cells selected from stem cells, progenitor cells and differentiated cells.

In one aspect, the formulation includes the bioactive agent at a concentration from about 0.1 mg/mL to about 300 mg/mL. In one aspect, the bioactive agent includes an antibody or antigen-binding antibody fragment with a concentration from about 10 mg/mL to about 200 mg/mL. In one aspect, the concentration of antibody or antigen-binding antibody fragment is at least about 10 mg/mL, about 15 mg/mL, about 20 mg/mL, about 25 mg/mL, about 50 mg/mL, about 75 mg/mL or about 100 mg/mL and up to about 125 mg/mL, about 150 mg/mL, about 175 mg/mL, about 200 mg/mL, about 250 mg/mL or about 300 mg/mL. In one aspect, the bioactive agent includes a therapeutic protein with a concentration from about 0.1 mg/mL, about 0.5 mg/mL, about 1 mg/mL, about 2 mg/mL, about 3 mg/mL, about 4 mg/mL or about 5 mg/mL and up to about 10 mg/mL, about 15 mg/mL, about 20 mg/mL, about 25 mg/mL, about 30 mg/mL, about 35 mg/mL, about 35 mg/mL, about 40 mg/mL, about 45 mg/mL or about 50 mg/mL.

In one aspect, the concentration of TPGS in the pharmaceutical formulation is from about 0.001% to about 1%. In one aspect, the concentration of TPGS is from about 0.001%, about 0.005%, about 0.01%, about 0.05% and up to about 0.1%, about 0.5% or about 1%. In one aspect, the concentration of TPGS is 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% or about 0.1%. In one aspect, the concentration of TPGS is about 0.01%. In one aspect, the concentration of TPGS is about 0.02%. In one aspect, the concentration of TPGS is about 0.03%. In one aspect, the concentration of TPGS is about 0.04%. In one aspect, the concentration of TPGS is about 0.05%. In one aspect, the concentration of TPGS is about 0.06%. In one aspect, the concentration of TPGS is about 0.07%. In one aspect, the concentration of TPGS is about 0.08%. In one aspect, the concentration of TPGS is about 0.09%. In one aspect, the concentration of TPGS is about 0.1%.

In one aspect, the pharmaceutical formulation includes a buffer. In one aspect, the buffer is selected from acetate, acetic acid, succinate, succinic acid, phosphate, phosphoric acid, ascorbate, ascorbic acid, lactate, lactic acid, tartartic acid, maleic acid, glycine, gluconate, citrate, histidine, imidazole, bicarbonate and carbonic acid, sodium benzoate, benzoic acid, edetate, malate, tris, glycylglycine and mixtures thereof. In one aspect, the buffer includes histidine/histidine hydrochloride (HCl). In one aspect, the buffer includes tris/tris hydrochloride (Tris HCl). In one aspect, the buffer includes citrate. In one aspect, the buffer includes sodium acetate. In one aspect, the buffer includes phosphate. In one aspect, the buffer has a concentration from about 0.1 mM to about 100 mM. In one aspect, the buffer concentration is from about 0.1 mM, about 0.5 mM, about 1 mM, about 5 mM, about 10 mM, about 20 mM or about 25 mM and up to about 30 mM, about 35 mM, about 40 mM, about 45 mM, or about 50 mM. In one aspect, the buffer includes about 5 mM histidine/histidine HCl. In one aspect, the buffer includes about 10 mM histidine/histidine HCl. In one aspect, the buffer includes about 15 mM histidine/histidine HCl. In one aspect, the buffer includes about 20 mM histidine/histidine HCl. In one aspect, the buffer includes about 25 mM histidine/histidine HCl. In one aspect, the buffer includes about 30 mM histidine/histidine HCl.

In one aspect, the formulation includes a tonicity agent. In one aspect, the tonicity agent includes a polyol, a saccharide, a carbohydrate, a salt, or a mixture thereof. In one aspect, the formulation includes the tonicity reagent at a concentration from about 1 mg/ml to about 300 mg/ml, about 10 mg/ml to about 200 mg/ml or about 50 mg/ml to about 100 mg/ml. In one aspect, the tonicity reagent includes a saccharide at a concentration of about 80 mg/ml to about 90 mg/ml. In one aspect, the tonicity reagent includes a salt at a concentration from about 1 mg/mL to about 20 mg/ml.

In one aspect, the formulation includes an amino acid as an excipient. In one aspect, the amino acid is selected from arginine, cysteine, glycine, lysine, ornithine, proline, alanine, glutamine, glutamic acid, histidine, valine or a combination thereof. In one aspect, the formulation includes an amino acid salt as an excipient. In one aspect, the formulation includes an amino acid salt selected from a salt form of arginine, cysteine, glycine, lysine, ornithine, proline, alanine, glutamine, glutamic acid, histidine, valine or a combination thereof. In one aspect, the formulation includes a chelating agent. In one aspect, the chelating agent is selected from aminopolycarboxylic acids, hydroxyaminocarboxylic acids, N-substituted glycines, 2-(2-amino-2-oxocthyl) aminoethane sulfonic acid (BES), deferoxamine (DEF), citric acid, niacinamide, and desoxycholates and mixtures thereof. In one aspect, the chelating agent is selected from ethylenediaminetetraacetic acid (EDTA), diethylenetriamine pentaacetic acid (DTPA), nitrilotriacetic acid (NTA), N-2-acetamido-2-iminodiacetic acid (ADA), bis(aminoethyl)glycolether, N,N,N′,N′-tetraacetic acid (EGTA), trans-diaminocyclohexane tetraacetic acid (DCTA), glutamic acid, and aspartic acid, N-hydroxyethyliminodiacetic acid (HIMDA), N,N-bis-hydroxyethylglycine (bicine) and N-(trishydroxymethylmethyl) glycine (tricine), glycylglycine, sodium desoxycholate, ethylenediamine; propylenediamine; diethylenetriamine; triethylenetetraamine (trien), ethylenediaminetetraaceto EDTA; disodium EDTA, calcium EDTA oxalic acid, malate, citric acid, citric acid monohydrate, and trisodium citrate-dihydrate, 8-hydroxyquinolate, amino acids, histidine, cysteine, methionine, peptides, polypeptides, and proteins and mixtures thereof. In one aspect, the concentration of the chelating agent is from about 0.01 mg/ml to about 50 mg/ml.

In one aspect, the stable pharmaceutical formulation has less than about 10,000, about 5,000, about 1,000, about 750, about 500, about 250, about 150, about 100, or about 50 particles greater than about about 2 μm, about 5 μm, about 10 μm, about 15 μm, about 20 μm or about 25 μm diameter/mL.

In one aspect, the formulation is stable at a temperature of about 40°C for up to 3 months. In one aspect, the formulation is stable at a temperature of about 40°C for up to 6 months. In one aspect, the formulation is stable at a temperature of about 25°C for up to 6 months. In one aspect, the formulation is stable at a temperature of about 25°C for up to 12 months. In one aspect, the formulation is stable at a temperature of about 2°C to about 8°C for up to 12 months. In one aspect,

the formulation is stable at a temperature of about 2°C to about 8°C for up to 24 months. In one aspect, the formulation is stable at a temperature of about 2°C to about 8°C for up to 36 months. In one aspect, the formulation is stable at a temperature of about -20°C for up to 6 months. In one aspect, the formulation is stable at a temperature of about -20°C for up to 12 months. In one aspect, the formulation is stable at a temperature of about -20°C for up to 24 months. In one aspect, the formulation is stable at a temperature of about -20°C for up to 36 months. In one aspect, the formulation is stable at a temperature of about -80°C for up to 6 months. In one aspect, the formulation is stable at a temperature of about -80°C for up to 12 months. In one aspect, the formulation is stable at a temperature of about -80°C for up to 24 months. In one aspect, the formulation is stable at a temperature of about -80°C for up to 36 months.

In one aspect, a method of reducing particle formation in an aqueous pharmaceutical formulation is provided. In one aspect, the method includes adding D-α-Tocopheryl polyethylene glycol succinate (TPGS) to the formulation. In one aspect, the method includes adding D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS 1000) to the formulation. In one aspect, the method includes adding D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS 1000) to the formulation in an amount ranging from about 0.001% to about 1%. In one aspect, less than about 10,000, about 5,000, about 1,000, about 750, about 500, about 250, about 150, about 100, or about 50 particles greater than about 2 μm, about 5 μm, about 10 μm, about 15 μm, about 20 μm or about 25 μm diameter/mL are formed. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL.

In one aspect, a method of reducing interference during antibody-drug (ADC) conjugation is provided. In one aspect, the method includes adding D-α-Tocopheryl polyethylene glycol succinate (TPGS) to a formulation comprising an unconjugated antibody intermediate. In one aspect, D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS 1000) is added to the formulation in an amount ranging from about 0.001% to about 1%.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic of the structure of D-α-Tocopheryl polyethylene glycol succinate (TPGS).

FIG. 2A is a graph showing the number of particles/ml with a diameter greater than or equal to 1 μm and less than 2 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, Kolliphor HS15, Kolliphor EL, Kolliphor RH40, PEG 300, PS80, P188, or no surfactant when assayed for stability after shipping simulation (SS) using micro-flow imaging (MFI).

FIG. 2B is a graph showing the number of particles/ml with a diameter greater than or equal to 2 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, Kolliphor HS15, Kolliphor EL, Kolliphor RH40, PEG 300, PS80, P188, or no surfactant when assayed for stability after shipping simulation (SS) using micro-flow imaging (MFI).

FIG. 2C is a graph showing the number of particles/ml with a diameter greater than or equal to 10 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, Kolliphor HS15, Kolliphor EL, Kolliphor RH40, PEG 300, PS80, P188, or no surfactant when assayed for stability after shipping simulation (SS) using micro-flow imaging (MFI).

FIG. 2D is a graph showing the number of particles/ml with a diameter greater than or equal to 25 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, Kolliphor HS15, Kolliphor EL, Kolliphor RH40, PEG 300, PS80, P188, or no surfactant when assayed for stability after shipping simulation (SS) using micro-flow imaging (MFI).

FIG. 3 is a graph showing the % monomer for four test molecules (mAb-1, mAb-2, mAb-4 and mAb-3) formulated with TPGS, Kolliphor HS15, Kolliphor EL, Kolliphor RH40, PEG 300, PS80, P188, or no surfactant and assayed for stability after shipping simulation (SS) using high pressure size exclusion chromatography (HPSEC).

FIG. 4A is a graph showing the number of particles/ml with a diameter greater than or equal to 1 μm and less than 2 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, PS80, and P188 and stored at 40°C for 4 weeks as determined by micro-flow imaging (MFI).

FIG. 4B is a graph showing the number of particles/ml with a diameter greater than or equal to 2 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, PS80, and P188 and stored at 40°C for 4 weeks as determined by micro-flow imaging (MFI).

FIG. 4C is a graph showing the number of particles/ml with a diameter greater than or equal to 10 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, PS80, and P188 and stored at 40°C for 4 weeks as determined by micro-flow imaging (MFI).

FIG. 4D is a graph showing the number of particles/ml with a diameter greater than or equal to 25 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, PS80, and P188 and stored at 40°C for 4 weeks as determined by micro-flow imaging (MFI).

FIG. 5A is a graph showing the % monomer for mAb-1 formulated with TPGS, PS80, and P188 and stored at 40°C for 0-4 weeks. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 5B is a graph showing the % monomer for mAb-2 formulated with TPGS, PS80, and P188 and stored at 40°C for 0-4 weeks. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 5C is a graph showing the % monomer for mAb-3 formulated with TPGS, PS80, and P188 and stored at 40°C for 0-4 weeks. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 5D is a graph showing the % monomer for mAb-4 formulated with TPGS, PS80, and P188 and stored at 40°C for 0-4 weeks. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 6A is a graph showing the number of particles/ml with a diameter greater than or equal to 1 μm and less than 2 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, PS80, and P188 and stored at 25°C for 6 months as determined by micro-flow imaging (MFI).

FIG. 6B is a graph showing the number of particles/ml with a diameter greater than or equal to 2 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, PS80, and P188 and stored at 25°C for 6 months as determined by micro-flow imaging (MFI).

FIG. 6C is a graph showing the number of particles/ml s with a diameter greater than or equal to 10 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, PS80, and P188 and stored at 25°C for 6 months as determined by micro-flow imaging (MFI).

FIG. 6D is a graph showing the number of particles/ml with a diameter greater than or equal to 25 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, PS80, and P188 and stored at 25°C for 6 months as determined by micro-flow imaging (MFI).

FIG. 7A is a graph showing the % monomer for mAb-1 formulated with TPGS, PS80, and P188 and stored at 25°C for 0-6 months. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 7B is a graph showing the % monomer for mAb-2 formulated with TPGS, PS80, and P188 and stored at 25°C for 0-6 months. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 7C is a graph showing the % monomer for mAb-3 formulated with TPGS, PS80, and P188 and stored at 25°C for 0-6 months. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 7D is a graph showing the % monomer for mAb-4 formulated with TPGS, PS80, and P188 and stored at 25°C for 0-6 months. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 8A is a graph showing the number of particles/ml with a diameter greater than or equal to 1 μm and less than 2 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, PS80, and P188 and stored at 5°C for 6 months as determined by micro-flow imaging (MFI).

FIG. 8B is a graph showing the number of particles/ml with a diameter greater than or equal to 2 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, PS80, and P188 and stored at 5°C for 6 months as determined by micro-flow imaging (MFI).

FIG. 8C is a graph showing the number of particles/ml s with a diameter greater than or equal to 10 μm for four test molecules (mAb-1 mAb-2, mAb-3 and mAb-4) formulated with TPGS, PS80, and P188 and stored at 5°C for 6 months as determined by micro-flow imaging (MFI).

FIG. 8D is a graph showing the number of particles/ml with a diameter greater than or equal to 25 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, PS80, and P188 and stored at 5°C for 6 months as determined by micro-flow imaging (MFI).

FIG. 9A is a graph showing the % monomer for mAb-1 formulated with TPGS, PS80, and P188 and stored at 5°C for 0-6 months. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 9B is a graph showing the % monomer for mAb-2 formulated with TPGS, PS80, and P188 and stored at 5°C for 0-6 months. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 9C is a graph showing the % monomer for mAb-3 formulated with TPGS, PS80, and P188 and stored at 5°C for 0-6 months. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 9D is a graph showing the % monomer for mAb-4 formulated with TPGS, PS80, and P188 and stored at 5°C for 0-6 months. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 10A is a graph showing the number of particles/ml with a diameter greater than or equal to 1 μm and less than 2 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, PS80, and P188 and stored at -80°C for 6 months as determined by micro-flow imaging (MFI).

FIG. 10B is a graph showing the number of particles/ml with a diameter greater than or equal to 2 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, PS80, and P188 and stored at -80°C for 6 months as determined by micro-flow imaging (MFI).

FIG. 10C is a graph showing the number of particles/ml with a diameter greater than or equal to 10 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with TPGS, PS80, and P188 and stored at -80°C for 6 months as determined by micro-flow imaging (MFI).

FIG. 10D is a graph showing the number of particles/ml with a diameter greater than or equal to 25 μm for four test molecules (mAb-1, mAb-2, mAb-3 and mAb-4) formulated with

TPGS, PS80, and P188 and stored at -80°C for 6 months as determined by micro-flow imaging (MFI).

FIG. 11A is a graph showing the % monomer for mAb-1 formulated with TPGS, PS80, and P188 and stored at -80°C for 0-6 months. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 11B is a graph showing the % monomer for mAb-2 formulated with TPGS, PS80, and P188 and stored at -80°C for 0-6 months. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 11C is a graph showing the % monomer for mAb-3 formulated with TPGS, PS80, and P188 and stored at -80°C for 0-6 months. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 11D is a graph showing the % monomer for mAb-4 formulated with TPGS, PS80, and P188 and stored at -80°C for 0-6 months. Stability was evaluated by determining % monomer using high pressure size exclusion chromatography (HPSEC).

FIG. 12A is a graph showing the number of particles/ml with a diameter greater than or equal to 1 μm and less than 2 μm for mAb-2 formulated with varying concentrations of TPGS, PS80, PS20 or P188 and subjected to a shipping simulation (SS). Stability was assessed via micro-flow imaging (MFI).

FIG. 12B is a graph showing the number of particles/ml with a diameter greater than or equal to 2 μm for mAb-2 formulated with varying concentrations of TPGS, PS80, PS20 or P188 and subjected to a shipping simulation (SS). Stability was assessed via micro-flow imaging (MFI).

FIG. 12C is a graph showing the number of particles/ml with a diameter greater than or equal to 10 μm for mAb-2 formulated with varying concentrations of TPGS, PS80, PS20 or P188 and subjected to a shipping simulation (SS). Stability was assessed via micro-flow imaging (MFI).

FIG. 12D is a graph showing the number of particles/ml with a diameter greater than or equal to 25 μm for mAb-2 formulated with varying concentrations of TPGS, PS80, PS20 or P188 and subjected to a shipping simulation (SS). Stability was assessed via micro-flow imaging (MFI).

FIG. 13 is a table showing stability of mAb-2 formulated with varying concentrations of TPGS, PS80, PS20 or P188 and subjected to a shipping simulation (SS). Stability was assessed via high-pressure size exclusion chromatography (HPSEC) and evaluated for % monomer (%Mon),

% high molecular weight species (%HMW), and % fragmentation (%Frag). *The apparent increase in %HMW observed for the sample containing 1.00% TPGS was due to interreference of TPGS with the HPSEC column, rather than aggregation of the molecule.

FIG. 14 shows overlaid HPSEC chromatograms of blank buffer containing different levels of TPGS. At 1.0% TPGS concentration, the area of the peak increases which results in interference with the mAb chromatograms. At the lower concentrations, the interreference of TPGS is negligible. This behaviour is found with other surfactants such as PS80.

FIG. 15 shows overlaid HPSEC chromatograms of blank buffer containing 1.0% TPGS with mAb-2 samples containing 0.2% or 1.0% TPGS. The TPGS eluted at a similar retention time as the aggregate peak of mAb-2 demonstrating that inclusion of 1.0% TPGS can result in an overestimation of aggregate level.

FIG. 16 is a table showing capillary gel electrophoresis (CGE) results for formulations of mAb-2 containing PS80, PS20, Poloxamer 188 and different concentrations of TPGS. CGE is not subject to interference from surfactants and the samples containing 1.00% TPGS had comparable results with other sample conditions.

FIG. 17A is a graph showing the number of particles/ml with a diameter greater than or equal to 1 μm and less than 2 μm for mAb-4 formulated with varying concentrations of TPGS, PS80, PS20 or P188 and subjected to a shipping simulation (SS). Stability was assessed via micro-flow imaging (MFI).

FIG. 17B is a graph showing the number of particles/ml with a diameter greater than or equal to 2 μm for mAb-4 formulated with varying concentrations of TPGS, PS80, PS20 or P188 and subjected to a shipping simulation (SS). Stability was assessed via micro-flow imaging (MFI).

FIG. 17C is a graph showing the number of particles/ml with a diameter greater than or equal to 10 μm for mAb-4 formulated with varying concentrations of TPGS, PS80, PS20 or P188 and subjected to a shipping simulation (SS). Stability was assessed via micro-flow imaging (MFI).

FIG. 17D is a graph showing the number of particles/ml with a diameter greater than or equal to 25 μm for mAb-4 formulated with varying concentrations of TPGS, PS80, PS20 or P188 and subjected to a shipping simulation (SS). Stability was assessed via micro-flow imaging (MFI).

FIG. 18 is a table showing stability of mAb-4 formulated with varying concentrations of TPGS, PS80, PS20 or P188 and subjected to a shipping simulation (SS). Stability was assessed via

high-pressure size exclusion chromatography (HPSEC) and evaluated for % monomer (%Mon), % high molecular weight species (%HMW), and % fragmentation (%Frag). *The apparent increase in %HMW observed for the sample containing 1.00% TPGS was due to interreference of TPGS with the HPSEC column, rather than aggregation of the molecule.

FIG. 19 is a table showing capillary gel electrophoresis (CGE) results for formulations of mAb-4 containing PS80, PS20, Poloxamer 188 and different concentrations of TPGS. CGE is not subject to interference from surfactants and the samples containing 1.00% TPGS had comparable results with other sample conditions.

FIG. 20A is a table showing stability of mAb-5 formulated with TPGS or control. Stability was assessed at time zero (T0), after 3 freeze-thaw cycles (3-FT) and post-shipping simulation (SS) via micro-flow imaging (MFI).

FIG. 20B is a table showing stability of mAb-6 formulated with TPGS or control. Stability was assessed at time zero (T0), after 3 freeze-thaw cycles (3-FT) and post-shipping simulation (SS) via micro-flow imaging (MFI).

FIG. 21A is a graph showing the number of particles/ml with a diameter greater than or equal to 1 μm and less than 2 μm for mAb-5 formulated with TPGS or control and subjected to a shipping simulation (SS). Stability was assessed at time zero (T0), after 3 freeze-thaw cycles (3-FT), via micro-flow imaging (MFI).

FIG. 21B is a graph showing the number of particles/ml with a diameter greater than or equal to 2 μm for mAb-5 formulated with TPGS or control and subjected to a shipping simulation (SS). Stability was assessed at time zero (T0), after 3 freeze-thaw cycles (3-FT), via micro-flow imaging (MFI).

FIG. 21C is a graph showing the number of particles/ml with a diameter greater than or equal to 10 μm for mAb-5 formulated with TPGS or control and subjected to a shipping simulation (SS). Stability was assessed at time zero (T0), after 3 freeze-thaw cycles (3-FT), via micro-flow imaging (MFI).

FIG. 21D is a graph showing the number of particles/ml with a diameter greater than or equal to 25 μm for mAb-5 formulated with TPGS or control and subjected to a shipping simulation (SS). Stability was assessed at time zero (T0), after 3 freeze-thaw cycles (3-FT), via micro-flow imaging (MFI).

FIG. 22A is a graph showing the number of particles/ml with a diameter greater than or equal to 1 μm and less than 2 μm for mAb-6 formulated with TPGS or control and subjected to a shipping simulation (SS). Stability was assessed at time zero (T0), after 3 freeze-thaw cycles (3-FT), via micro-flow imaging (MFI).

FIG. 22B is a graph showing the number of particles/ml with a diameter greater than or equal to 2 μm for mAb-6 formulated with TPGS or control and subjected to a shipping simulation (SS). Stability was assessed at time zero (T0), after 3 freeze-thaw cycles (3-FT), via micro-flow imaging (MFI).

FIG. 22C is a graph showing the number of particles/ml with a diameter greater than or equal to 10 μm for mAb-6 formulated with TPGS or control and subjected to a shipping simulation (SS). Stability was assessed at time zero (T0), after 3 freeze-thaw cycles (3-FT), via micro-flow imaging (MFI).

FIG. 22D is a graph showing the number of particles/ml with a diameter greater than or equal to 25 μm for mAb-6 formulated with TPGS or control and subjected to a shipping simulation (SS). Stability was assessed at time zero (T0), after 3 freeze-thaw cycles (3-FT), via micro-flow imaging (MFI).

FIG. 23 is a table showing stability of mAb-5 and mAb-6 formulated with TPGS or control and subjected to a shipping simulation (SS). Stability was assessed stability at time zero (T0), after 3 freeze-thaw cycles (3-FT), and post-shipping simulation (SS) via high-pressure size exclusion chromatography (HPSEC).

DETAILED DESCRIPTION

Definitions

Generally, nomenclatures used in connection with, and techniques of, cell and tissue culture, molecular biology, and protein and oligo- or polynucleotide chemistry and hybridization described herein are those well-known and commonly used in the art. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

As used herein, "a" or "an" may mean one or more. As used herein in the specification and claims, when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one. As used herein, "another" or "a further" may mean at least a second or more.

Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the method/device being employed to determine the value, or the variation that exists among the study subjects. Typically, the term is meant to encompass approximately or less than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% or 20% variability, depending on the situation.

The use of the term "or" in the disclosure and claims is used to mean "and/or" unless explicitly indicated to refer only to alternatives or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or."

As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited, elements or method steps. It is contemplated that any embodiment discussed in this specification can be implemented with respect to any method, system, host cells, expression vectors, or composition of the present disclosure.

The use of the term "for example" and its corresponding abbreviation "e.g." (whether italicized or not) means that the specific terms recited are representative examples and embodiments of the disclosure that are not intended to be limited to the specific examples referenced or cited unless explicitly stated otherwise.

The term "pharmaceutical formulation" refers to a composition that includes a bioactive agent and a pharmaceutically acceptable carrier, excipient, diluent or other pharmaceutically acceptable additive. The formulation can be suitable for diagnostic, therapeutic, or preventive use. The formulation can be suitable for use in *vitro*, *in vivo*, or *ex vivo*. In one aspect, the pharmaceutical formulation is administered parenterally, including, but not limited to, intravenously, intramuscularly or subcutaneously. Pharmaceutical formulations can include liquid formulations, for example, aqueous solutions, or lyophilized powders that can be

reconstituted into a suitable solution for administration, for example, by adding an appropriate diluent before administration.

A "stable" formulation refers to a formulation in which the bioactive agent retains its physical stability, chemical stability or biological activity during storage. "Chemical stability" can be assessed by detecting and quantifying chemically altered forms of the bioactive agent, including, for example, deamidation, including, for example, asparagine (Asn) deamidation; isomerization, including, for example, aspartate (Asp) isomerization; oxidation, including for example methionine (Met) oxidation; clipping/hydrolysis/fragmentation, including, for example, antibody hinge region fragmentation; succinimide formation; racemization; beta-elimination; glycation; adduct formation; disulfide scrambling; N-terminal extension; C-terminal processing; and glycosylation differences. "Physical stability" can be assessed by detecting and quantifying physically altered forms of the bioactive agent, including, but not limited to, physically altered forms due to denaturation, aggregation, precipitation or particle formation, and surface adsorption. The term “biological activity” refers to a function of a molecule and can encompass, biological function, biochemical function, physical function, and chemical function. Examples of biological activity include, but are not limited to, enzymatic activity; ability to interact with or bind to another molecule; ability to activate, promote, stabilize, inhibit, suppress, or destabilize a function of another molecule; and ability to localize to a specific position in a cell. As used herein, “biological function,” with regard to a polynucleotide, for example, a gene or polypeptide related thereto, can refer to a specific function that the polynucleotide, gene, or polypeptide can have in a living body. Examples include, but are not limited to, production of a specific protein, enzymatic activity, impartation of resistance, and the like. Biological activity can be measured using technique known in the art. "Improved stability" or "increased stability" refers to a pharmaceutical formulation in which the physical stability, chemical stability or biological activity of the bioactive agent is qualitatively or quantitatively evaluated and which is increased in comparison to the physical stability, chemical stability or biological activity of a reference composition of the bioactive agent.

Stability can be evaluated qualitatively or quantitatively in a variety of different ways, including, but not limited to, evaluation of aggregate formation (for example using size exclusion chromatography, by measuring turbidity, or by visual inspection); by assessing charge

heterogeneity using cation exchange chromatography, image capillary isoelectric focusing (icIEF) or capillary zone electrophoresis; amino-terminal or carboxy-terminal sequence analysis; mass spectrometric analysis; SDS-PAGE; peptide map analysis; or by evaluating biological activity, for example, via an *in vitro*, *in vivo* or *in situ* assay indicative of bioactive agent activity.

In one aspect, a bioactive agent is "stable" in a pharmaceutical formulation, if the physical stability, chemical stability or biological activity of the bioactive agent at a given time is within about 0.1%, about 0.5%, about 1%, about 5%, about 10%, about 15%, about 20% or about 25% (within a standard of error) of the physical stability, chemical stability or biological activity of the bioactive agent exhibited at an initial time point, for example, at the time the pharmaceutical formulation was prepared.

The term "bioactive agent" can refer to any substance that is suitable for therapeutic, prophylactic, or diagnostic use and can be used interchangeably with the term "therapeutic agent." A bioactive agent can include "biotherapeutic" products in which the active substance is obtained from a biological source. Examples of biotherapeutics include macromolecules, for example, therapeutic polynucleotides or polypeptides; and cell-based therapeutics. Examples of biotherapeutics include, but are not limited to recombinant proteins and hormones, monoclonal antibodies (mAbs), cytokines, growth factors, gene therapy products, vaccines, gene-silencing/editing therapies, cell-based therapeutics, tissue-engineered products, and stem cell therapies. Bioactive agents include, but are not limited to, naturally occurring or recombinantly produced cells or macromolecules, including polypeptides and polynucleotides; and cells, including, for example, stem cells, progenitor cells or differentiated cells. The term "polypeptide" and "protein" are used interchangeably to refer to a molecule having two or more amino acid residues joined to each other by peptide bonds and includes antibodies, antigen-binding antibody fragments, non-antibody binding proteins, therapeutic proteins and therapeutic peptides. In one aspect, the bioactive agent is of therapeutic, scientific or commercial interest.

A "therapeutically effective amount" of a bioactive agent refers to an amount effective in the prevention or treatment of a disease or disorder in a subject. A "therapeutically effective amount" can vary depending on factors such as disease state, age, sex, and weight of subject. In one aspect, a "therapeutically effective amount" refers to an amount of the bioactive agent sufficient to ameliorate at least one symptom associated with a disease or disorder. In one aspect,

a therapeutically effective amount encompasses an amount in which any toxic or detrimental effects of the bioactive agent are outweighed by the therapeutically beneficial effects. A "therapeutically effective amount" can be administered in one or more administrations.

"Subject" can refer to any subject in need of treatment or that is at risk for, or is predisposed to, developing a pathologic condition. The term "subject" includes mammals, such as humans, dogs, cows, horses, pigs, sheep, goats, cats, mice, rabbits, rats, and transgenic non-human animals. In one aspect, the subject is human.

"Pharmaceutically acceptable" means that a compound can safely be administered to a subject to provide an effective dose of the bioactive agent.

The term "recombinant" when used in reference to a polynucleotide, peptide, polypeptide, protein or cell means of, or resulting from, a new combination of material that is not known to exist in nature.

"Excipient" refers to a therapeutically inactive substance in a pharmaceutical formulation. Excipients can be included in a formulation for a wide variety of purposes, including, for example, as a diluent, vehicle, buffer, stabilizer, tonicity agent, bulking agent, surfactant, cryoprotectant, lyoprotectant, anti-oxidant, metal ion source, chelating agent or preservative.

"Amino acid salt" refers to a cationic or anionic form of an amino acid in combination with a counter ion with an opposite charge. In one aspect, the amino acid salt is a pharmacologically acceptable salt. In one aspect, the amino acid salt is an inorganic salt. In one aspect, the amino acid salt is an organic salt. In one aspect, the amino acid salt includes a sodium salt, potassium salt, calcium salt, magnesium salt, ammonium salt, hydrochloride salt, sulfate salt, nitrate salt, or phosphate salt. In one aspect, the amino acid salt includes an organic acid salt such as acetate, citrate, maleate, malate, or oxalate salt. In one aspect, the formulation includes an amino acid salt selected from a salt form of arginine, cysteine, glycine, lysine, ornithine, proline, alanine, glutamine, glutamic acid, histidine, valine or a combination thereof. In one aspect, the amino acid salt includes arginine, lysine or histidine.

"Surfactant" refers to a surface-active agent that lowers the surface tension of a liquid in which it is dissolved. Surfactants can be included in a pharmaceutical formulation for a variety of reasons including, for example, to prevent or control aggregation, particle formation or surface adsorption in liquid formulations or to prevent or control these phenomena during lyophilization

or reconstitution of lyophilized formulations. Surfactants include, for example, amphipathic organic compounds that exhibit partial solubility in both organic solvents and aqueous solutions. General characteristics of surfactants include their ability to reduce the surface tension of water, reduce the interfacial tension between oil and water and to form micelles. Surfactants can be anionic, non-ionic, cationic, amphoteric, zwitterionic, and combinations thereof. In one aspect, the surfactant includes vitamin E (alpha-tocopherol) polyethylene glycol succinate (TPGS). In one aspect, the surfactant includes D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS 1000).

As used herein, "buffer" refers to a composition that resists changes in pH due to the presence of acid-base conjugate components. Examples of buffers include, but are not limited to, acetate, acetic acid, succinate, succinic acid, phosphate, phosphoric acid, ascorbate, ascorbic acid, lactate, lactic acid, tartartic acid, maleic acid, glycine, gluconate, citrate, histidine, imidazole, bicarbonate and carbonic acid, sodium benzoate, benzoic acid, edetate, malate, tris, glycylglycine and mixtures thereof. In one aspect, the pharmaceutical formulation has a pH in the range from about 3.0 to about 9.0, from about 4.0 to about 8.0, or from about 5.5 to about 7.5, or at least about 3.0, about 3.5, about 4.0, about 4.5, about 5.0, about 5.5, about 6.0, about 6.5 or about 7.0 and up to about 7.0, about 7.5, about 8.0, about 8.5, or about 9.0. In one aspect, the pharmaceutical formulation has a pH of about 3.0, about 3.5, about 4.0, about 4.5, about 5.0, about 5.5, about 6.0, about 6.5, about 7.0, about 7.5, about 8.0, about 8.5, or about 9.0.

"Tonicity agent" or "tonicifier" refers to an excipient that can adjust the osmotic pressure of a pharmaceutical formulation. In one aspect, the tonicity agent is used to adjust the osmotic pressure of a pharmaceutical formulation to isotonic so that the formulation is physiologically compatible with cells and tissues of the subject. In one aspect, an "isotonic" formulation is one that has a similar osmotic pressure as human blood, generally from about 250 mOsm to about 350 mOsm. "Hypotonic" describes a formulation with an osmotic pressure below that of human blood and "hypertonic" describes a formulation with an osmotic pressure above that of human blood. Methods for determining isotonicity are known and include, for example, measuring a vapor pressure or use of an ice-freezing type osmometer. In one aspect, the "tonicity agent" improves the stability of a therapeutic macromolecule in a pharmaceutical formulation.

A "chelating agent" is a chemical compound capable of forming a complex with a metal ion. Generally, chelating agents include organic compounds that include a ring-like center

capable of forming bonds with the metal atom and are included in pharmaceutical formulations to convert metal ions in the formulation to a chemically inert form.

In one aspect, the pharmaceutical formulation includes an amino acid excipient. In one aspect, the pharmaceutical formulation includes an amino acid salt as an excipient. In one aspect, the pharmaceutical formulation includes an amino acid excipient as an aggregation inhibitor. While not wishing to be bound by theory, it is believed that inclusion of one or more amino acids as an excipient in a pharmaceutical formulation can stabilize a proteinaceous bioactive agent by mechanisms such as preferential hydration or by direct interactions between the protein and ions.

The term "diluent" refers to a pharmaceutically acceptable solvent that can be used to prepare a liquid pharmaceutical formulation and includes, but is not limited to, sterile water and bacteriostatic water for injection (BWFI).

Overview

Provided herein are stable pharmaceutical formulations for bioactive agents. In one aspect, the bioactive agent is a biotherapeutic. In one aspect, the biotherapeutic is a therapeutic macromolecule, such as a therapeutic polynucleotide or a therapeutic protein. In one aspect, the biotherapeutic includes cells. Long-term stability of a biotherapeutic can impact safety, consistency and efficacy of treatment. Loss of functionality within a preparation can decrease the effective concentration of the therapeutic for a given administration. Similarly, undesired modifications of a biotherapeutic can affect the activity or the safety of a formulation, which can result in loss of efficacy and increased risk of adverse side effects.

Non-ionic surfactants such as polysorbate 80, polysorbate 20 and poloxamer 188 are widely used in biotherapeutic formulations to protect against aggregation and particle formation. However, polysorbate 80 and polysorbate 20 are known to be susceptible to degradation during storage by oxidative and hydrolytic pathways, which can result in aggregation and particle formation when included in a pharmaceutical formulation. Additionally, these non-ionic surfactants are not easily removed from formulations, for example, using processes such as diafiltration. This can be problematic in the case of antibody intermediates used to generate antibody drug conjugates (ADC) because the presence of non-ionic surfactants may interfere with the conjugation process. Therefore, there is a need for alternate surfactants that can protect against aggregation and particle formation.

Provided herein is an alternate surfactant that can protect against aggregation and particle formation in therapeutic biomolecule formulations. In particular, provided herein are stable pharmaceutical formulations that contain D-α-Tocopheryl polyethylene glycol succinate (TPGS) as a surfactant. As demonstrated herein, inclusion of TPGS can reduce particle formation in a therapeutic macromolecule formulation even as compared formulations that include non-ionic surfactants such as polysorbate 80, polysorbate 20 or poloxamer 188.

D-α-tocopheryl polyethylene glycol succinate (TPGS)

D-α-tocopheryl polyethylene glycol succinate (TPGS) (also referred to as tocophersolan) is a water-soluble synthetic derivative of natural α-tocopherol (vitamin E), formed by covalently joining tocopherol succinate, an ester formed through esterification of tocopherol and succinic acid, to a polyethylene glycol (PEG) moiety via an esterification reaction. A schematic of the structure for TPGS is shown in FIG. 1. TPGS has amphiphilic properties due to the presence of a polar hydrophilic head (polyethylene glycol) and a lipophilic tail (phytyl chain of d-α-tocopherol). TPGS has a melting point from about 37 °C to about 41 °C, or about 38°C, is stable at a pH from about 4.5 to about 7.5 and has a solubility in water of about 20% at 20°C. Vitamin E TPGS is a highly stable form of Vitamin E. See, PMC Isochem. (2015). *Vitamin E TPGS:NF and Food Grade*. Available at pmcisochem.fr/page/info-center. TPGS is generally regarded as safe (GRAS) and has been approved by the Federal Drug Administration (FDA) as an inactive ingredient for oral and topical formulations.

TPGS compositions typically contain a mixture of monomers and dimers, where a monomer includes a single vitamin E molecule covalently joined to a water-soluble moiety, such as a polyethylene glycol (PEG), through a linker, in which the water-soluble moiety, e.g., PEG, has a free, unreacted, terminal reactive group, e.g., a free terminal hydroxyl group. A dimer includes two vitamin E molecules covalently joined to a water-soluble moiety, such as a polyethylene glycol (PEG), through one or more linkers, where both ends of the water-soluble moiety, e.g., both terminal hydroxyl groups of a PEG moiety, have reacted with a linker that is joined to a vitamin E molecule so that there are no free terminal reactive groups, e.g., hydroxyl groups. The monomers and dimers are formed during the esterification reaction. In one aspect, the TPGS compositions includes at least about 70%, about 75%, about 80%, about 85%, about 86%, about 87%, about 88%, about 89% or about 90% TPGS monomer and less than about 30%, about

25%, about 20%, about 15%, about 14%, about 13%, about 12%, about 11% or about 10% TPGS dimer. In one aspect, the TPGS composition includes at least about 85% TPGS monomer and less than about 15% TPGS dimer. The TPGS surfactant can include PEG moieties with a variety of molecular weights. In one aspect, the PEG moiety of TPGS has a molecular weight of about 1000 Da and the TPGS molecule is referred to D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS 1000). As used herein, the term TPGS includes TPGS 1000.

Surfactants are typically amphiphilic molecules that contain both hydrophilic and lipophilic groups. The hydrophile-lipophile balance (HLB) number can be used as a measure of the ratio between these groups and can have a value between 0-60, which defines the affinity of a surfactant for water or oil. Molecules with a HLB number greater than 10 have an affinity for water (hydrophilic) and molecules with a HLB number less than 10 have an affinity for oil (lipophilic). Non-ionic surfactants have HLB numbers ranging from 0-20. TPGS 1000 has a HLB number of 13.2. See, Wu and Hopkins. (1999). *Characteristics of D-alpha-tocopheryl PEG1000 succinate for applications as an absorption enhancer in drug delivery systems.* Pharm Tech. 23:52-60. The critical micelle concentration (CMC) is the concentration at and above which the surfactant forms micelles. Below the CMC, the surface tension decreases with increasing surfactant concentration. Above the CMC, additional surfactant added to the system forms micelles. TPGS 1000 has a CMC of 0.02% (w/w) at 37% C. See, Wu and Hopkins. (1999). *Characteristics of D-alpha-tocopheryl PEG1000 succinate for applications as an absorption enhancer in drug delivery systems.* Pharm Tech. 23:52-60.

Stable pharmaceutical formulation

In one aspect, a stable pharmaceutical formulation is provided. In one aspect, the pharmaceutical formulation has an improved stability against stress, including, for example, mechanical stress, thermal stress or stress resulting from freezing and thawing. In one aspect, the bioactive agent in the stable pharmaceutical formulation retains its physical stability, chemical stability or biological activity during storage.

In one aspect, the pharmaceutical formulation includes D-α-Tocopheryl polyethylene glycol succinate (TPGS). In one aspect, the pharmaceutical formulation includes D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS 1000). In one aspect, the pharmaceutical formulation includes D-α-Tocopheryl polyethylene glycol succinate (TPGS) as a surfactant. In one aspect, the

pharmaceutical formulation includes D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS 1000) as a surfactant. Surfactants can be included in a pharmaceutical formulation for a variety of reasons including, for example, to prevent or control aggregation, particle formation or surface adsorption in liquid formulations or to prevent or control these phenomena during lyophilization or reconstitution of lyophilized formulations. In one aspect, the stable pharmaceutical formulation does not include a non-ionic surfactant. In one aspect, the stable pharmaceutical formulation does not include a poly-oxy-ethylene (PEO) based surfactant such as polysorbate 80, polysorbate 20, poloxamer 188, or combinations thereof.

In one aspect, the physical stability, chemical stability or biological activity of the bioactive agent in the pharmaceutical formulation at a given time is within about 0.1%, about 0.5%, about 1%, about 5%, about 10%, about 15%, about 20% or about 25% (within a standard of error) of the physical stability, chemical stability or biological activity of the bioactive agent exhibited at an initial time point, for example, at the time the pharmaceutical formulation was prepared (T0).

Generally, stability is determined with respect to a selected temperature and a selected time-period. In one aspect, the formulation is stable at a temperature of about 30°C ± 10°C, about 30°C ± 5°C or about 30°C ± 2°C, or at about 30°C, about 35°C or about 40°C for at least about 2 weeks, about 1 month, about 2 months or about 3 months and up to about 6 months. In one aspect, the formulation is stable at a temperature of about 20°C ± 10°C, about 20°C ± 5°C or about 20°C ± 2°C, or at about 15°C, about 20°C, about 25°C or about 30°C for at least about 1 month, about 2 months or about 3 months and up to about 6 months, about 9 months or about 12 months. In one aspect, the formulation is stable at a temperature from about 5°C ± 5°C or about 5°C ± 3°C, or between about 2°C to about 8°C, or at about 2°C, about 4°C, about 6°C or about 8°C for at least about 1 month, about 3 months, or about 6 months and up to about 9 months, about 12 months, about 24 months or about 36 months. In one aspect, the formulation is stable at a temperature from about -20 °C ± 10°C or about -20 °C ± 5°C, or at about -25°C, about -20 °C, about -15 °C or about -10°C for at least about 1 month, about 3 months, or about 6 months and up to about 9 months, about 12 months, about 24 months or about 36 months. In one aspect, the formulation is stable at a temperature from about -70°C ± 10°C, or about -70 °C ± 5°C or at about -70°C , about -75°C or about -80°C for at least about 1 month, about 3 months, or about 6 months and up to about 9 months, about 12 months, about 24 months or about 36 months.

In one aspect, the formulation is stable at 40°C for up to about 3 months. In one aspect, the formulation is stable at 40°C for up to about 6 months. In one aspect, the formulation is stable at 25°C for up to about 6 months. In one aspect, the formulation is stable at 25°C for up to about 12 months. In one aspect, the formulation is stable at a temperature from about 2°C to about 8°C for up to about 6 months. In one aspect, the formulation is stable at a temperature from about 2°C to about 8°C for up to about 12 months. In one aspect, the formulation is stable at a temperature from about 2°C to about 8°C for up to about 24 months. In one aspect, the formulation is stable at a temperature from about 2°C to about 8°C for up to about 36 months. In one aspect, the formulation is stable at -20°C for up to about 6 months. In one aspect, the formulation is stable at -20°C for up to about 12 months. In one aspect, the formulation is stable at -20°C for up to about 24 months. In one aspect, the formulation is stable at -20°C for up to about 36 months. In one aspect, the formulation is stable at -80°C for up to about 6 months. In one aspect, the formulation is stable at -80°C for up to about 12 months. In one aspect, the formulation is stable at -80°C for up to about 24 months. In one aspect, the formulation is stable at -80°C for up to about 36 months.

In one aspect, the bioactive agent in the pharmaceutical formulation is chemically stable. In one aspect, there is an increase of less than about 25%, about 20%, about 15%, about 10% or about 5% in chemically altered forms of the bioactive agent in the pharmaceutical formulation when stored for at least about 2 weeks, about 1 month, about 2 months, about 3 months or about 6 months and up to about 9 months, about 12 months, about 24 months or about 36 months at a temperature of about 40°C, about 25°C, about 2°C to about 8°C, about -20°C or about -80°C. In one aspect, there is less than about 25%, about 20%, about 15%, about 10% or about 5% increase in one or more chemically altered forms of the bioactive agent, including, for example, chemically altered forms due to deamidation, including, for example, asparagine (Asn) deamidation; oxidation, including for example methionine, cysteine, histidine, tyrosine, tryptophan or phenylalanine oxidation; intra- and inter-residue cyclization (aspartic and glutamic acid, asparagine, glutamine, N-terminal dipeptidyl motifs); clipping/hydrolysis/fragmentation; β-elimination; glycation; and disulfide scrambling.

In one aspect, the bioactive agent in the pharmaceutical formulation is physically stable. In one aspect, there is an increase of less than about 25%, about 20%, about 15%, about 10% or

about 5% in physically altered forms of the bioactive agent in the pharmaceutical formulation when stored for at least about 2 weeks, about 1 month, about 2 months, about 3 months or about 6 months and up to about 9 months, about 12 months, about 24 months or about 36 months at a temperature of about 40°C, about 25°C, about 2°C to about 8°C, about -20°C or about -80°C. In one aspect, there is less than about 25%, about 20%, about 15%, about 10% or about 5% increase in one or more physically altered forms of the bioactive agent, including, for example, physically altered forms due to denaturation, aggregation, precipitation, or particulate formation.

In one aspect, the bioactive agent in the biopharmaceutical formulation is biologically stable. In one aspect, there is a decrease of less than about 25%, about 20%, about 15%, about 10% or about 5% in a biological activity of the bioactive agent in the pharmaceutical formulation when stored for at least about 2 weeks, about 1 month, about 2 months, about 3 months or about 6 months and up to about 9 months, about 12 months, about 24 months or about 36 months at a temperature of about 40°C, about 25°C, about 2°C to about 8°C, about -20°C or about -80°C. In one aspect, there is a decrease of less than about 25%, about 20%, about 15%, about 10% or about 5% in a biological activity including, but not limited to, enzymatic activity; ability to interact with or bind to another molecule; ability to activate, promote, stabilize, inhibit, suppress, or destabilize a function of another molecule; ability to produce a specific protein; ability to impart resistance; and ability to localize at a specific position in a cell.

In one aspect, the stability of a pharmaceutical formulation is evaluated by measuring the amount of particle impurities. Particle impurities can include visible, subvisible and submicron impurities. Visible impurities have a diameter of greater than about 100 μm or about 150 μm and can be detected by visual inspection. Sub-visible particles generally range in size from about 1 μm to about 100 μm or about 150 μm. Submicron particles have a diameter of less than about 1 μm. Sub-visible particles generally pose the greatest risk when present in pharmaceutical formulations, particularly when present in pharmaceutical formulations for parenteral administration, including subcutaneous, intravenous or intramuscular administration, due, in some cases, to the ability of sub-visible particles to elicit an adverse immunogenic response. Sub-visible particles with a diameter of greater than or equal to about 10 μm or greater than or equal to about 5 μm can block blood vessels in the lungs following vascular infusion. Methods for detecting and quantifying particulate impurities are known and include Dynamic Light Scattering (DLS) or Static Light

Scattering (SLS), Nanoparticle Tracking Analysis (NTA), light microscopy, Electrical Sensing Zone (ESZ), Flow-Imaging Technology, Resonant Mass measurement, Electron Microscopy, Fourier Transform Infrared (FTIR) Microscopy, and Raman Microscopy. In one aspect, sub-visible particles are detected using Micro Flow Imaging (MFI).

In one aspect, a stable pharmaceutical formulation is provided that includes less than about 10,000, about 5,000, about 1,000, about 750, about 500, about 250, about 150, about 100, or about 50 particles greater than about 2 μm, about 5 μm, about 10 μm, about 15 μm, about 20 μm or about 25 μm diameter/mL when stored for at least about 2 weeks, about 1 month, about 2 months, about 3 months or about 6 months and up to about 9 months, about 12 months, about 24 months or about 36 months at a temperature of about 30°C ± 10°C, about 30°C ± 5°C, about 20°C ± 10°C, about 20°C ± 5°C, about 20°C ± 2°C, about 5°C ± 5°C, about 5°C ± 3°C, about -20 °C ± 10°C, about -20 °C ± 5°C, about -70°C ± 10°C, or about -70 °C ± 5°C.

In one aspect, a stable pharmaceutical formulation is provided that includes less than about 5,000 particles greater than about 2 μm diameter/mL when stored for at least about 2 weeks, about 1 month, about 2 months, about 3 months or about 6 months and up to about 9 months, about 12 months, about 24 months or about 36 months at a temperature of about 30°C ± 10°C, about 20°C ± 10°C, about 5°C ± 3°C, about -20°C ± 10°C or about -70°C ± 10°C. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 30°C ± 10°C for up to about 3 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 30°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 20°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 20°C ± 5°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 5°C ± 3°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 5°C ± 3°C for up to about 24 months. In one

aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 5°C ± 3°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 36 months.

In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 40°C for up to about 3 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 40°C for up to 6 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 25°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 25°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of

about 2°C to about 8°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 2°C to about 8°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 2°C to about 8°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -80°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -80°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -80°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -80°C for up to about 36 months.

In one aspect, a stable pharmaceutical formulation is provided that includes less than about 1,000 particles greater than about 2 μm diameter/mL when stored for at least about 2 weeks, about 1 month, about 2 months, about 3 months or about 6 months and up to about 9 months, about 12 months, about 24 months or about 36 months at a temperature of about 30°C ± 10°C, about 20°C ± 10°C, about 5°C ± 3°C, about -20°C ± 10°C or about -70°C ± 10°C. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 30°C ± 10°C for up to about 3 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles

greater than about 2 μm in diameter/mL when stored at a temperature of about 30°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 20°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 20°C ± 5°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 5°C ± 3°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 5°C ± 3°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 5°C ± 3°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 36 months.

In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 40°C for up to about 3 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 40°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 25°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 25°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 2°C to about 8°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 2°C to about 8°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about 2°C to about 8°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -20°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -80°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -80°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -80°C for up to about 24 months. In

one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL when stored at a temperature of about -80°C for up to about 36 months.

In one aspect, a stable pharmaceutical formulation is provided that includes less than about 6,000 particles greater than about 10 μm diameter/mL when stored for at least about 2 weeks, about 1 month, about 2 months, about 3 months or about 6 months and up to about 9 months, about 12 months or about 24 months at a temperature of about 30°C ± 10°C, about 20°C ± 10°C, about 5°C ± 3°C, about -20°C ± 10°C or about -70°C ± 10°C. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about 30°C ± 10°C for up to about 3 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about 30°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about 20°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about 20°C ± 5°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about 5°C ± 3°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about 5°C ± 3°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about 5°C ± 3°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes

less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 36 months.

In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about 40°C for up to about 3 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about 40°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about 25°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about 25°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about 2°C to about 8°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about 2°C to about 8°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about 2°C to about 8°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -20°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -

20°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -20°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -20°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -80°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -80°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -80°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL when stored at a temperature of about -80°C for up to about 36 months.

In one aspect, a stable pharmaceutical formulation is provided that includes less than about 600 particles greater than about 25 μm diameter/mL when stored for at least about 2 weeks, about 1 month, about 2 months, about 3 months or about 6 months and up to about 9 months, about 12 months or about 24 months at a temperature of about 30°C ± 10°C, about 20°C ± 10°C, about 5°C ± 3°C, about -20°C ± 10°C or about -70°C ± 10°C. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about 30°C ± 10°C for up to about 3 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about 30°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about 20°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about 20°C ± 5°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about 5°C ± 3°C for up to about 12 months. In one aspect, the pharmaceutical formulation

includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about 5°C ± 3°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about 5°C ± 3°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -20°C ± 10°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -70°C ± 10°C for up to about 36 months.

In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about 40°C for up to about 3 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about 40°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about 25°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of

about 25°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about 2°C to about 8°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about 2°C to about 8°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about 2°C to about 8°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -20°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -20°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -20°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -20°C for up to about 36 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -80°C for up to about 6 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -80°C for up to about 12 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -80°C for up to about 24 months. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL when stored at a temperature of about -80°C for up to about 36 months.

In one aspect, a method of reducing particle formation in an aqueous pharmaceutical formulation is provided. In one aspect, the method includes adding D-α-Tocopheryl polyethylene glycol succinate (TPGS) to the formulation. In one aspect, the method includes adding D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS 1000) to the formulation. In one aspect, less than about 10,000, about 5,000, about 1,000, about 750, about 500, about 250, about 150,

about 100, or about 50 particles greater than about 2 μm, about 5 μm, about 10 μm, about 15 μm, about 20 μm or about 25 μm diameter/mL are formed after at least about 2 weeks, about 1 month, about 2 months, about 3 months or about 6 months and up to about 9 months, about 12 months, about 24 months or about 36 months at a temperature of about 30°C ± 10°C, about 30°C ± 5°C, about 20°C ± 10°C, about 20°C ± 5°C, about 20°C ± 2°C, about 5°C ± 5°C, about 5°C ± 3°C, about -20 °C ± 10°C, about -20 °C ± 5°C, about -70°C ± 10°C, or about -70 °C ± 5°C. In one aspect, the pharmaceutical formulation includes less than about 5,000 particles greater than about 2 μm in diameter/mL. In one aspect, the pharmaceutical formulation includes less than about 1,000 particles greater than about 2 μm in diameter/mL. In one aspect, the pharmaceutical formulation includes less than about 6,000 particles greater than about 10 μm in diameter/mL. In one aspect, the pharmaceutical formulation includes less than about 600 particles greater than about 25 μm in diameter/mL.

In one aspect, the pharmaceutical formulation includes TPGS at a concentration from about 0.001% to about 1%, or from about 0.01% to about 1% or from about 0.01% to about 0.1%. In one aspect, the pharmaceutical formulation includes TPGS at a concentration from about 0.001%, about 0.005%, about 0.01%, about 0.02%, about 0.03%, about 0.04%, or about 0.05% and up to about 0.1%, about 0.2%, about 0.3%, about 0.4%, about 0.5%, or about 1%. In one aspect, the pharmaceutical formulation includes TPGS in an amount of about 0.01%, about 0.02%, about 0.03%, about 0.04%, or about 0.05%. In one aspect, the pharmaceutical formulation includes TPGS in an amount of about 0.01%. In one aspect, the pharmaceutical formulation includes TPGS in an amount of about 0.02%. In one aspect, the pharmaceutical formulation includes TPGS in an amount of about 0.03%. In one aspect, the pharmaceutical formulation includes TPGS in an amount of about 0.04%. In one aspect, the pharmaceutical formulation includes TPGS in an amount of about 0.05%.

In one aspect, the pharmaceutical formulation includes TPGS 1000 at a concentration from about 0.001% to about 1%, or from about 0.01% to about 1% or from about 0.01% to about 0.1%. In one aspect, the pharmaceutical formulation includes TPGS 1000 at a concentration from about 0.001%, about 0.005%, about 0.01%, about 0.02%, about 0.03%, about 0.04%, or about 0.05% and up to about 0.1%, about 0.2%, about 0.3%, about 0.4%, about 0.5%, or about 1%. In one aspect, the pharmaceutical formulation includes TPGS 1000 in an amount of about 0.01%, about

0.02%, about 0.03%, about 0.04%, or about 0.05%. In one aspect, the pharmaceutical formulation includes TPGS 1000 in an amount of about 0.01%. In one aspect, the pharmaceutical formulation includes TPGS 1000 in an amount of about 0.02%. In one aspect, the pharmaceutical formulation includes TPGS 1000 in an amount of about 0.03%. In one aspect, the pharmaceutical formulation includes TPGS 1000 in an amount of about 0.04%. In one aspect, the pharmaceutical formulation includes TPGS 1000 in an amount of about 0.05%.

In one aspect, the pharmaceutical formulation is lyophilized. "Lyophilization" refers to a dehydration process in which a liquid formulation is frozen and the surrounding pressure is reduced to allow for sublimation of the frozen solvent. Lyophilization methods are known in the art. Lyophilization results in a cake or powder which can be stored over a long time-period. Prior to administration, the lyophilized composition is reconstituted using a solvent, for example, sterile water for injection. A "reconstituted formulation" refers to a lyophilized composition after solubilization.

In one aspect the pharmaceutical formulation is a liquid formulation. In one aspect, a "liquid formulation" includes reconstituted lyophilized compositions. In one aspect, the liquid formulation is not lyophilized and has not been subjected to lyophilization. In one aspect, the liquid formulation is an aqueous formulation, in which the solvent includes water.

In one aspect, the pharmaceutical formulation includes a bioactive agent at a concentration from about 0.1 mg/mL to about 300 mg/mL. In one aspect, the pharmaceutical formulation includes an antibody or antigen-binding antibody fragment as a bioactive agent at a concentration from about 10 mg/mL to about 200 mg/mL. In one aspect, the concentration of antibody or antigen-binding antibody fragment is at least about 10 mg/mL, about 15 mg/mL, about 20 mg/mL, about 25 mg/mL, about 50 mg/mL, about 75 mg/mL or about 100 mg/mL and up to about 125 mg/mL, about 150 mg/mL, about 175 mg/mL, about 200 mg/mL, about 250 mg/mL or about 300 mg/mL.

In one aspect, the pharmaceutical formulation includes a therapeutic protein as a bioactive agent at a concentration from about 0.1 mg/mL to about 100 mg/mL, or about 1 mg/mL to about 50 mg/mL. In one aspect, the pharmaceutical formulation includes a therapeutic protein as a bioactive agent at a concentration from about 0.1 mg/mL, about 0.5 mg/mL, about 1 mg/mL, about 2 mg/mL, about 3 mg/mL, about 4 mg/mL or about 5 mg/mL and up to about 10 mg/mL, about 15

mg/mL, about 20 mg/mL, about 25 mgL, about 30 mg/mL, about 35 mg/mL, about 35 mg/mL, about 40 mg/mL, about 45 mg/mL, about 50 mg/mL, about 75 mg/mL or about 100 mg/mL.

In one aspect, the formulation includes a buffer at a concentration from about 0.1 mM to about 100 mM, about 0.5 mM to about 50 mM, about 10 mM to about 50 mM, or about 10 mM to about 30 mM. In one aspect, the formulation includes a buffer at a concentration from about 0.1 mM, about 0.5 mM, about 1 mM, about 5 mM, about 10 mM, about 20 mM or about 25 mM and up to about 30 mM, about 35 mM, about 40 mM, about 45 mM, about 50 mM, about 75 mM or about 100 mM. In one aspect, the formulation includes a buffer at a concentration of about 10 mM. In one aspect, the formulation includes a buffer at a concentration of about 15 mM. In one aspect, the formulation includes a buffer at a concentration of about 20 mM. In one aspect, the formulation includes a buffer at a concentration of about 30 mM. In one aspect, the formulation includes a buffer at a concentration of about 40 mM. In one aspect, the formulation includes a buffer at a concentration of about 50 mM. In one aspect, the buffer includes histidine/histidine hydrochloride. In one aspect, the buffer includes tris/tris hydrochloride. In one aspect, the buffer includes citrate. In one aspect, the buffer includes sodium acetate. In one aspect, the buffer includes phosphate. In one aspect, the buffer includes from about 10 mM to about 50 mM histidine/histidine HCl. In one aspect, the buffer includes from about 10 mM to about 30 mM histidine/histidine HCl. In one aspect, the buffer includes from about 10 mM to about 15 mM histidine/histidine HCl. In one aspect, the buffer includes from about 15 mM to about 20 mM histidine/histidine HCl. In one aspect, the buffer includes from about 20 mM to about 30 mM histidine/histidine HCl. In one aspect, the buffer includes about 5 mM histidine/histidine HCl. In one aspect, the buffer includes about 10 mM histidine/histidine HCl. In one aspect, the buffer includes about 15 mM histidine/histidine HCl. In one aspect, the buffer includes about 20 mM histidine/histidine HCl. In one aspect, the buffer includes about 25 mM histidine/histidine HCl. In one aspect, the buffer includes about 30 mM histidine/histidine HCl.

In one aspect, a stable pharmaceutical formulation is provided that includes a bioactive agent and D-α-Tocopheryl polyethylene glycol succinate (TPGS) as a surfactant. In one aspect, a stable pharmaceutical formulation is provided that includes from about 0.1 mg/mL to about 300 mg/mL bioactive agent, and from about 0.01% to about 0.1% TPGS. In one aspect, a stable pharmaceutical formulation is provided that includes from about 0.1 mg/mL to about 300 mg/mL

bioactive agent, from about 0.01% to about 0.1% TPGS, and from about 10 mM to about 50 mM buffer. In one aspect, a stable pharmaceutical formulation is provided that includes from about 0.1 mg/mL to about 300 mg/mL bioactive agent, from about 0.01% to about 0.1% TPGS, and from about 10 mM to about 50 mM histidine/histidine HCl. In one aspect, a stable pharmaceutical formulation is provided that includes from about 0.1 mg/mL to about 300 mg/mL bioactive agent, from about 0.01% to about 0.1% TPGS, and about 20 mM histidine/histidine HCl. In one aspect, the pharmaceutical formulation has a pH from about 3 to about 9. In one aspect, the pharmaceutical formulation has a pH from about 4 to about 8. In one aspect, the pharmaceutical formulation has a pH from about 5.5 to about 7.5.

In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of an antibody or antigen-binding antibody fragment, and from about 0.01% to about 0.1% TPGS. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of an antibody or an antigen-binding antibody fragment, from about 0.01% to about 0.1% TPGS, and from about 10 mM to about 50 mM buffer. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of an antibody or an antigen-binding antibody fragment, from about 0.01% to about 0.1% TPGS, and from about 10 mM to about 50 mM histidine/histidine HCl. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of an antibody or an antigen-binding antibody fragment, about 0.02% TPGS, and about 20 mM histidine/histidine HCl. In one aspect, the pharmaceutical formulation has a pH from about 3 to about 9. In one aspect, the pharmaceutical formulation has a pH from about 4 to about 8. In one aspect, the pharmaceutical formulation has a pH from about 5.5 to about 7.5.

In one aspect, a stable pharmaceutical formulation is provided that includes from about 0.1 mg/mL to about 50 mg/mL of a therapeutic protein, and from about 0.01% to about 0.1% TPGS. In one aspect, a stable pharmaceutical formulation is provided that includes from about 0.1 mg/mL to about 50 mg/mL of a therapeutic protein, from about 0.01% to about 0.1% TPGS, and from about 10 mM to about 50 mM buffer. In one aspect, a stable pharmaceutical formulation is provided that includes from about 0.1 mg/mL to about 50 mg/mL of a therapeutic protein, from about 0.01% to about 0.1% TPGS, and from about 10 mM to about 50 mM histidine/histidine HCl. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL

to about 200 mg/mL of an antibody or an antigen-binding antibody fragment, about 0.02% TPGS, and about 20 mM histidine/histidine HCl. In one aspect, the pharmaceutical formulation has a pH from about 3 to about 9. In one aspect, the pharmaceutical formulation has a pH from about 4 to about 8. In one aspect, the pharmaceutical formulation has a pH from about 5.5 to about 7.5.

In one aspect, a stable pharmaceutical formulation is provided that includes a bioactive agent and D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS 1000) as a surfactant. In one aspect, a stable pharmaceutical formulation is provided that includes from about 0.1 mg/mL to about 300 mg/mL bioactive agent, and from about 0.01% to about 0.1% TPGS 1000. In one aspect, a stable pharmaceutical formulation is provided that includes from about 0.1 mg/mL to about 300 mg/mL bioactive agent, from about 0.01% to about 0.1% TPGS 1000, and from about 10 mM to about 50 mM buffer. In one aspect, a stable pharmaceutical formulation is provided that includes from about 0.1 mg/mL to about 300 mg/mL bioactive agent, from about 0.01% to about 0.1% TPGS 1000, and from about 10 mM to about 50 mM histidine/histidine HCl. In one aspect, a stable pharmaceutical formulation is provided that includes from about 0.1 mg/mL to about 300 mg/mL bioactive agent, from about 0.01% to about 0.1% TPGS 1000, and about 20 mM histidine/histidine HCl. In one aspect, the pharmaceutical formulation has a pH from about 3 to about 9. In one aspect, the pharmaceutical formulation has a pH from about 4 to about 8. In one aspect, the pharmaceutical formulation has a pH from about 5.5 to about 7.5.

In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of an antibody or antigen-binding antibody fragment, and from about 0.01% to about 0.1% TPGS 1000. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of an antibody or an antigen-binding antibody fragment, from about 0.01% to about 0.1% TPGS 1000, and from about 10 mM to about 50 mM buffer. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of an antibody or an antigen-binding antibody fragment, from about 0.01% to about 0.1% TPGS 1000, and from about 10 mM to about 50 mM histidine/histidine HCl. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of an antibody or an antigen-binding antibody fragment, about 0.02% TPGS 1000, and about 20 mM histidine/histidine HCl. In one aspect, the pharmaceutical formulation has a pH from about 3 to about 9. In one aspect, the pharmaceutical

formulation has a pH from about 4 to about 8. In one aspect, the pharmaceutical formulation has a pH from about 5.5 to about 7.5.

In one aspect, a stable pharmaceutical formulation is provided that includes from about 0.1 mg/mL to about 50 mg/mL of a therapeutic protein, and from about 0.01% to about 0.1% TPGS 1000. In one aspect, a stable pharmaceutical formulation is provided that includes from about 0.1 mg/mL to about 50 mg/mL of a therapeutic protein, from about 0.01% to about 0.1% TPGS 1000, and from about 10 mM to about 50 mM buffer. In one aspect, a stable pharmaceutical formulation is provided that includes from about 0.1 mg/mL to about 50 mg/mL of a therapeutic protein, from about 0.01% to about 0.1% TPGS 1000, and from about 10 mM to about 50 mM histidine/histidine HCl. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of an antibody or an antigen-binding antibody fragment, about 0.02% TPGS 1000, and about 20 mM histidine/histidine HCl. In one aspect, the pharmaceutical formulation has a pH from about 3 to about 9. In one aspect, the pharmaceutical formulation has a pH from about 4 to about 8. In one aspect, the pharmaceutical formulation has a pH from about 5.5 to about 7.5.

In one aspect, the pharmaceutical formulation includes a tonicity agent. In one aspect, the tonicity agent includes a polyol, a saccharide, a carbohydrate, a salt, such as sodium chloride, or mixtures thereof. In one aspect, the polyol has a molecular weight that is less than about 600 kD, or from about 120 to about 400 kD. In one aspect, the tonicity agent is selected from mannitol, trehalose, sorbitol, erythritol, isomalt, lactitol, maltitol, xylitol, glycerol, lactitol, propylene glycol, polyethylene glycol, inositol, or mixtures thereof. In one aspect, the tonicity agent is a saccharide or carbohydrate selected from monosaccharides, disaccharides and polysaccharides or mixtures thereof. In one aspect, the saccharide or carbohydrate is selected from fructose, glucose, mannose, sucrose, sorbose, xylose, lactose, maltose, dextran, pullulan, dextrin, cyclodextrins, soluble starch, hydroxyethyl starch, water-soluble glucans, and mixtures thereof. In one aspect, the tonicity agent includes a reducing sugar or a non-reducing sugar or a mixture thereof.

In one aspect, the formulation includes the tonicity agent at a concentration from about 1 mg/ml to about 300 mg/ml, about 10 mg/ml to about 200 mg/ml, about 50 mg/ml to about 100 mg/ml, or about 80 mg/ml to about 90 mg/ml. In one aspect, the concentration of tonicity agent in the formulation is from about 1 mg/ml, about 5 mg/ml, about 10 mg/ml, about 15 mg/ml, about 20

mg/ml, about 25 mg/ml or about 50 mg/ml and up to about 100 mg/ml, about 150 mg/ml, about 200 mg/ml, about 250 mg/ml or about 300 mg/ml. In one aspect, the concentration of tonicity agent in the formulation is about 60 mg/ml, about 65 mg/ml, about 70 mg/ml, about 75 mg/ml, about 80 mg/ml, about 81 mg/ml, about 82 mg/ml, about 83 mg/ml, about 84 mg/ml, about 85 mg/ml, about 86 mg/ml, about 87 mg/ml, about 88 mg/ml, about 89 mg/ml, about 90 mg/ml, about 91 mg/ml, about 92 mg/ml, about 93 mg/ml, about 94 mg/ml, about 95 mg/ml, about 96 mg/ml, about 97 mg/ml, about 98 mg/ml, about 99 mg/ml, or about 100 mg/ml. In one aspect, the concentration of the tonicity agent is about 80 mg/ml. In one aspect, the concentration of the tonicity agent is about 81 mg/ml. In one aspect, the concentration of the tonicity agent is about 82 mg/ml. In one aspect, the concentration of the tonicity agent is about 83 mg/ml. In one aspect, the concentration of the tonicity agent is about 84 mg/ml. In one aspect, the concentration of the tonicity agent is about 85 mg/ml.

In one aspect, the formulation includes a non-reducing sugar as tonicity agent at a concentration from about 1 mg/ml to about 300 mg/ml, about 10 mg/ml to about 200 mg/ml, about 50 mg/ml to about 100 mg/ml, or about 80 mg/ml to about 90 mg/ml. In one aspect, the concentration of non-reducing sugar in the formulation is from about 1 mg/ml, about 5 mg/ml, about 10 mg/ml, about 15 mg/ml, about 20 mg/ml, about 25 mg/ml or about 50 mg/ml and up to about 100 mg/ml, about 150 mg/ml, about 200 mg/ml, about 250 mg/ml or about 300 mg/ml. In one aspect, the concentration of non-reducing sugar in the formulation is about 60 mg/ml, about 65 mg/ml, about 70 mg/ml, about 75 mg/ml, about 80 mg/ml, about 81 mg/ml, about 82 mg/ml, about 83 mg/ml, about 84 mg/ml, about 85 mg/ml, about 86 mg/ml, about 87 mg/ml, about 88 mg/ml, about 89 mg/ml, about 90 mg/ml, about 91 mg/ml, about 92 mg/ml, about 93 mg/ml, about 94 mg/ml, about 95 mg/ml, about 96 mg/ml, about 97 mg/ml, about 98 mg/ml, about 99 mg/ml, or about 100 mg/ml. In one aspect, the concentration of the non-reducing sugar is about 80 mg/ml. In one aspect, the concentration of the non-reducing sugar is about 81 mg/ml. In one aspect, the concentration of the non-reducing sugar is about 82 mg/ml. In one aspect, the concentration of the non-reducing sugar is about 83 mg/ml. In one aspect, the concentration of the non-reducing sugar is about 84 mg/ml. In one aspect, the concentration of the non-reducing sugar is about 85 mg/ml. In one aspect, the tonicity reagent includes a saccharide. In one aspect, the tonicity agent includes sucrose.

In one aspect, the formulation includes a salt as a tonicity agent. In one aspect, the salt is a pharmaceutically acceptable salt selected from sodium chloride, sodium succinate, sodium sulfate, potassium chloride, magnesium chloride, magnesium sulfate, calcium chloride, and mixtures thereof. In one aspect, the formulation includes a salt as a tonicity agent at a concentration from about 1 mg/ml to about 20 mg/ml. In one aspect, the salt is at a concentration from about 1 mg/ml, about 2 mg/ml, about 3 mg/ml, about 4 mg/ml, about 5 mg/ml, about 6 mg/ml, about 7 mg/ml, about 8 mg/ml, about 9 mg/ml or about 10 mg/ml and up to about 11 mg/ml, about 12 mg/ml, about 13 mg/ml, about 14 mg/ml, about 15 mg/ml, about 16 mg/ml, about 17 mg/ml, about 18 mg/ml, about 19 mg/ml or about 20 mg/ml. In one aspect, the salt is at a concentration of about 1 mg/ml, about 2 mg/ml, about 3 mg/ml, about 4 mg/ml, about 5 mg/ml, about 6 mg/ml, about 7 mg/ml, about 8 mg/ml, about 9 mg/ml, about 10 mg/ml, about 11 mg/ml, about 12 mg/ml, about 13 mg/ml, about 14 mg/ml, about 15 mg/ml, about 16 mg/ml, about 17 mg/ml, about 18 mg/ml, about 19 mg/ml and about 20 mg/ml.

In one aspect, the pharmaceutical formulation includes an amino acid excipient. In one aspect, the pharmaceutical formulation includes an amino acid excipient to reduce protein aggregation. In one aspect, the pharmaceutical formulation includes a charged amino acid as a protein stabilizing excipient. In one aspect, the pharmaceutical formulation includes a positively charged amino acid as a stabilizing excipient, such as lysine, histidine or arginine. In one aspect, the pharmaceutical formulation includes a negatively charged amino acid, such as glutamic acid. In one aspect, the pharmaceutical formulation includes an uncharged polar amino acid such as serine, threonine or glutamine. In one aspect, the pharmaceutical formulation includes a hydrophobic amino acid such as alanine, valine, leucine, isoleucine, methionine or phenylalanine. In one aspect, a mixture of amino acids is included in the pharmaceutical formulation. In one aspect, the formulation includes a mixture of one or more of a positively charged amino acid, a negatively charged amino acid, an uncharged polar amino, and a hydrophobic amino acid as an excipient. In one aspect, the amino acid excipient is selected from arginine, cysteine, glycine, lysine, ornithine, proline or histidine. In one aspect, the amino acid excipient is selected from arginine, cysteine, glycine, or lysine. In one aspect, the amino acid is in the L-form. In one aspect, L-arginine hydrochloride (L-ArgHCl) is included in the pharmaceutical formulation. In one aspect, the pharmaceutical formulation includes from about 1 mg/ml to about 50 mg/ml, about 5 mg/ml

to about 25 mg/ml, about 10 mg/ml to about 20 mg/ml, or about 1 mg/ml to about 10 mg/ml of at least one amino acid as an excipient. In one aspect, the pharmaceutical formulation includes from about 1 mg/ml, about 5 mg/ml, about 10 mg/ml, about 15 mg/ml, about 20 mg/ml or about 25 mg/ml and up to about 30 mg/ml, about 35 mg/ml, about 40 mg/ml, about 45 mg/ml or about 50 mg/ml of at least one amino acid as an excipient. In one aspect, the pharmaceutical formulation includes about 1 mg/ml, about 5 mg/ml, about 10 mg/ml, about 15 mg/ml, about 20 mg/ml, about 25 mg/ml, about 30 mg/ml, about 35 mg/ml, about 40 mg/ml, about 45 mg/ml or about 50 mg/ml of at least one amino acid as an excipient.

In one aspect, the pharmaceutical formulation includes a chelating agent. In one aspect, the chelating agent is selected from aminopolycarboxylic acids, hydroxyaminocarboxylic acids, N-substituted glycines, 2-(2-amino-2-oxocthyl) aminoethane sulfonic acid (BES), deferoxamine (DEF), citric acid, niacinamide, and desoxycholates and mixtures thereof. In one aspect, the chelating agent is selected from ethylenediaminetetraacetic acid (EDTA), diethylenetriamine pentaacetic acid (DTPA), nitrilotriacetic acid (NTA), N-2-acetamido-2-iminodiacetic acid (ADA), bis(aminoethyl)glycolether, N,N,N′,N′-tetraacetic acid (EGTA), trans-diaminocyclohexane tetraacetic acid (DCTA), glutamic acid, and aspartic acid, N-hydroxyethyliminodiacetic acid (HIMDA), N,N-bis-hydroxyethylglycine (bicine) and N-(trishydroxymethylmethyl) glycine (tricine), glycylglycine, sodium desoxycholate, ethylenediamine; propylenediamine; diethylenetriamine; triethylenetetraamine (trien), ethylenediaminetetraaceto EDTA; disodium EDTA, calcium EDTA oxalic acid, malate, citric acid, citric acid monohydrate, and trisodium citrate-dihydrate, 8-hydroxyquinolate, amino acids, histidine, cysteine, methionine, peptides, polypeptides, and proteins and mixtures thereof. In one aspect, the chelating agent is selected from salts of EDTA including dipotassium edetate, disodium edetate, edetate calcium disodium, sodium edetate, trisodium edetate, and potassium edetate; and a suitable salt of deferoxamine (DEF) is deferoxamine mesylate (DFM), or mixtures thereof. Chelating agents can be included in a pharmaceutical formulation as a free acid, free base form or salt form of the compound, or as an anhydrous, solvated or hydrated form of the compound. In one aspect, the pharmaceutical formulation includes a chelating agent at a concentration from about 0.01 mg/ml to about 50 mg/ml, from about 1 mg/ml to about 10 mg/ml, from about 1.5 mg/ml to about 5.0 mg/ml, or from about 0.1 mg/ml, about 0.5 mg/ml, about 1 mg/ml or about 5 mg/ml and up to about 10 mg/ml,

about 20 mg/ml, about 30 mg/ml, about 40 mg/ml or about 50 mg/ml. In one aspect, the concentration of chelating agent is about 0.01 mg/ml, about 0.02 mg/ml, about 0.03 mg/ml, about 0.04 mg/ml, about 0.05 mg/ml, about 0.06 mg/ml, about 0.07 mg/ml, about 0.08 mg/ml, about 0.09 mg/ml, or about 0.1 mg/ml. In one aspect, the concentration of chelating agent is 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, or about 1 mg/ml.

Bioactive agents

In one aspect, the pharmaceutical formulation includes a bioactive agent. In one aspect, the bioactive agent is a biotherapeutic. In one aspect, the bioactive agent includes a therapeutic macromolecule, for example, a polynucleotide or polypeptide. In one aspect, the bioactive agent includes a naturally occurring macromolecule. In one aspect, the bioactive agent includes a recombinantly produced macromolecule. In one aspect, the bioactive agent includes a polypeptide. In one aspect, the bioactive agent includes a polynucleotide. In one aspect, the bioactive agent includes a cell. In one aspect, the bioactive agent includes a polypeptide with a modified glycosylation profile. In one aspect, the bioactive agent includes an un-glycosylated polypeptide. In one aspect, the bioactive agent includes a chemically modified macromolecule, for example, a pegylated macromolecule or a macromolecule to which a diagnostic, targeting or therapeutic moiety has been appended. In one aspect, the bioactive agent is of therapeutic, scientific or commercial interest.

In one aspect, the bioactive agent includes an antibody or an antigen-binding fragment of an antibody. In one aspect, the bioactive agent includes an antibody-like protein. In one aspect, the bioactive agent includes a therapeutic antibody or antigen-binding antibody fragment. As used herein, the terms "antibody" and “immunoglobulin” can be used interchangeably and refer to a polypeptide or group of polypeptides that include at least one binding domain that is formed from the folding of polypeptide chains having three-dimensional binding spaces with internal surface shapes and charge distributions complementary to the features of an antigenic determinant of an antigen. A naturally occurring antibody typically has a tetrameric form, with two pairs of polypeptide chains, each pair having one "light" and one "heavy" chain. The variable regions of each light/heavy chain pair form an antibody binding site. Typically, each light chain is linked to a heavy chain by one covalent disulfide bond although the number of

disulfide linkages between the heavy chains of different immunoglobulin isotypes can vary. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has a variable domain (VH) at one end and constant domains (CH) at the other end. Each light chain has a variable domain at one end (VL) and a constant domain (CL) at the other end. The constant domain of the light chain is typically aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Light chains can be classified as either *lambda* chains or *kappa* chains based on the amino acid sequence of the light chain constant region. The variable domain of a *kappa* light chain may also be denoted as V_K. Similarly, the variable domain of a *lambda* light chain may also be denoted as V_λ.

Bioactive agents can include immunoglobulin molecules of any isotype (e.g., IgG, IgE, IgM, IgD, IgA and IgY), subisotype (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or allotype (e.g., Gm, e.g., G1m(f, z, a or x), G2m(n), G3m(g, b, or c), Am, Em, and Km(1, 2 or 3)). In one aspect, the bioactive agent is an antibody, an antigen-binding antibody fragment or an antibody-like molecule, such as a monoclonal antibody, for example, a full-length monoclonal antibody; a polyclonal antibody; a multispecific antibody that includes at least two different epitope binding fragments, for example, a bispecific antibody; a human antibody; a humanized antibody; a camelised antibody; a chimeric antibody; an anti-idiotypic (anti-Id) antibody; an intrabody; a diabody; a peptibody; a linear antibody; a single-chain antibody molecule; or an antigen binding fragment thereof. The terms "antigen-binding fragment" or "immunologically active fragment" refer to fragments of an antibody that contain at least one antigen-binding site and retain the ability to specifically bind to an antigen. Examples of antibody fragments that can be recombinantly produced include, but are not limited to, antibody fragments that include variable heavy- and light-chain domains, such as Fv, single-chain Fvs (scFv), single-chain antibodies, Fab fragments, Fab' fragments, $F(ab')_2$ fragments. Antibody fragments can also include epitope-binding fragments or derivatives of any of the antibodies enumerated above. In one aspect, the antibody is part of a fusion protein. In one aspect, the antibody is a covalently modified antibody. The antibody may be murine, rat, human, or any other origin and can include chimeric and humanized antibodies. In one aspect, the antibody is an antibody fragment that retains one or

more functional characteristics of the original antibody such as ligand binding or antagonist or agonist activity.

In one aspect, the bioactive agent includes the fragment crystallizable (Fc) region or domain of an IgG, which includes a paired set of antibody heavy chain domains, each of which include a heavy chain constant domain 2 (CH2) and a heavy chain constant domain 3 (CH3), which form a structure of about 50 kDa. The Fc region interacts with cell surface receptors called Fc receptors and some proteins of the complement system, allowing an antibody to activate the immune system.

In one aspect, the bioactive agent includes an antibody-drug conjugate (ADC) or an unconjugated antibody intermediate that is used to make an ADC. As used herein, "unconjugated antibody intermediate" refers to an antibody, for example, a monoclonal antibody, to which a biologically active molecule can be conjugated to form the ADC. Typically, the biologically active molecule is conjugated to the antibody intermediate through a linker. In one aspect, the linker is cleavable. In one aspect, the linker is non-cleavable. In one aspect, the biologically active molecule is conjugated to the antibody intermediate using site-specific conjugation. In one aspect, the biologically active molecule is conjugated to the antibody intermediate using non-site-specific conjugation. There are generally six types of biologically active molecules that are used in making ADC: small molecules, for example, highly potent cytotoxic agents that can be used to kill tumor cells; protein toxins, such as *Pseudomonas* or diphtheria toxin, that can be used to kill tumor cells; cytolytic immunomodulatory proteins, such as Fas ligand, that can be used to kill targeted cells; biologically active peptides, such as GLP-1, that can be used to extend the pharmacological half-life of the antibody; enzymes, such as urease, that can be used to modify the biochemistry of the targeted microenvironment; and radionuclides, such as ^{90}Y or ^{111}In, that can be used either to kill or image tumor cells.

In one aspect, the biologically active molecule conjugated to the antibody intermediate is a cytotoxic agent. As used herein, the term "cytotoxic agent" refers to a molecule that is detrimental to the growth, viability or propagation of cells. Examples of cytotoxic agents include, but are not limited to, mitomycin, doxorubicin, aminopterin, actinomycin, bleomycin, 9-amino-camptothecin, N^8-acetyl spermidine, 1-(2-chloroethyl)-1,2-dimethanesulfonyl hydrazide, tallysomycin, cytarabine, etoposide, camptothecin, taxol, esperamicin, Podophyllotoxin,

anguidine, vincristine, vinblastine, morpholine-doxorubicin, n-(5,5-diacetoxy-pentyl) doxorubicin, and derivatives thereof. Other cytotoxic agents are known and include, for example, those listed in Sapra et al., (2013) "Monoclonal antibody-based therapies in cancer: advances and challenges". *Pharmacol. & Therapeutics*, 138:452-469.

In one aspect, the ADC includes a monoclonal antibody that specifically binds to a cell surface antigen. In one aspect, the ADC includes a monoclonal antibody that specifically binds to a tumor-associated antigen. In one aspect, the tumor-associated antigen is selected from HER2, EGFR, CD70 (CD27L), CD33, CD19, Mesothelin, CD22, CEACAM5, Trop-2 (M1S1, TACSTD2 or GA733-1), PSMA, CD37, CD30, DLL3, GPNMB, CD79b, GCC, NaPi2b, CA6, CD74, CD138, BCMA, myeloma antigen, SLAMF7 (CS1), CD56, ENPP3 (CD203c), TF (CD142), TIM1, FOLR1, MUC16(CA-125), CanAg, Ckit (CD117 or SCFR), EphA2, Nectin 4(PVRL4), SLTRK6, HGFR (cMet), FGFR2, C4.41 (LYPD3), p-Cadherin (Cadherin 3), 5T4 (TPBG), STEAP1, PTK7, Ephrin-A4 (EFNA4) LIV1 (SLC39A6 or ZIP6), TENB2, ETBR, integrin V3, crypto antigen, AGS-5 (SLC44A4), LY6E, AXL (UFO), CD205, CD25 (IL-2R alpha), LAMP-1 or MN/CAIX.

In one aspect, the ADC includes a biologically active agent conjugated to a monoclonal antibody through a linker. In one aspect, the linker is non-cleavable. In one aspect, the linker is cleavable. Generally, cleavable linkers exploit the differences in intracellular pH, reduction potential or enzyme concentration to trigger the release of the biologically active agent in the target cell. Cleavable linkers can include chemically labile linkers such as acid-cleavable linkers or reducible linkers; and enzyme-cleavable linkers such as peptide-based linkers or β-glucuronide linkers. Cleavable linkers include, but are not limited to, linkers that include cleavable hydrazone, disulfide, peptide or thioether bonds. For example, cleavable linkers can include linker chemistries, including, but not limited to, hydrazone and hydrazide moieties, disulfide containing linkers such as N-succinimidyl-4-(2-pyridyldithio)pentanoate (SPP) and N-succinimidyl-4-(2-pyridyldithio)butyrate (SPDB), 4-(4'-acetylphenoxy)butanoic acid (AcBut) linkers, dipeptides valine-citrulline (Val-Cit) and phenylalanine-lysine (Phe-Lys) type linkers. Non-cleavable linkers rely on lysosomal proteolytic degradation of the antibody after internalization to release the biologically active molecule.

ADC present unique formulation challenges. Conjugation of a biologically active molecule to an antibody intermediate results in a molecule (the ADC) that can have different properties from the unconjugated antibody intermediate. For example, an antibody that exhibits acceptable aggregation behavior in its unconjugated form may behave differently in its conjugated form, for example, because of changes in surface properties or because the conjugate has altered the higher-order structure of the antibody. Although non-ionic surfactants such as polysorbate 80, polysorbate 20 and poloxamer 188 are widely used in biotherapeutic formulations to protect against aggregation and particle formation, these non-ionic surfactants are not easily removed from formulations, for example, using processes such as diafiltration. This can be problematic in the case of antibody intermediates used to generate antibody drug conjugates (ADC) because the presence of non-ionic surfactants may interfere with the conjugation process. Consequently, non-ionic surfactants such as polysorbate 80, polysorbate 20, and poloxamer 188 are often avoided in antibody intermediate formulations, despite the fact that formulations that lack a surfactant may be prone to particle formation. Advantageously, it has been found that inclusion of TPGS in an unconjugated antibody intermediate formulation does not interfere with the ADC conjugation process. In one aspect, a formulation is provided that includes an unconjugated antibody intermediate and TPGS as a surfactant.

In one aspect, the bioactive agent is an unconjugated antibody intermediate. In one aspect, the bioactive agent is a monoclonal antibody intermediate for use in forming an antibody drug conjugate (ADC). In one aspect, the bioactive agent is an antibody intermediate that specifically targets a tumour antigen that can be used to form an antibody drug conjugate (ADC) with a cytotoxic agent for use as an anti-cancer therapeutic.

In one aspect, the bioactive agent includes a therapeutic protein. In one aspect, the bioactive agent includes a naturally occurring therapeutic protein. In one aspect, the bioactive agent includes a recombinantly produced therapeutic protein. In one aspect, the bioactive agent includes a therapeutic protein such as an enzyme or enzymatically active polypeptide, a soluble receptor or receptor ligand, hormone, neurotransmitter, growth factor, integrin, interferon, an antigen, a secreted protein, or a fragment thereof. In one aspect, the therapeutic protein includes a fusion protein or a proteolysis targeting chimera (protac). In one aspect, the bioactive agent includes a therapeutic peptide. As used herein "therapeutic peptide" refers to a bioactive

polypeptide that includes less than about 500, about 250, about 100, about 50 or about 20 amino acids and has a molecular weight below about 100 kDa, or about 50 kDa.

In one aspect, the bioactive agent includes cells. In one aspect, the bioactive agent includes cells for use in cellular therapy. In one aspect, the bioactive agent includes whole, viable cells such as stem cells, progenitor cells or differentiated cells. In one aspect, the cells include pluripotent cells. In one aspect, the cells include multipotent cells. Examples of cells suitable for use in cellular therapy include, but are not limited to, hematopoietic stem cells (HSC), skeletal muscle stem cells, mesenchymal stem cells, lymphocytes, T-cells, dendritic cells, and pancreatic islet cells. In one aspect, the cells originate from the subject to be treated (i.e., autologous cells). In one aspect, the cells originate from a donor (i.e., allogeneic cells).

In one aspect, the bioactive agent includes a therapeutic polynucleotide, for example, a single-stranded or double-stranded polynucleotide such as deoxyribonucleotides (DNA), for example, cDNA or ribonucleotides (RNA) including, for example, RNA-based therapeutics such as antisense RNA, microRNAs (miRNAs), shorthairpin RNAs (shRNAs), RNA interference (RNAi), small interfering RNA (siRNA) and ribozymes. In one aspect, the bioactive agent includes plasmid DNA or an expression vector. In one aspect, the bioactive agent includes a plasmid, phagemid, cosmid or yeast artificial chromosomes (YAC). In one aspect, the bioactive agent includes a viral vector, including for example, retroviral, adenoviral, poxviral (e.g., vaccinia), adeno-associated viral (AAV), Newcastle disease viral (NDV) or a herpes simplex viral vector.

In one aspect, a pharmaceutical formulation is provided that includes an antibody or antigen binding fragment thereof. In one aspect, a pharmaceutical formulation is provided that includes a monoclonal antibody or antigen binding fragment thereof that specifically binds to IL-5Rα. The term "specifically binds" refers to the ability of an antibody or antigen binding fragment to recognize and bind to a target antigen, set of antigens or a domain or amino acid sequence within a target antigen with no or insignificant binding to other molecules in the sample. In one aspect, a pharmaceutical formulation is provided that includes Benralizumab or an antigen binding fragment thereof. As used herein, the term "Benralizumab", also known by its trade name Fasenra™ (MedImmune), refers to a human IgG antibody that binds IL-5Rα.

In one aspect, a pharmaceutical formulation is provided that includes a monoclonal antibody that specifically binds to IL-5Rα. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of a monoclonal antibody or antigen-binding antibody fragment that specifically binds to IL-5Rα; and from about 0.01% to about 0.1% TPGS. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL Benralizumab; and from about 0.01% to about 0.1% TPGS.

In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of a monoclonal antibody or antigen-binding antibody fragment that specifically binds to IL-5Rα; from about 0.01% to about 0.1% TPGS; and from about 10 mM to about 50 mM buffer. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of a monoclonal antibody or antigen-binding antibody fragment that specifically binds to IL-5Rα; from about 0.01% to about 0.1% TPGS; and from about 10 mM to about 50 mM histidine/histidine HCl. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of a monoclonal antibody or antigen-binding antibody fragment that specifically binds to IL-5Rα; about 0.02% TPGS; and about 20 mM histidine/histidine HCl. In one aspect, the pharmaceutical formulation has a pH from about 3 to about 9. In one aspect, the pharmaceutical formulation has a pH from about 4 to about 8. In one aspect, the pharmaceutical formulation has a pH from about 5.5 to about 7.5.

In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL Benralizumab; from about 0.01% to about 0.1% TPGS; and from about 10 mM to about 50 mM buffer. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL Benralizumab; from about 0.01% to about 0.1% TPGS; and from about 10 mM to about 50 mM histidine/histidine HCl. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL Benralizumab; about 0.02% TPGS; and about 20 mM histidine/histidine HCl. In one aspect, the pharmaceutical formulation has a pH from about 3 to about 9. In one aspect, the pharmaceutical formulation has a pH from about 4 to about 8. In one aspect, the pharmaceutical formulation has a pH from about 5.5 to about 7.5.

In one aspect, a pharmaceutical formulation is provided that includes a monoclonal antibody that specifically binds to IL-5Rα. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of a monoclonal antibody or antigen-binding antibody fragment that specifically binds to IL-5Rα; and from about 0.01% to about 0.1% TPGS 1000. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL Benralizumab; and from about 0.01% to about 0.1% TPGS 1000.

In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of a monoclonal antibody or antigen-binding antibody fragment that specifically binds to IL-5Rα; from about 0.01% to about 0.1% TPGS 1000; and from about 10 mM to about 50 mM buffer. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of a monoclonal antibody or antigen-binding antibody fragment that specifically binds to IL-5Rα; from about 0.01% to about 0.1% TPGS 1000; and from about 10 mM to about 50 mM histidine/histidine HCl. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL of a monoclonal antibody or antigen-binding antibody fragment that specifically binds to IL-5Rα; about 0.02% TPGS 1000; and about 20 mM histidine/histidine HCl. In one aspect, the pharmaceutical formulation has a pH from about 3 to about 9. In one aspect, the pharmaceutical formulation has a pH from about 4 to about 8. In one aspect, the pharmaceutical formulation has a pH from about 5.5 to about 7.5.

In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL Benralizumab; from about 0.01% to about 0.1% TPGS 1000; and from about 10 mM to about 50 mM buffer. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL Benralizumab; from about 0.01% to about 0.1% TPGS 1000; and from about 10 mM to about 50 mM histidine/histidine HCl. In one aspect, a stable pharmaceutical formulation is provided that includes from about 10 mg/mL to about 200 mg/mL Benralizumab; about 0.02% TPGS 1000; and about 20 mM histidine/histidine HCl. In one aspect, the pharmaceutical formulation has a pH from about 3 to about 9. In one aspect, the pharmaceutical formulation has a pH from about 4 to about 8. In one aspect, the pharmaceutical formulation has a pH from about 5.5 to about 7.5.

In one aspect, a method for preventing, treating, or managing an eosinophil mediated disorder or disease or one or more symptoms thereof, is provided. In one aspect, the method includes administering to a subject a pharmaceutical formulation that includes an antibody that specifically binds to human interleukin-5 receptor (IL-5Rα) and D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS 1000) as surfactant. In one aspect, the subject suffers from chronic obstructive pulmonary disease (COPD). In one aspect, the subject suffers from mild persistent or mild intermittent asthma.

Method of use

In one aspect, a method of treating or preventing a disease or disorder is provided. In one aspect, the method includes administering to a subject in need thereof a therapeutically effective amount of a pharmaceutical formulation that includes a bioactive agent and a surfactant that includes D-α-Tocopheryl polyethylene glycol succinate (TPGS) as described herein. In one aspect, the method includes administering to a subject in need thereof a therapeutically effective amount of a pharmaceutical formulation that includes a bioactive agent and a surfactant that includes D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS 1000). In one aspect, a pharmaceutical formulation that includes a bioactive agent and TPGS is provided for use as a medicament. In one aspect, a pharmaceutical formulation that includes a bioactive agent and TPGS is provided for the preparation of a medicament for treating a disease or disorder in a subject. In one aspect, the pharmaceutical formulation is a liquid, for example, an aqueous solution. In one aspect, the pharmaceutical formulation is a lyophilized powder that can be reconstituted into a suitable solution for administration by adding an appropriate diluent.

In one aspect, the subject is selected from a rodent (such as a mouse or rat), rabbit, a companion animal (such as a cat, dog and horse), a farm animal (such as a cow, sheep, pig and goat), or a primate (such as a human or non-human primate). In one aspect, the subject is a human.

In one aspect, the pharmaceutical formulation is administered parenterally. In one aspect, the pharmaceutical formulation is administered intravenously, intramuscularly or subcutaneously. In one aspect, the pharmaceutical formulation is administered intravenously as a bolus or as continuous infusion over a period of time. Suitable devices for parenteral administration include needles (including microneedles, microprojections, soluble needles and

other micropore formation techniques), injectors, needle-free injectors and other infusion techniques.

Article of Manufacture

In one aspect, an article of manufacture is provided. In one aspect, the article of manufacture includes a device or container that contains a pharmaceutical formulation that includes a bioactive agent and a surfactant that includes D-α-Tocopheryl polyethylene glycol succinate (TPGS) as described herein. In one aspect, the article of manufacture includes a device or container that contains a pharmaceutical formulation that includes a bioactive agent and a surfactant that includes D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS 1000). In one aspect, the container or device is a syringe, for example, a pre-filled syringe; an auto injector; bottle; vial; or test tube. In one aspect, the article of manufacture includes a device or container that contains the pharmaceutical formulation and a label on, or associated with, the device or container that provides directions for use. In one aspect, the article of manufacture further includes other materials desirable from a commercial or user standpoint, including buffers, diluents, filters, needles, syringes, or package inserts with instructions for use.

In one aspect, a kit is provided. In one aspect, the kit includes at least one device or container that contains a pharmaceutical formulation that includes a bioactive agent and a surfactant that includes D-α-Tocopheryl polyethylene glycol succinate (TPGS) as described herein. In one aspect, the kit includes at least one device or container that contains a pharmaceutical formulation as described herein and an injection device. In one aspect, the injection device is adapted for intravenous, intramuscular or subcutaneous administration. In one aspect, the kit includes instructions for administration of the composition.

Incorporation by Reference

All references cited herein, including patents, patent applications, papers, text books and the like, and the references cited therein, to the extent that they are not already, are hereby incorporated herein by reference in their entirety.

EXAMPLES

Example 1 – surfactant screening

In this example, the stability of four different antibodies was evaluated using three different surfactants and a no-surfactant control.

(mAb-1) a human anti-Ang2-TNFa bispecific antibody;

(mAb-2) a bivalent, bispecific human IgG1κ monoclonal antibody that binds to both the *Pseudomonas aeruginosa* PcrV type III secretion (T3S) protein involved in host cell cytotoxicity and the Psl exopolysaccharide involved in *Psuedomonas aeruginosa* colonization and tissue adherence;

(mAb-3) an anti-interleukin 13 IgG1 monoclonal antibody with YTE amino acid substitutions in the crystallizable fragment (Fc) domain; and

(mAb-4) an anti-influenza A IgG1monoclonal antibody that specifically binds to influenza A hemagglutinin stalk.

Each were formulated in 3cc glass vials (1.1 mL volume) as described in the table below.

Molecule	**Formulation**	**Concentration**	**Molecule type**
mAb-1	25mM His/His-HCl, 235mM Sucrose, pH 6	50 mg/mL	Bi-specific mAb
mAb-2	25mM His/His-HCl, 8% Glycine, 4% Arg-HCl, pH 6	100 mg/mL	Bi-specific mAb
mAb-3	25 mM His/His-HCl, 190 mM Arg-HCl, pH 6.0	150 mg/mL	mAb IgG1
mAb-4	25 mM His/His-HCl 235 mM Sucrose, pH 6.0	50 mg/ml	mAb IgG1

In separate 1.1 mL aliquots of the formulations containing test molecules, 0.02% TPGS (Vitamin E succinate with PEG-1000), 0.02% PS80 (polysorbate 80), 0.02% Poloxamer 188 (P188), 0.02% Kolliphor HS15 (Polyoxyl 15 Hydroxystearate), 0.02% Kolliphor EL (PEG-35 Castor Oil), 0.02% Kolliphor RH40 (PEG-40 Hydrogenated Castor Oil), or 1% PEG 300 (Polyethylene glycol 300) were added. A separate vial in which no surfactant was added was

used as a control. Vials were subjected to three freeze-thaw cycles (3FT), and a shipping simulation procedure or vortex shaking for 2.5 h, respectively. Test molecule stability was assessed by visual inspection, micro-flow imaging (MFI), and high-pressure size exclusion chromatography (HPSEC).

Visual inspection of the samples was performed by examining the samples in their respective container for particles, color, and clarity using appropriate standards.

Detection of non-visible particles was performed using Micro Flow Imaging (MFI) using the 5200 series Micro-Flow Imaging system (available from Protein Simple) in accordance with the supplier's instructions.

HPSEC was performed using an Agilent HPLC system (Santa Clara, CA) with a TSK-Gel G3000 column for analysis. Briefly, 100 µg of antibody was loaded on the column by injecting 25 µl of the antibody diluted to 10 mg/ml in phosphate buffered saline. Standard integration parameters were used for automatic integration of aggregate, monomer and fragment peaks.

Test molecule stability was assessed by visual inspection, MFI, and HPSEC. Results from time zero (T0) and post shipping simulation (SS) are shown in FIGS. 2 (MFI) and 3 (HPSEC) as examples.

In the initial stability screen for the test molecules formulated in TPGS, Kolliphor HS15, Kolliphor EL, Kolliphor RH40, PEG 300, PS80, P188, or no surfactant (control), the MFI results in FIG. 2 demonstrate that among all particle sizes assessed (≥1 µm <2 µm, ≥2 µm, ≥10 µm, and ≥25 µm), the control group containing no surfactant consistently displayed the highest numbers of particles/mL compared to TGPS, PS80, and P188. The performance of the individual surfactants varied depending on the size of particle assessed. TPGS showed consistently low amounts of particles/mL compared to other surfactants across the entire range of particle sizes, most notably in the ≥1 µm <2 µm and ≥2 µm categories.

FIG. 3 shows the percentage of monomers (a metric of stability, or non-aggregation) of each test molecule at T0 or after SS as measured by HPSEC. Based on these data, for all test molecules, the formulations containing different surfactant types showed comparable HPSEC monomer percentages (%monomer) compared to the control.

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Example 2 – Stressed, accelerated and long-term stability

To determine the effects of different surfactants on the stressed, accelerated and long term stability of the four test molecules, formulations of mAb-1, mAb-2, mAb-3, and mAb-4 at a concentration range of 50-150 mg/mL were spiked with 0.02% TPGS, PS80, or P188 and stored in 3cc glass vials (with 1.3 mL fill) and stored at four different temperatures: 40°C, 25°C, 2-8°C, and -80°C. The stability of the formulations of test molecules containing surfactants was assessed via visual inspection, MFI, and HPSEC.

Test molecule stability was assessed by visual inspection, MFI, and HPSEC. Results are reported in FIGS. 4-11.

In FIG. 4A-D, the stressed stability of mAb-1, mAb-2, mAb-3, and mAb-4 formulated with TPGS, PS80, and P188 is shown as assessed by MFI after storage at 40°C for 4 weeks. Most notably, mAb-3 appears to be less stable (high particles/mL at all measured sizes) when formulated with PS80 under these conditions, while all four of the test molecules are stable when formulated with TPGS.

FIG. 5 A-D show HPSEC data for mAb-1, mAb-2, mAb-3, and mAb-4 formulated with TPGS, PS80, and P188 and stored at 40°C over a period of 0-4 weeks. mAb-4 formulated in TPGS shows minimal %monomer loss (0.27% loss per month) after 4 weeks stored at 40°C (reported as % monomers) compared to approximately 1% and 1.6% monomer loss per month when formulated in PS80 and P188, respectively. The type of surfactant used in the formulation of mAb-1, mAb-2, and mAb-3 did not appear to significantly affect the decrease in % monomers present in the solution after 4 weeks storage.

In FIG. 6 A-D, the accelerated stability of mAb-1, mAb-2, mAb-3, and mAb-4 formulated with TPGS, PS80, and P188 is shown as assessed by MFI after storage at 25°C for 6 months. As in Figure 4, mAb-3 appears to be highly unstable when formulated with PS80 under these conditions, while all four of the test molecules are highly stable when formulated with TPGS. Decreased levels of stability were also observed with P188, most notably mAb-2 and mAb-4.

In FIG. 7A-D, HPSEC data are shown for mAb-1, mAb-2, mAb-3 and mAb-4 formulated with TPGS, PS80, and P188 and stored at 25°C over a period of 0-6 months. The type of

surfactant used in the formulation of mAb-1, mAb-2, mAb-3, and mAb-4 did not appear to significantly affect % monomers present in the solution at 6 months.

In FIG. 8A-D, MFI data are shown for mAb-1, mAb-2, mAb-3, and mAb-4 formulated with TPGS, PS80, and P188 after storage at 5°C for 6 months. As in the previous Figures, the TPGS formulations of the four test molecules appeared to be more stable compared to the PS80 and P188 surfactant groups, especially for mAb-2 and mAb-3.

FIG. 9A-D shows the HPSEC data for mAb-1, mAb-2, mAb-3, and mAb-4 formulated with TPGS, PS80, and P188 after storage at 5°C for 0-6 months. The TPGS formulations showed comparable stability to PS80 and P188.

In FIG. 10A-D, the long-term stability of mAb-1, mAb-2, mAb-3, and mAb-4 formulated with TPGS, PS80, and P188 after storage at -80°C for 6 months as assessed by MFI are shown. In each surfactant group, mAb-3 displayed a slightly higher number of particles/mL compared to other test molecules.

FIG. 11 A-D show the HPSEC data for mAb-1, mAb-2, mAb-3, and mAb-4 formulated with TPGS, PS80, and P188 after storage at -80°C for 0-6 months. The TPGS formulations showed comparable stability to PS80 and P188.

Example 3 – Concentration range of TPGS formulation for mAb-2 and mAb-4

To determine the effects of different concentrations of TPGS on stability, mAb-2 (100 mg/mL, formulated in 25 mM His-His/HCl, 8% Gly, 4% Arg-HCl) and mAb-4 (50 mg/mL, formulated in 25 mM His-His/HCl, 235, mM sucrose) were spiked with 0.001%, 0.01%, 0.02%, 0.05%, 0.10%, 1.00% or 5.00% of TPGS, 0.02% PS80, 0.02% PS20, or 0.02% P188and stored in 3cc glass vials with 1.3 mL fill. The formulations were subjected to three freeze-thaw cycles and shipping simulation (SS). Stability of the formulations was assessed via visual inspection, MFI, HPSEC, and Capillary Gel Electrophoresis (CGE).

Test molecule stability was assessed by visual inspection, MFI, Horizon, HPSEC, and CGE. Results are reported in FIGS. 12-16.

In FIG. 12A-D, data comparing the stability of surfactant formulations of mAb-2 at time zero (T0) to post-shipping simulation (SS) as assessed via MFI are shown. TPGS started to show significant effect in reducing particles from a level as low as 0.01%. The particle count of the formulation with 0.01% TPGS is significantly less than formulations containing PS80, PS20 and

P188 with a higher surfactant level (0.02%). The effect of TPGS was demonstrated at higher concentrations from 0.02% to 5.00%. All showed a reduced level of particles, which were significantly better than the other surfactants.

FIG. 13 shows data comparing the stability of surfactant formulations of mAb-2 at time zero (T0) to post-shipping simulation (SS) as assessed via HPSEC. The TPGS formulation ranges of 0.01-0.10% displayed comparable purity profiles among themselves and also to the formulations containing 0.02% of PS80, PS20 and P188. However, at the higher concentration of TPGS (1%), the percentage of monomers appeared to decrease with an increased level of %HMW. However, this apparent increase in %HMW is not, in fact, due to an increase in aggregation, but rather to interference of TPGS eluting at similar retention time.

FIG. 14 shows overlaid HPSEC chromatograms of blank formulation buffer containing different levels of TPGS (from 0.001% to 1.00%). As the TPGS concentration increases, the area of the peak increases which results in an increased interference with the mAb chromatograms (e.g. 1.00% TPGS). At lower concentrations (e.g., 0.001% to 0.10%), the interreference from TPGS is negligible.

FIG. 15 shows overlaid HPSEC chromatograms of blank formulation buffer containing 1.00% TPGS with mAb-2 samples containing 0.02% or 1.00% of TPGS. The TPGS eluted at similar retention times as the aggregate peak of the mAb. When TPGS was used at 1.00%, it interfered with the mAb chromatograms leading to an overestimation of %HMW and an apparent decrease in % monomer.

Capillary gel electrophoresis (CGE) was used to assess the stability of the surfactant formulations as an orthogonal method. As shown in FIG. 16, formulations containing different levels of TPGS (from 0.001% to 1.00%) all had similar values, and were similar to formulations containing 0.02% of PS80, PS20 and P188 in both the non-reduced and reduced conditions. The CGE data confirmed that the apparent reduction in % monomer for the 1.00% TPGS formulation observed by HPSEC was, in fact, due to interreference of TPGS and not aggregation.

In FIG.17A-D, data comparing the stability of surfactant formulations of mAb-4 at time zero (T0) to post-shipping simulation (SS) as assessed via MFI are shown. The presence of TPGS in the formulation reduced particles starting from 0.01% TPGS. At 0.02% TPGS, the formulations showed less or comparable particle counts compared to formulation containing

PS80 at the same level, and significantly less particle counts than formulations containing PS20 or P188. As % TPGS was increased, the particle numbers decreased further showing a promising effect of TPGS overall in reducing particles.

FIG. 18 shows data comparing the stability of surfactant formulations of mAb-4 at time zero (T0) to post-shipping simulation (SS) as assessed via HPSEC. Similar to mAb-2, the TPGS formulation ranges of 0.01-0.10% displayed comparable purity profiles among themselves and also to the formulations containing 0.02% of PS80, PS20 and P188. At 1.00% TPGS, the formulation appeared to show a decrease in % monomer. However, this was due to interreference of TPGS with the chromatogram, rather than an increase in aggregation.

FIG. 19 shows the results from capillary gel electrophoresis (CGE) that was used to assess the stability of the surfactant formulations of mAb-4 and similar to mAb-2 above, formulations containing different levels of TPGS (from 0.001% to 1.00%) all showed similar values, and were similar to formulations containing 0.02% of PS80, PS20 and P188 in both non-reduced and reduced conditions.

Example 4 – Evaluation of stability of mAb-5 and mAb-6 formulated in TPGS

In this example, the stability of two antibodies was determined:
(mAb-5) a biparatopic monospecific antibody intermediate targeting HER2; and
(mAb-6) an antibody intermediate targeting B7H4.

To determine the stability of mAb-5 and mAb-6 (20 mg/mL, formulated in 20 mM His-His HCL, 240 mM sucrose, pH 6), C-Pak bags (30 mL) containing each test molecule were spiked with 0.02% TPGS. The formulations were subjected to three freeze-thaw (3xFT) cycles and shipping simulation. Stability of the formulations was assessed at time zero (T0), post-3xFT, and post-shipping simulation via visual inspection, MFI, HPSEC, and UV absorption at 280 nm (A280).

Stability of mAb-5 and mAb-6 were assessed by visual inspection, MFI, and HPSEC. Key results are reported in FIGS. 20-23.

FIG. 20A-B shows the comparative stability of test molecules mAb-5 and mAb-6 formulated in 0.02% TPGS or control at time zero (T0), after 3 freeze-thaw cycles (3xFT) and post-shipping simulation (SS) as measured by MFI. In each of the three time points for both test

molecules, the TPGS sample showed a marked decrease in the number of particles compared to the control sample.

FIG. 21 represents the mAb-5 stability MFI data from FIG. 20A in graphical format.

FIG. 22 represents the mAb-6 stability MFI data from FIG. 20B in graphical format.

FIG. 23 shows the stability of mAb-5 and mAb-6as measured by HPSEC at T0, after 3xFT, and post-SS. The presence of TPGS in the formulation of either test molecule did not appear to significantly influence the stability compared to control at any point in the study.

WHAT IS CLAIMED IS:

1. A stable pharmaceutical formulation comprising:

(a) a bioactive agent;

(b) a buffer; and

(c) a surfactant comprising D-α-Tocopheryl polyethylene glycol succinate (TPGS),

wherein the formulation has a pH from about 3 to about 9, about 4 to about 8 or about 5.5 to about 7.5.

2. The formulation of claim 1, comprising a liquid formulation or a lyophilized formulation.

3. The formulation of claim 1, wherein the bioactive agent comprises a therapeutic polypeptide.

4. The formulation of claim 3, wherein the therapeutic polypeptide is selected from an antibody or antigen-binding antibody fragment, an enzyme or enzymatically-active polypeptide, a soluble receptor or receptor ligand, hormone, neurotransmitter, growth factor, integrin, interferon or an antigen.

5. The formulation of claim 4, wherein the antibody or antigen-binding antibody fragment comprises a monoclonal antibody.

6. The formulation of claim 3 or 4, wherein the antibody or antigen-binding antibody fragment is selected from human, humanized, chimeric, multispecific, bispecific, Fab', F(ab')2, Fv, single chain Fv (scFv), diabodies, peptibodies, linear antibodies and single-chain antibodies.

7. The formulation of claim 3 or 4, wherein the therapeutic polypeptide comprises a fusion polypeptide, a proteolysis targeting chimera (protac), or an antibody-drug conjugate (ADC).

8. The formulation of claim 1, wherein the bioactive agent comprises a therapeutic polynucleotide.

9. The formulation of claim 8, wherein the therapeutic polynucleotide comprises single-stranded or double-stranded DNA or RNA.

10. The formulation of claim 8 or 9, wherein the therapeutic polynucleotide is selected from cDNA, antisense RNA, microRNA (miRNA), shorthairpin RNA (shRNA), RNA interference (RNAi), small interfering RNA (siRNA) and ribozymes.

11. The formulation of any of claims 8 to 10, wherein the therapeutic polynucleotide comprises a plasmid, phagemid, cosmid or yeast artificial chromosomes (YAC).

12. The formulation of any of claims 8 to 11, wherein the therapeutic polynucleotide comprises a retroviral, adenoviral, poxviral, adeno-associated viral (AAV), Newcastle disease viral (NDV) or herpes simplex viral vector.

13. The formulation of claim 1, wherein the bioactive agent comprises cells selected from stem cells, progenitor cells and differentiated cells.

14. The formulation of claim 1, wherein the formulation comprises the bioactive agent at a concentration from about 0.1 mg/mL to about 300 mg/mL.

15. The formulation of claim 14, wherein the bioactive agent comprises an antibody or antigen-binding antibody fragment with a concentration from about 10 mg/mL to about 200 mg/mL.

16. The formulation of claim 15, wherein the concentration of antibody or antigen-binding antibody fragment is at least about 10 mg/mL, about 15 mg/mL, about 20 mg/mL, about 25 mg/mL, about 50 mg/mL, about 75 mg/mL or about 100 mg/mL and up to about 125 mg/mL, about 150 mg/mL, about 175 mg/mL, about 200 mg/mL, 2 about 50 mg/mL or 3 about 00 mg/mL.

17. The formulation of claim 14, wherein the bioactive agent comprises a therapeutic protein with a concentration from about 0.1 mg/mL, about 0.5 mg/mL, about 1 mg/mL, about 2 mg/mL, about 3 mg/mL, about 4 mg/mL or about 5 mg/mL and up to about 10 mg/mL, about 15 mg/mL, about 20 mg/mL, about 25 mg/mL, about 30 mg/mL, about 35 mg/mL, about 35 mg/mL, about 40 mg/mL, about 45 mg/mL or about 50 mg/mL.

18. The formulation of any of the preceding claims, wherein the concentration of TPGS is from about 0.001% to about 1%.

19. The formulation of claim 18, wherein the concentration of TPGS is from about 0.001%, about 0.005%, about 0.01%, about 0.05% and up to about 0.1%, about 0.5% or about 1%.

20. The formulation of claim 18 or 19, wherein the concentration of TPGS is 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% or about 0.1%.

21. The formulation of any of the preceding claims, wherein the buffer is selected from acetate, acetic acid, succinate, succinic acid, phosphate, phosphoric acid, ascorbate, ascorbic acid, lactate, lactic acid, tartartic acid, maleic acid, glycine, gluconate, citrate, histidine, imidazole, bicarbonate and carbonic acid, sodium benzoate, benzoic acid, edetate, malate, tris, glycylglycine and mixtures thereof.

22. The formulation of claim 21, wherein the buffer comprises histidine/histidine hydrochloride, tris/tris hydrochloride, citrate, sodium acetate, phosphate, or a combination thereof.

23. The formulation of the preceding claims, wherein the buffer has a concentration from about 0.1 mM to about 100 mM.

24. The formulation according to claim 23, wherein the buffer concentration is from about 0.1 mM, about 0.5 mM, about 1 mM, about 5 mM, about 10 mM, about 20 mM or about 25 mM and up to about 30 mM, about 35 mM, about 40 mM, about 45 mM, or about 50 mM.

25. The formulation according to claim 23 or 24, wherein the buffer comprises about 0.5, about 1 mM, about 5 mM, about 10 mM, about 20 mM, about 25 mM, about 30 mM, about 35 mM, about 40 mM, about 45 mM or about 50 mM histidine/histidine HCl.

26. The formulation of any of the preceding claims, further comprising a tonicity agent.

27. The formulation of claim 26, wherein the tonicity agent comprises a polyol, a saccharide, a carbohydrate, a salt, or a mixture thereof.

28. The formulation of claim 26 or 27, wherein the formulation includes the tonicity reagent at a concentration from about 1 mg/ml to about 300 mg/ml, about 10 mg/ml to about 200 mg/ml or about 50 mg/ml to about 100 mg/ml.

29. The formulation of any of claims 26 to 28, wherein the tonicity reagent comprises a saccharide at a concentration of about 80 mg/ml to about 90 mg/ml.

30. The formulation of any of claims 26 to 28, wherein the tonicity reagent comprises a salt at a concentration from about 1 mg/mL to about 20 mg/ml.

31. The formulation of any of the preceding claims, further comprising an amino acid as an excipient.

32. The formulation of claim 31, wherein the amino acid is selected from arginine, cysteine, glycine, lysine, glycine, ornithine, proline, alanine, glutamine, glutamic acid, histidine, or a salt or combination thereof.

33. The formulation of any of the preceding claims, further comprising a chelating agent.

34. The formulation of claim 17, wherein the chelating agent is selected from aminopolycarboxylic acids, hydroxyaminocarboxylic acids, N-substituted glycines, 2-(2-amino-2-oxocthyl) aminoethane sulfonic acid (BES), deferoxamine (DEF), citric acid, niacinamide, and desoxycholates and mixtures thereof.

35. The formulation of claim 17, wherein the chelating agent is selected from ethylenediaminetetraacetic acid (EDTA), diethylenetriamine pentaacetic acid (DTPA), nitrilotriacetic acid (NTA), N-2-acetamido-2-iminodiacetic acid (ADA), bis(aminoethyl)glycolether, N,N,N′,N′-tetraacetic acid (EGTA), trans-diaminocyclohexane tetraacetic acid (DCTA), glutamic acid, and aspartic acid, N-hydroxyethyliminodiacetic acid (HIMDA), N,N-bis-hydroxyethylglycine (bicine) and N-(trishydroxymethylmethyl) glycine (tricine), glycylglycine, sodium desoxycholate, ethylenediamine; propylenediamine; diethylenetriamine; triethylenetetraamine (trien), ethylenediaminetetraaceto EDTA; disodium EDTA, calcium EDTA oxalic acid, malate, citric acid, citric acid monohydrate, and trisodium citrate-dihydrate, 8-hydroxyquinolate, amino acids, histidine, cysteine, methionine, peptides, polypeptides, and proteins and mixtures thereof.

36. The formulation of claim 33 or 34, wherein the concentration of the chelating agent is from about 0.01 mg/ml to about 50 mg/ml.

37. A stable pharmaceutical formulation comprising:

(a) from about 0.1 mg/mL to about 300 mg/mL bioactive agent;

(b) from about 10 mM to about 50 mM buffer;

(c) from about 0.01% to about 0.1% TPGS,

wherein the formulation has a pH from about 3 to about 9.

38. The formulation of claim 37, wherein the buffer comprises histidine/histidine HCl.

39. The formulation of claim 38, comprising from about 20 mM to about 30 mM histidine/histidine HCl.

40. The formulation of claim 37, wherein the formulation has a pH from about 4 to about 8.

41. The formulation of claim 37, wherein the formulation has a pH from about 5.5 to about 7.5.

42. The formulation of claim 37, comprising from about 10 mg/mL to about 200 mg/mL of an antibody or antigen-binding antibody fragment.

43. The formulation of claim 37, comprising from about 0.1 mg/mL to about 50 mg/mL of a therapeutic protein.

44. The formulation of any of the preceding claims, comprising less than about 10,000, about 5,000, about 1,000, about 750, about 500, about 250, about 150, about 100, or about 50 particles greater than about 2 μm, about 5 μm, about 10 μm, about 15 μm, about 20 μm or about 25 μm diameter/mL.

45. The formulation of any of the preceding claims, wherein the formulation is stable at a temperature of about 40°C for up to about 3 months.

46. The formulation of any of the preceding claims, wherein the formulation is stable at a temperature of about 40°C for up to about 6 months.

47. The formulation of any of the preceding claims, wherein the formulation is stable at a temperature of about 25°C for up to about 6 months.

48. The formulation of any of the preceding claims, wherein the formulation is stable at a temperature of about 25°C for up to about 12 months.

49. The formulation of any of the preceding claims, wherein the formulation is stable at a temperature of about 2°C to about 8°C for up to about 12 months.

50. The formulation of any of the preceding claims, wherein the formulation is stable at a temperature of about 2°C to about 8°C for up to about 24 months.

51. The formulation of any of the preceding claims, wherein the formulation is stable at a temperature of about 2°C to about 8°C for up to about 36 months.

52. The formulation of any of the preceding claims, wherein the formulation is stable at a temperature of about -20°C for up to about 6 months.

53. The formulation of any of the preceding claims, wherein the formulation is stable at a temperature of about -20°C for up to about 12 months.

54. The formulation of any of the preceding claims, wherein the formulation is stable at a temperature of about -20°C for up to 2 about 4 months.

55. The formulation of any of the preceding claims, wherein the formulation is stable at a temperature of about -20°C for up to about 36 months.

56. The formulation of any of the preceding claims, wherein the formulation is stable at a temperature of about -80°C for up to about 6 months.

57. The formulation of any of the preceding claims, wherein the formulation is stable at a temperature of about -80°C for up to about 12 months.

58. The formulation of any of the preceding claims, wherein the formulation is stable at a temperature of about -80°C for up to about 24 months.

59. The formulation of any of the preceding claims, wherein the formulation is stable at a temperature of about -80°C for up to about 36 months.

60. A method of reducing particle formation in an aqueous pharmaceutical formulation, the method comprising adding D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS) to the formulation, wherein less than about 10,000, about 5,000, about 1,000, about 750, about 500, about 250, about 150, about 100, or about 50 particles greater than about 2 μm, about 5 μm, about 10 μm, about 15 μm, about 20 μm or about 25 μm diameter/mL are formed.

61. The method according to claim 60, wherein the pharmaceutical formulation comprises less than about 5,000 particles greater than about 2 μm in diameter/mL.

62. The method according to claim 60, wherein the pharmaceutical formulation comprises less than about 1,000 particles greater than about 2 μm in diameter/mL.

63. The method according to claim 60, wherein the pharmaceutical formulation comprises less than about 6,000 particles greater than about 10 μm in diameter/mL.

64. The method according to claim 60, wherein the pharmaceutical formulation comprises less than about 600 particles greater than about 25 μm in diameter/mL.

65. The method according to claim 60, wherein D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS) is added to the formulation in an amount ranging from about 0.001% to about 1%.

66. A method of reducing interference during antibody-drug (ADC) conjugation comprising, adding D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS) to a formulation comprising an unconjugated antibody intermediate.

67. The method according to claim 66, wherein D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS) is added to the formulation in an amount ranging from about 0.001% to about 1%.

FIG. 1

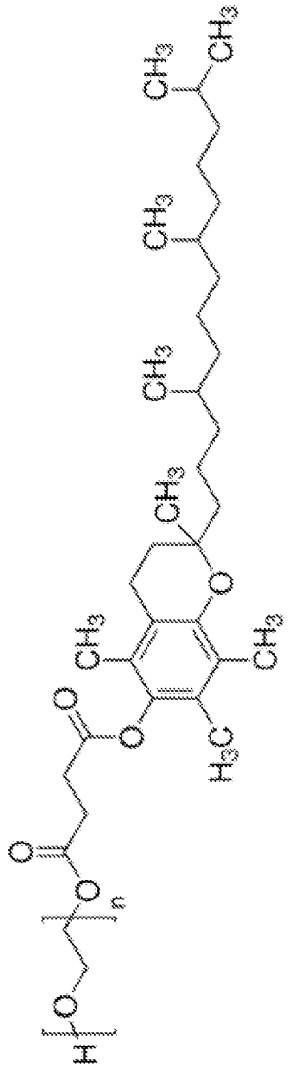


FIG. 2A

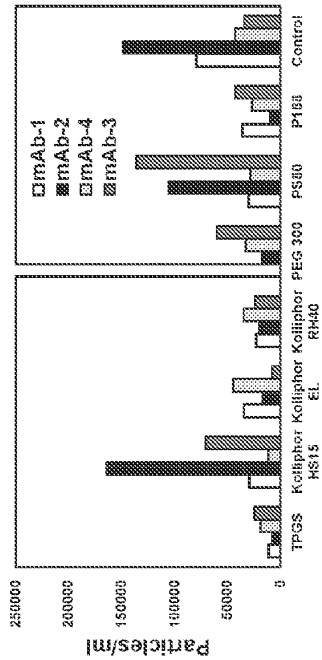


FIG. 2B

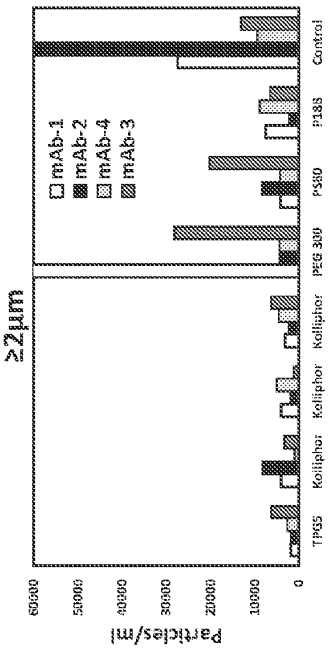


FIG. 2C

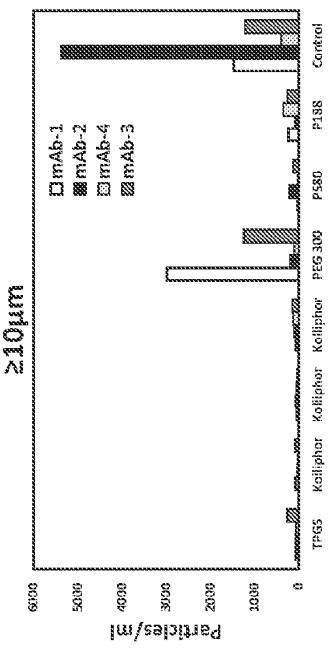
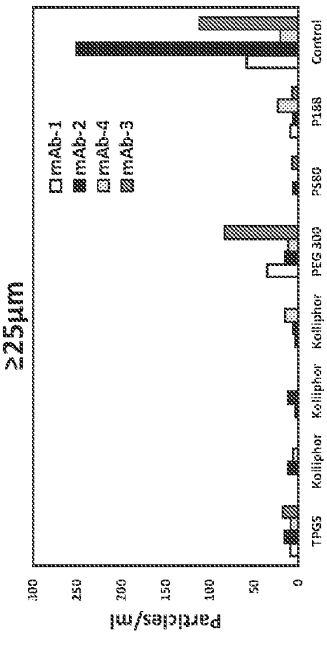


FIG. 2D



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FIG. 3

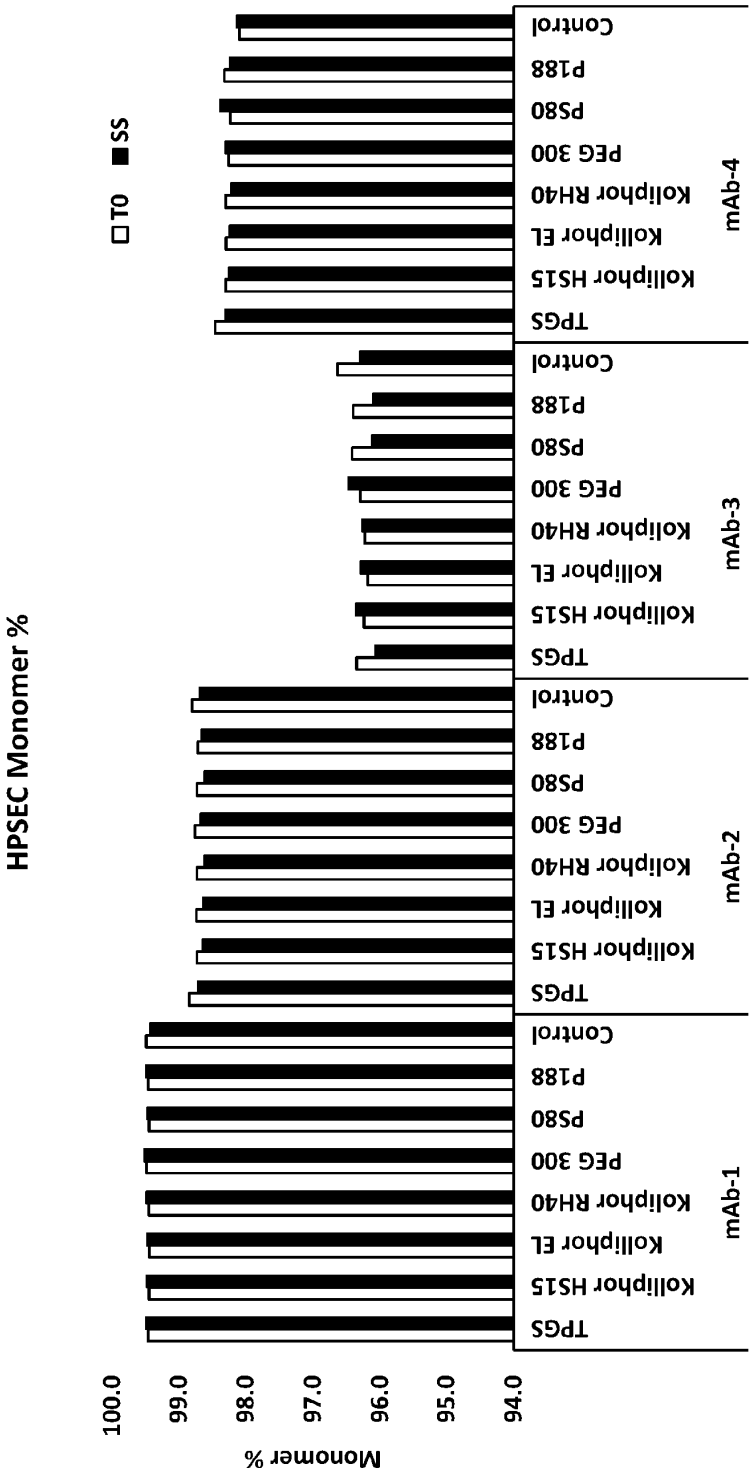

HPSEC Monomer %
T0
SS
Monomer %
100.0
99.0
98.0
97.0
96.0
95.0
94.0
mAb-1
mAb-2
mAb-3
mAb-4
TPGS
Kolliphor HS15
Kolliphor EL
Kolliphor RH40
PEG 300
PS80
P188
Control

FIG. 4A

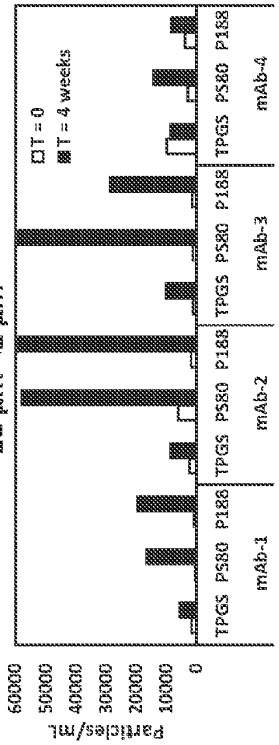


FIG. 4B

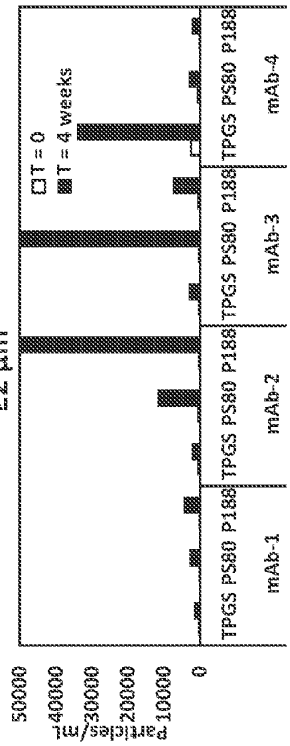


FIG. 4C

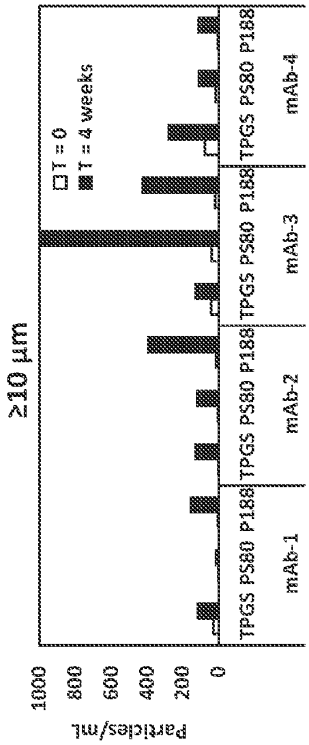
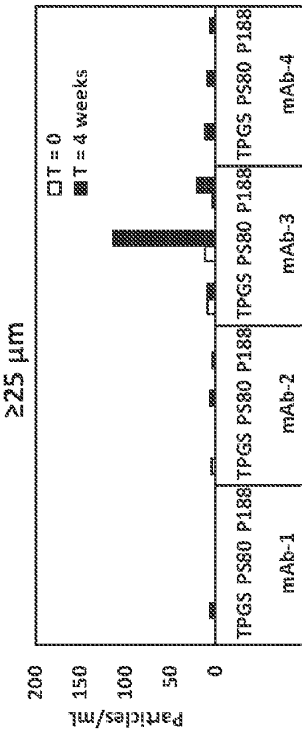


FIG. 4D



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FIG. 5A

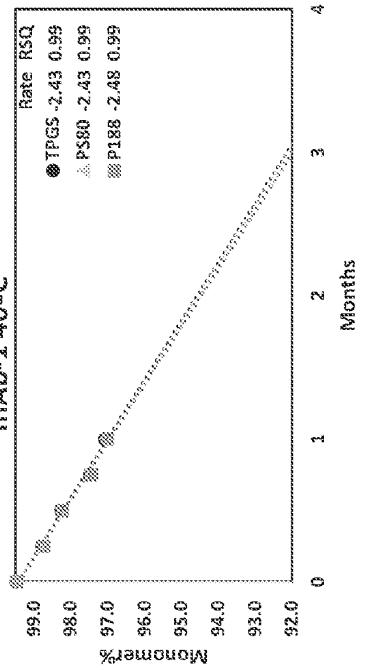


FIG. 5B

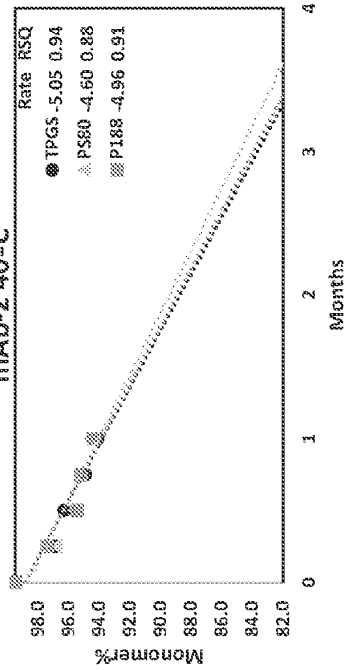


FIG. 5C

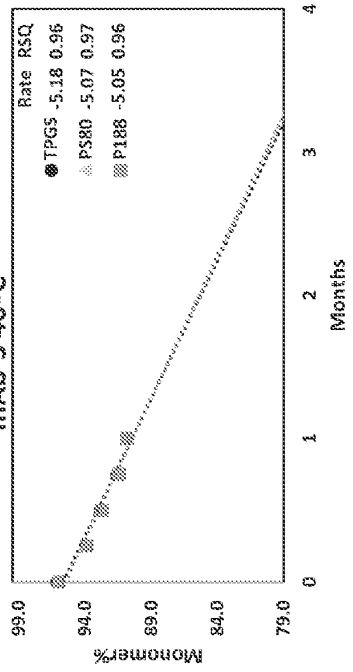


FIG. 5D

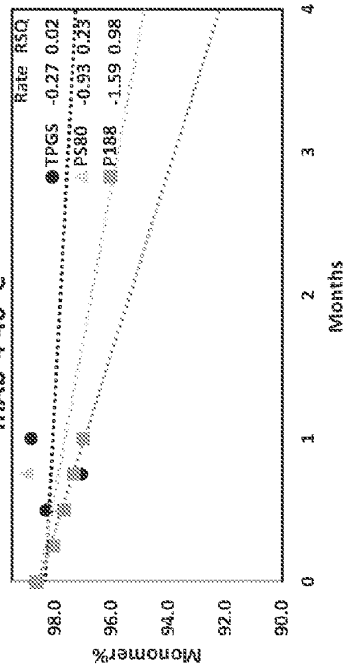


FIG. 6A

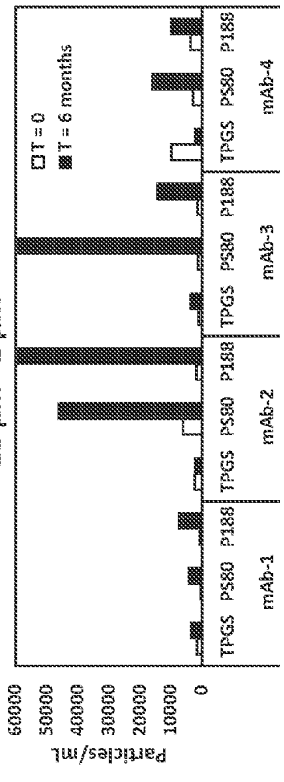


FIG. 6B

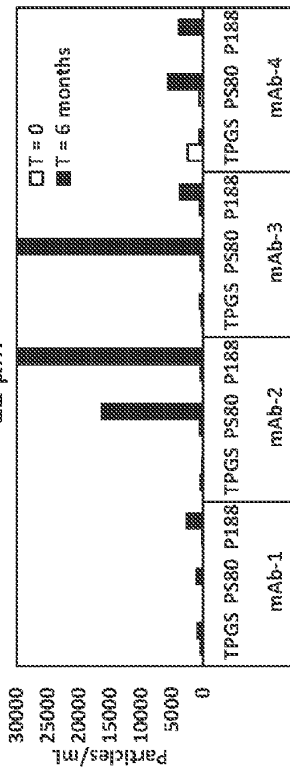


FIG. 6C

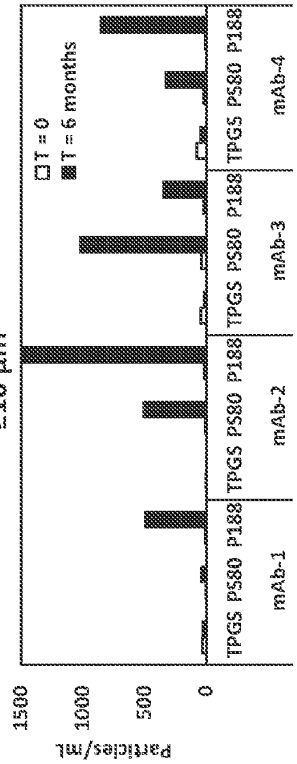


FIG. 6D

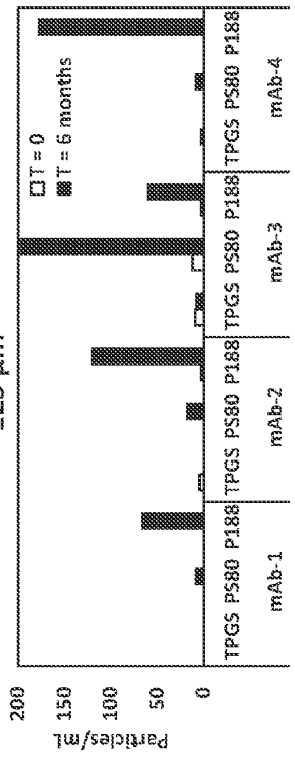


FIG. 7A

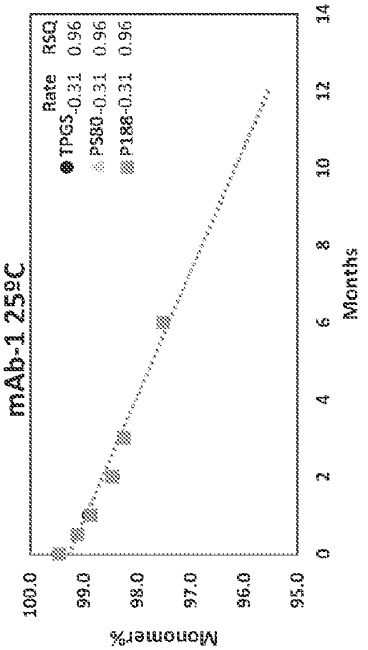


FIG. 7B

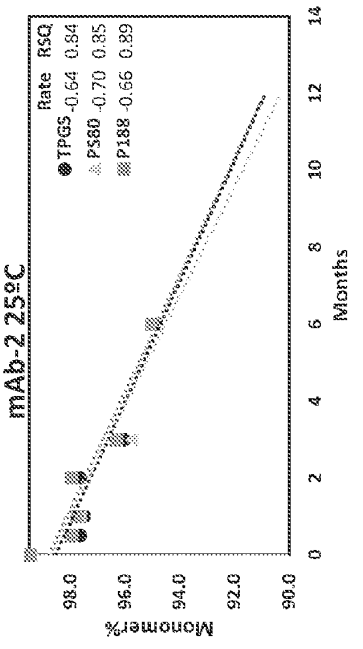


FIG. 7C

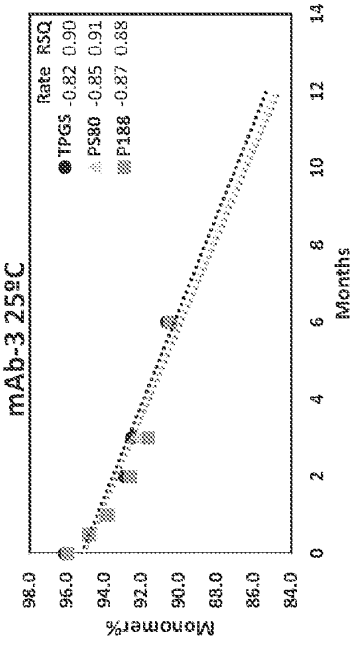


FIG. 7D

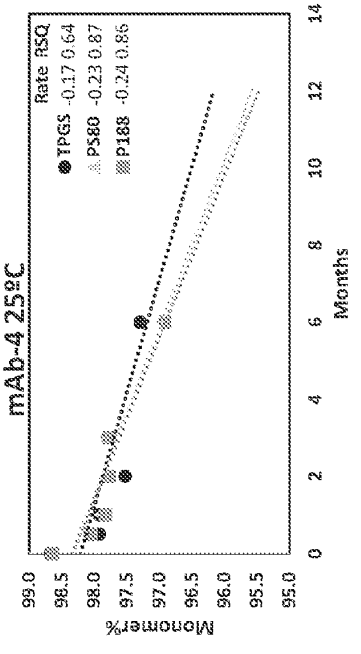


FIG. 8A

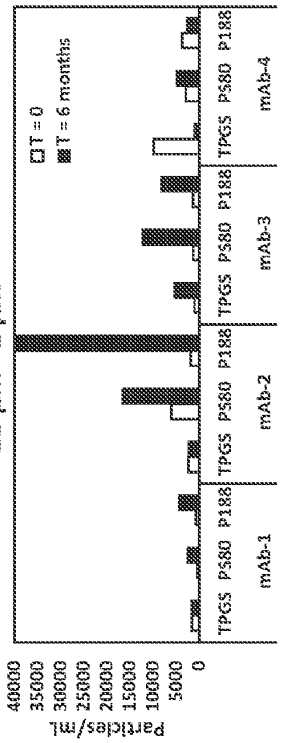


FIG. 8B

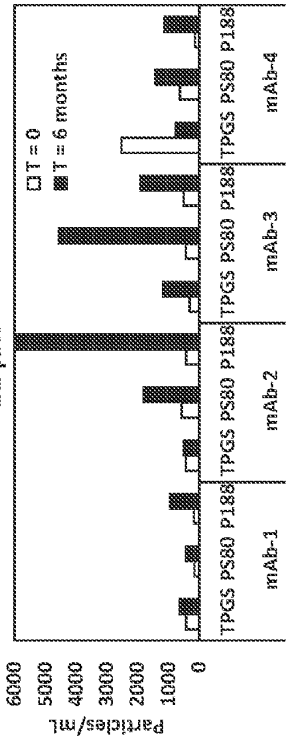


FIG. 8C

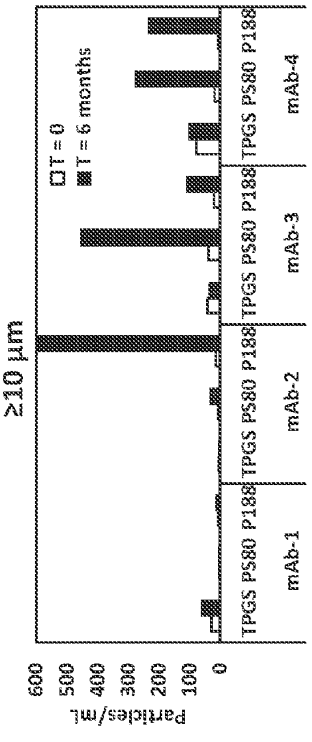


FIG. 8D

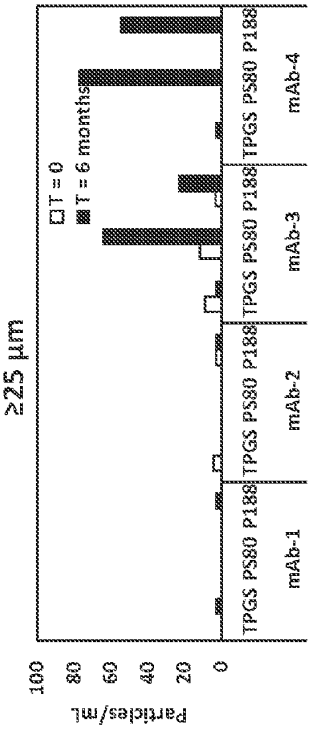


FIG. 9A

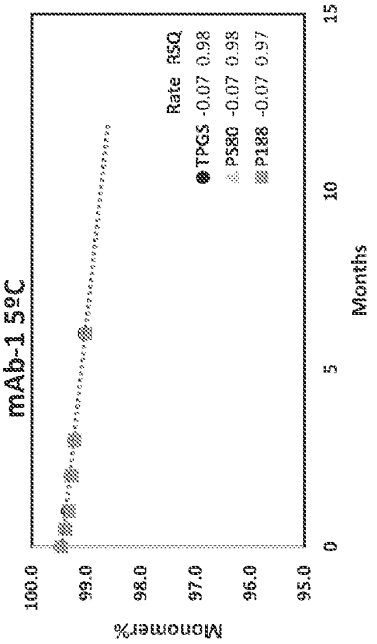


FIG. 9B

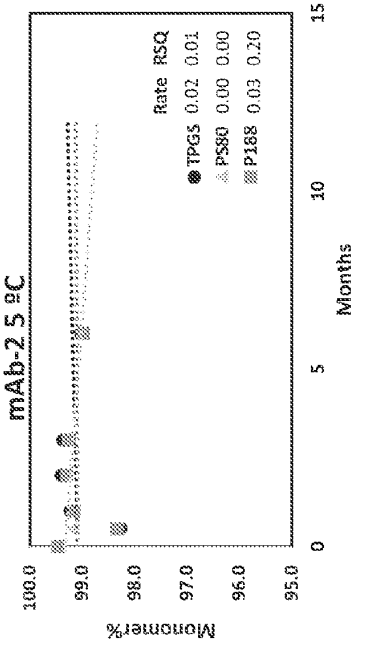


FIG. 9C

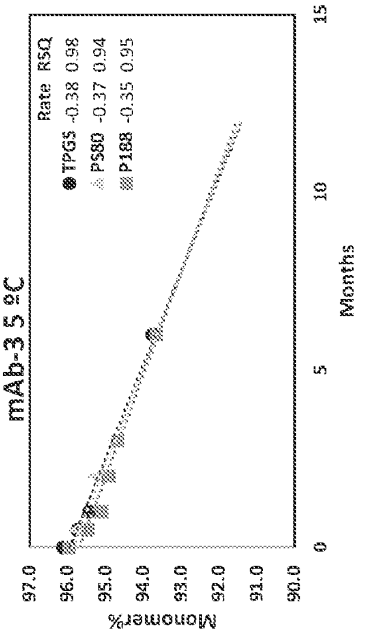


FIG. 9D

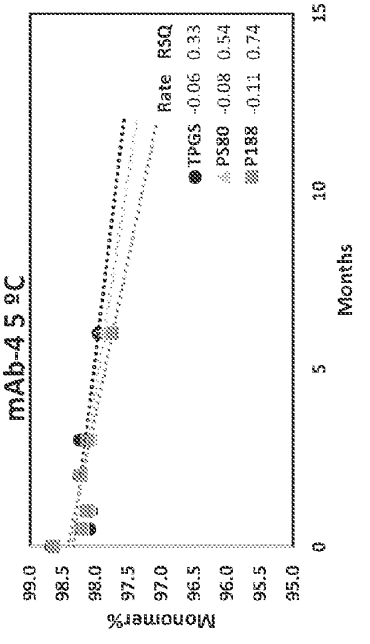


FIG. 10A

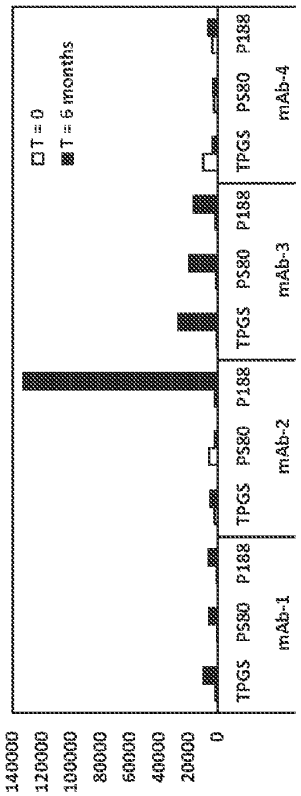


FIG. 10B

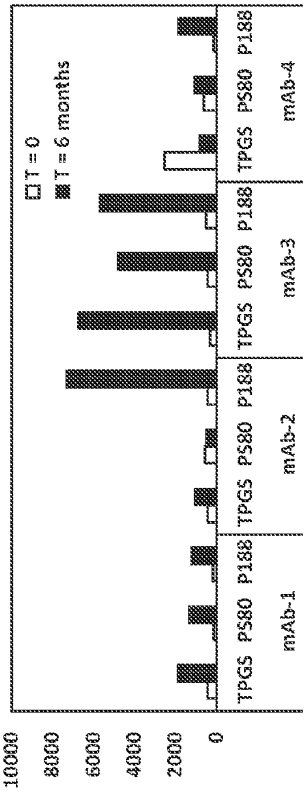


FIG. 10C

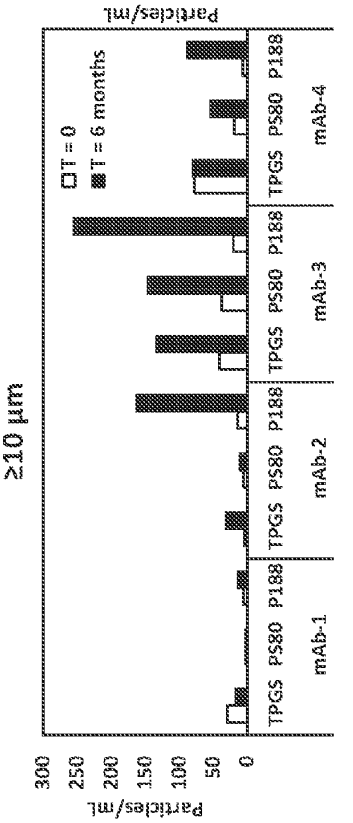


FIG. 10D

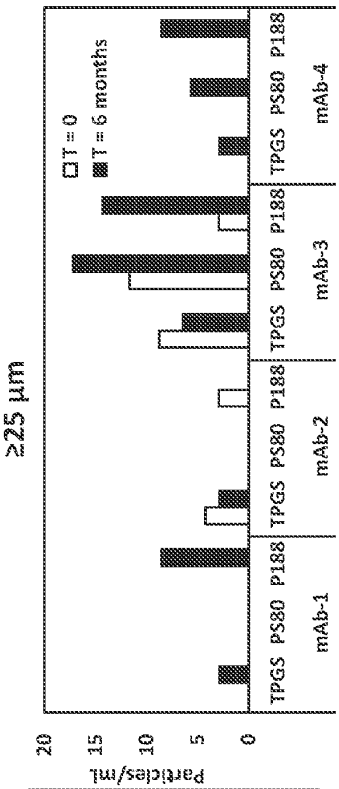


FIG.11A

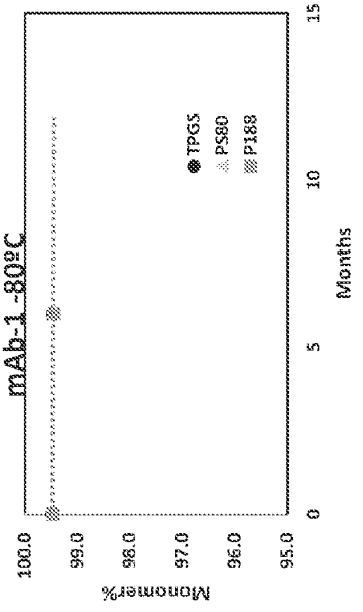


FIG.11B

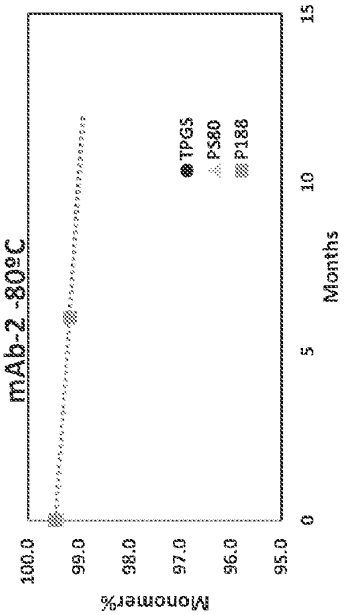


FIG.11C

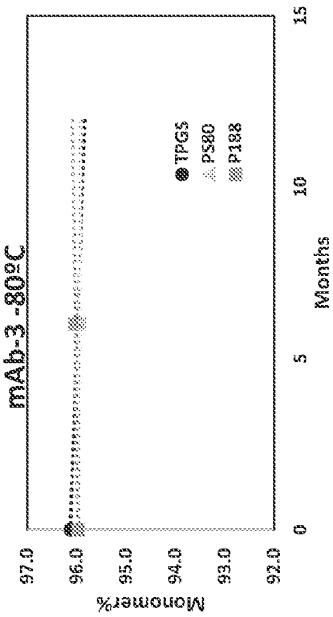
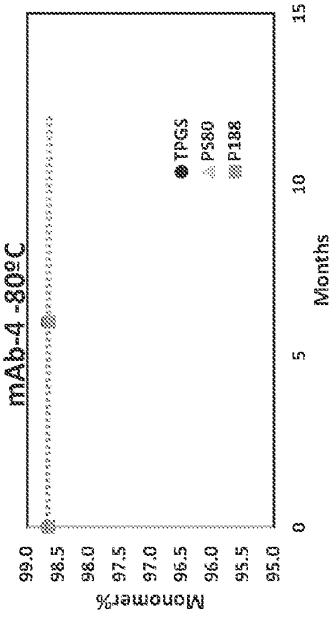


FIG.11D



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FIG.12A

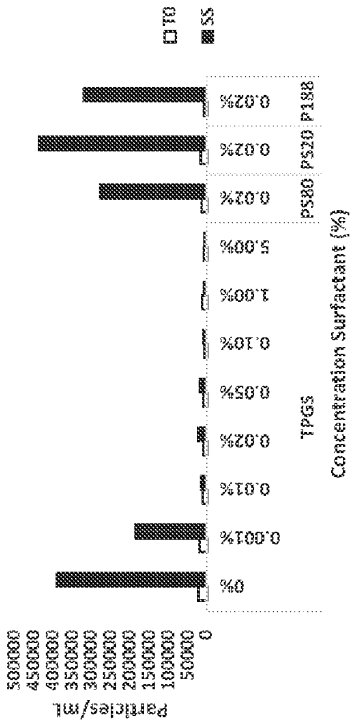


FIG.12B

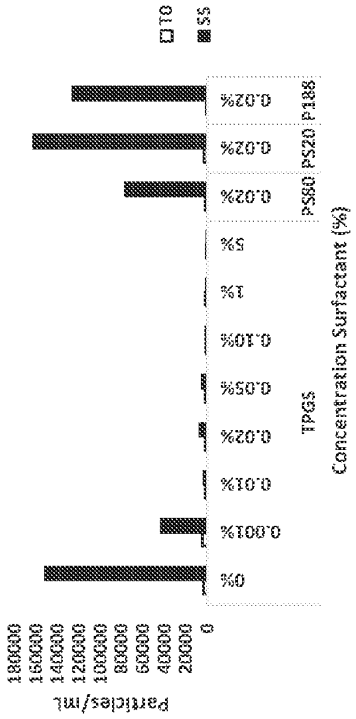


FIG.12C

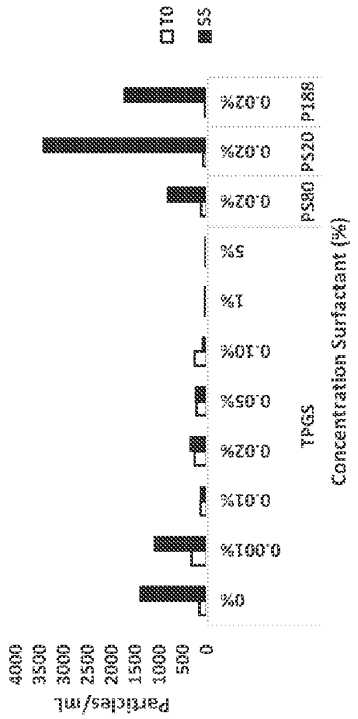
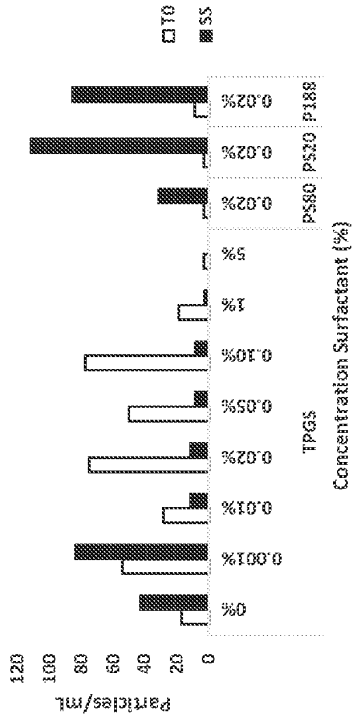


FIG.12D



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FIG.13

Concentration range study mAb-2 (HPSEC)

		T0			SS		
		%Mon	%HMW	%Frag	%Mon	%HMW	%Frag
Control	0%	98.39	0.00	1.61	98.45	0.00	1.55
TPGS	0.001%	98.43	0.00	1.57	98.45	0.00	1.55
	0.01%	98.43	0.00	1.57	98.12	0.00	1.88
	0.02%	98.43	0.00	1.57	98.52	0.00	1.48
	0.05%	98.48	0.00	1.52	98.29	0.00	1.71
	0.10%	98.51	0.00	1.49	98.51	0.00	1.49
	1.00%*	94.98	3.59	1.44	94.65	3.70	1.65
PS80	0.02%	98.08	0.02	1.90	98.46	0.05	1.49
PS20	0.02%	98.47	0.00	1.53	98.26	0.00	1.74
P188	0.02%	98.11	0.00	1.89	98.50	0.00	1.50

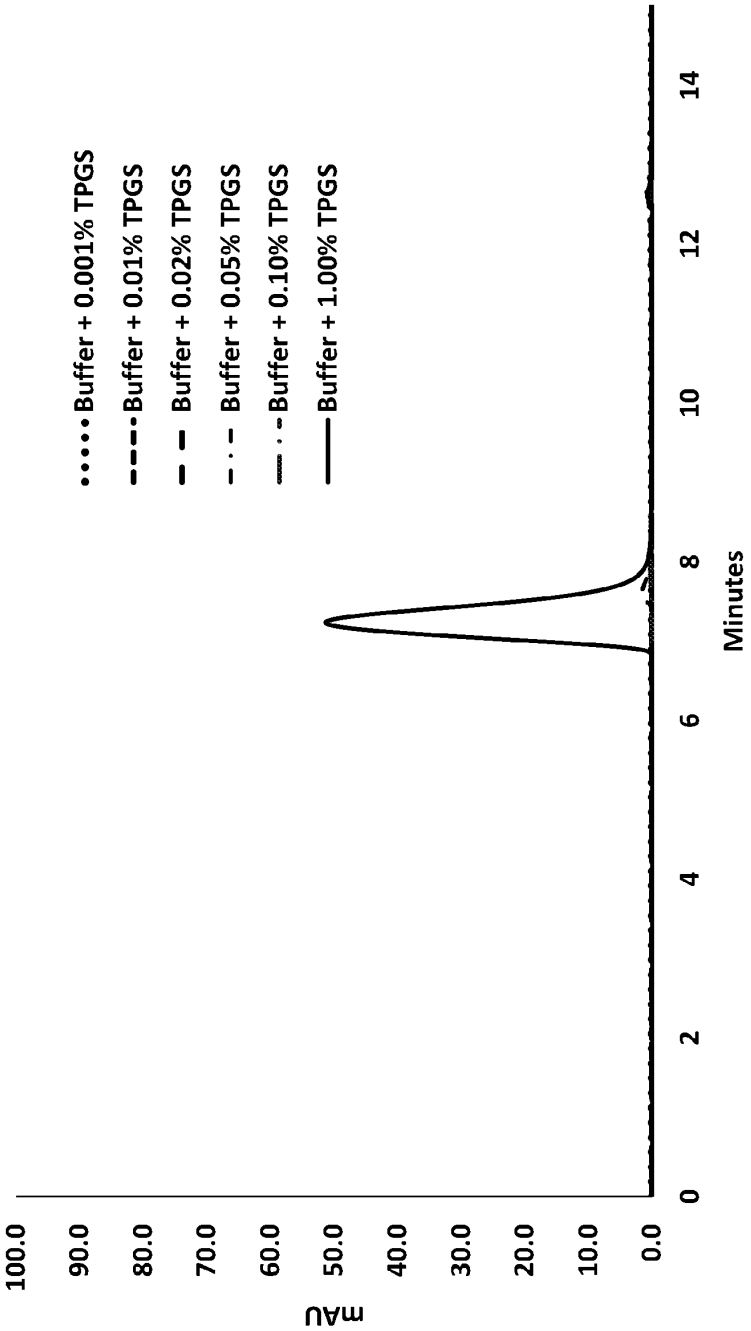

Buffer + 0.001% TPGS
Buffer + 0.01% TPGS
Buffer + 0.02% TPGS
Buffer + 0.05% TPGS
Buffer + 0.10% TPGS
Buffer + 1.00% TPGS
mAU
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
2
4
6
8
10
12
14
Minutes

FIG. 14

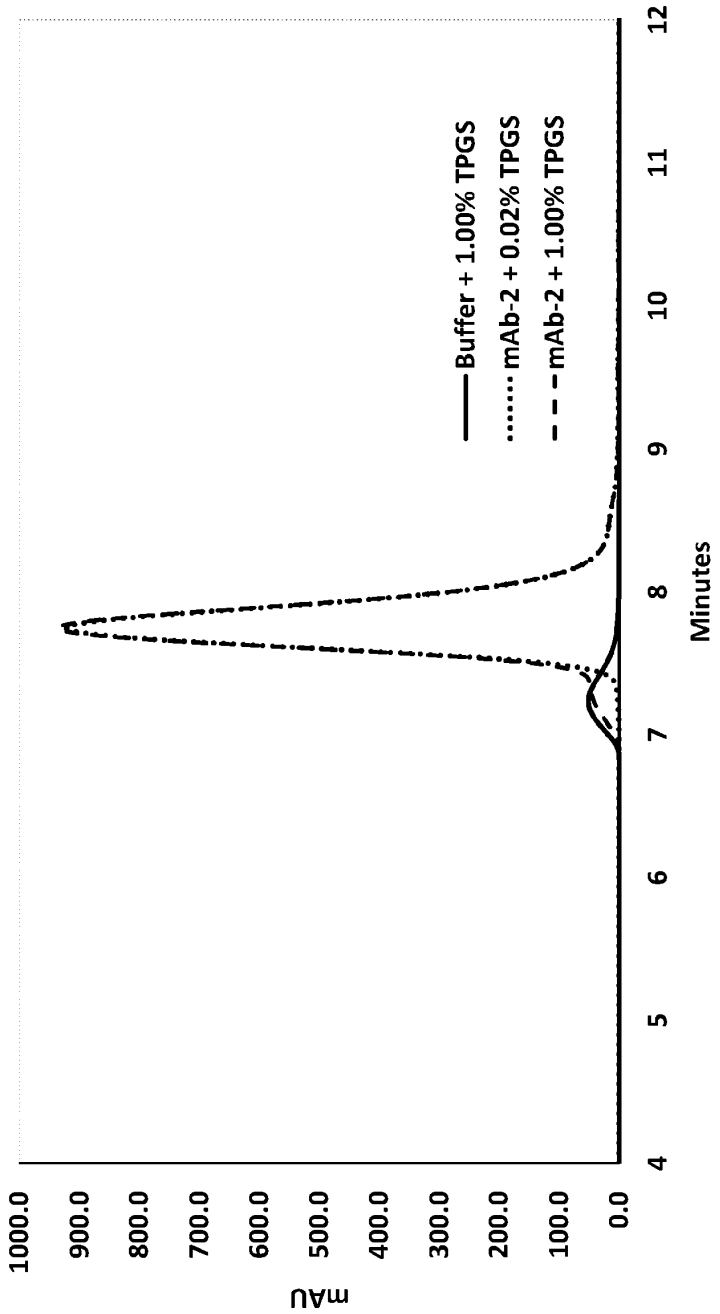

Buffer + 1.00% TPGS
mAb-2 + 0.02% TPGS
mAb-2 + 1.00% TPGS
mAU
1000.0
900.0
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
4
5
6
7
8
9
10
11
12
Minutes

FIG. 15

		Non-reduced				Reduced							
		T0		SS		T0				SS			
		Total Main peak	Total LMW	Total Main peak	Total LMW	Total Light Chain	Total Heavy Chain	Total LMW	Total HMW	Total Light Chain	Total Heavy Chain	Total LMW	Total HMW
Control	0%	98.4	1.5	98.2	1.9	26.1	70.8	2.6	0.5	26.6	71.2	2.7	0.6
TPGS	0.001%	98.2	1.9	98.3	1.7	26.1	71.0	2.3	0.5	25.1	71.4	2.9	0.6
	0.01%	98.3	1.8	98.2	1.9	25.8	71.1	2.4	0.5	25.2	71.4	2.7	0.6
	0.02%	98.3	1.8	98.4	1.6	25.8	71.1	2.4	0.5	26.0	71.0	2.4	0.6
	0.05%	98.2	1.8	98.2	1.8	26.0	71.2	2.4	0.4	26.0	70.9	2.6	0.6
	0.10%	98.3	1.7	98.2	1.9	25.5	71.4	2.5	0.5	25.6	71.4	2.5	0.5
	1.00%	98.3	1.6	98.3	1.8	26.2	70.8	2.4	0.5	26.5	70.7	2.4	0.4
	5.00%	98.4	1.6	98.3	1.8	26.1	70.9	2.4	0.6	25.2	71.5	2.7	0.5
PS80	0.02%	98.4	1.6	98.2	1.9	26.1	70.8	2.5	0.6	25.2	71.6	2.8	0.5
P520	0.02%	98.5	1.4	98.3	1.8	26.1	70.9	2.3	0.5	25.1	71.5	2.7	0.6
P188	0.02%	98.3	1.7	98.2	1.8	26.1	71.1	2.3	0.5	25.0	71.6	2.7	0.6

FIG. 16

FIG.17A

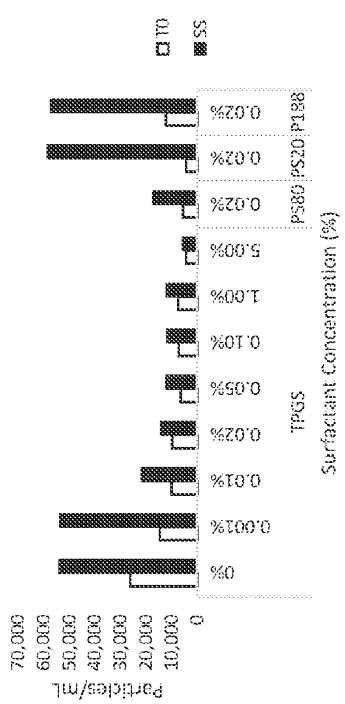


FIG.17B

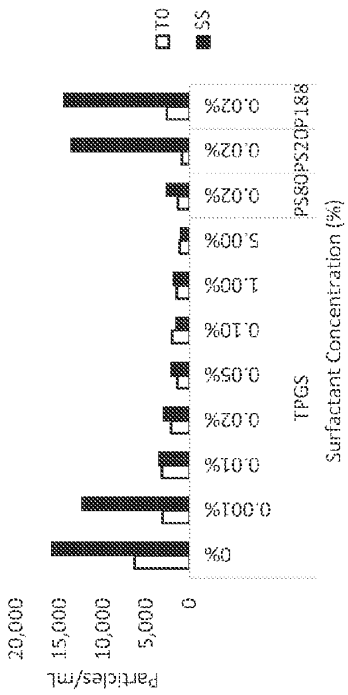


FIG.17C

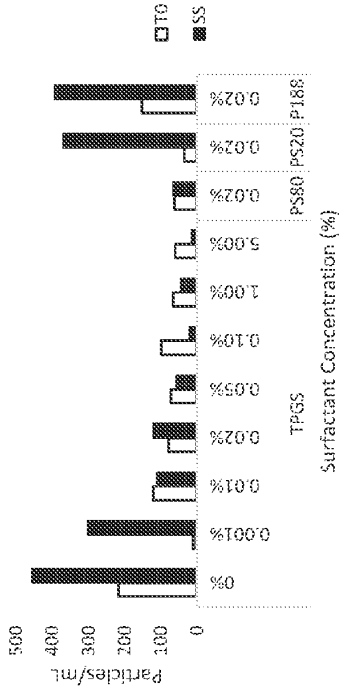


FIG.17D

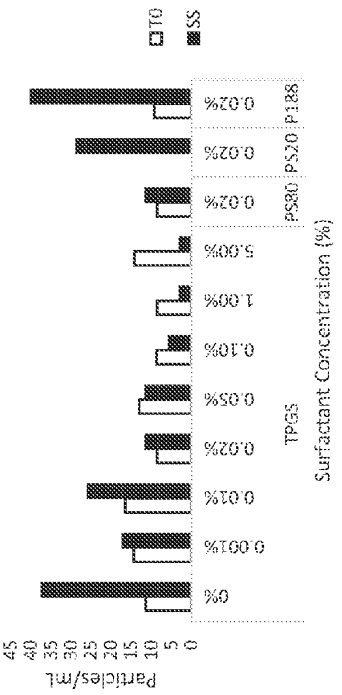
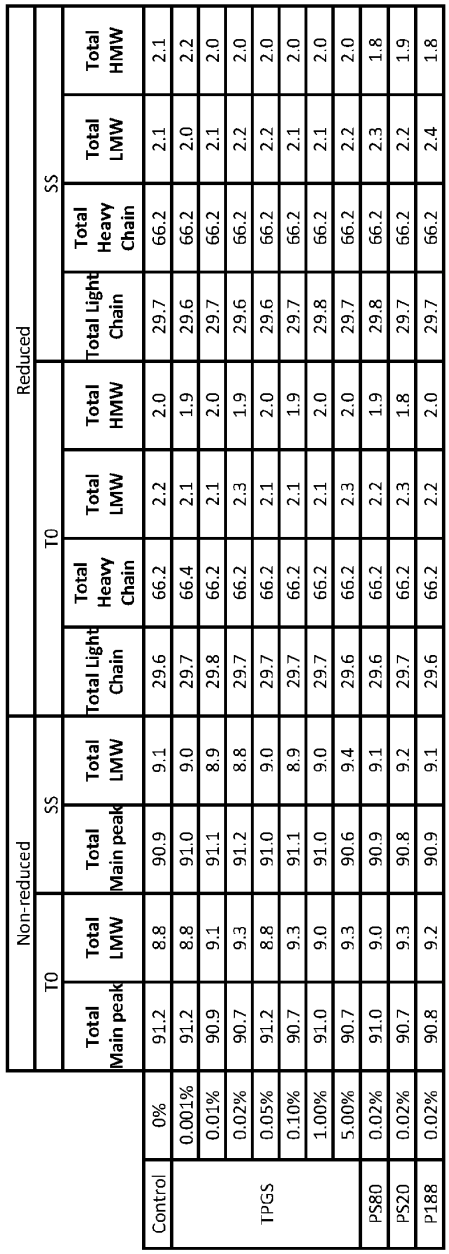


FIG.18

Concentration range study mAb-4 (HPSEC)

		T0			SS		
		%Mon	%HMW	%Frag	%Mon	%HMW	%Frag
Control	0%	97.67	2.10	0.23	97.68	2.09	0.23
TPGS	0.001%	97.69	2.08	0.23	97.69	2.08	0.23
	0.01%	97.63	2.14	0.23	97.61	2.16	0.23
	0.02%	97.47	2.30	0.23	97.41	2.36	0.23
	0.05%	97.44	2.34	0.22	97.41	2.36	0.23
	0.10%	96.26	3.52	0.23	96.17	3.61	0.22
	1.00%*	83.12	16.69	0.19	83.07	16.73	0.20
PS80	0.02%	97.50	2.27	0.23	97.50	2.27	0.23
PS20	0.02%	97.64	2.13	0.23	97.65	2.12	0.23
P188	0.02%	97.65	2.12	0.23	97.67	2.10	0.23



		Non-reduced				Reduced							
		T0		SS		T0				SS			
		Total Main peak	Total LMW	Total Main peak	Total LMW	Total Light Chain	Total Heavy Chain	Total LMW	Total HMW	Total Light Chain	Total Heavy Chain	Total LMW	Total HMW
Control	0%	91.2	8.8	90.9	9.1	29.6	66.2	2.2	2.0	29.7	66.2	2.1	2.1
TPGS	0.001%	91.2	8.8	91.0	9.0	29.7	66.4	2.1	1.9	29.6	66.2	2.0	2.2
	0.01%	90.9	9.1	91.1	8.9	29.8	66.2	2.1	2.0	29.7	66.2	2.1	2.0
	0.02%	90.7	9.3	91.2	8.8	29.7	66.2	2.3	1.9	29.6	66.2	2.2	2.0
	0.05%	91.2	8.8	91.0	9.0	29.7	66.2	2.1	2.0	29.6	66.2	2.2	2.0
	0.10%	90.7	9.3	91.1	8.9	29.7	66.2	2.1	1.9	29.7	66.2	2.1	2.0
	1.00%	91.0	9.0	91.0	9.0	29.7	66.2	2.1	2.0	29.8	66.2	2.1	2.0
	5.00%	90.7	9.3	90.6	9.4	29.6	66.2	2.3	2.0	29.7	66.2	2.2	2.0
PS80	0.02%	91.0	9.0	90.9	9.1	29.6	66.2	2.2	1.9	29.8	66.2	2.3	1.8
PS20	0.02%	90.7	9.3	90.8	9.2	29.7	66.2	2.3	1.8	29.7	66.2	2.2	1.9
P188	0.02%	90.8	9.2	90.9	9.1	29.6	66.2	2.2	2.0	29.7	66.2	2.4	1.8

FIG. 19

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FIG. 20A

mAb-5

Condition	Sample	≥1µm <2µm	≥2µm	≥10µm	≥25µm	≥50µm	≥100µm
T0	TPGS	2326	520	24	0	0	0
	Control	10398	5084	405	18	3	3
3xFT	TPGS	5096	1595	96	10	0	0
	Control	25315	9576	616	28	3	0
SS	TPGS	38229	3317	284	9	0	0
	Control	254164	59670	1414	67	3	0

FIG. 20B

mAb-6

Condition	Sample	≥1µm <2µm	≥2µm	≥10µm	≥25µm	≥50µm	≥100µm
T0	TPGS	3004	736	69	12	0	0
	Control	18201	5811	249	3	0	0
3xFT	TPGS	9074	2637	244	47	3	0
	Control	12945	3799	188	22	13	0
SS	TPGS	10595	3268	312	14	0	0
	Control	40878	6397	129	3	0	0

FIG.21A

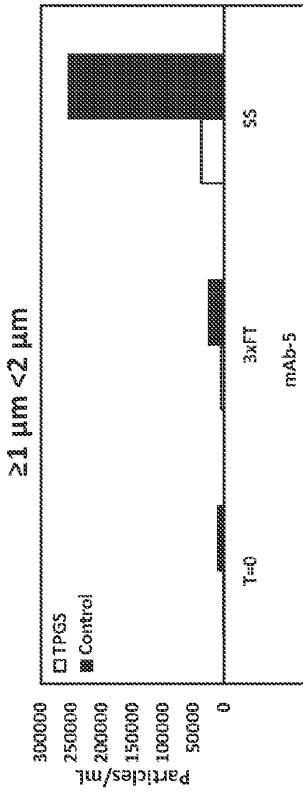


FIG.21B

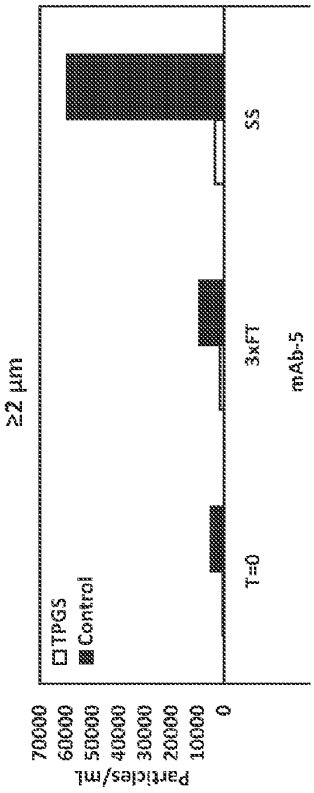


FIG.21C

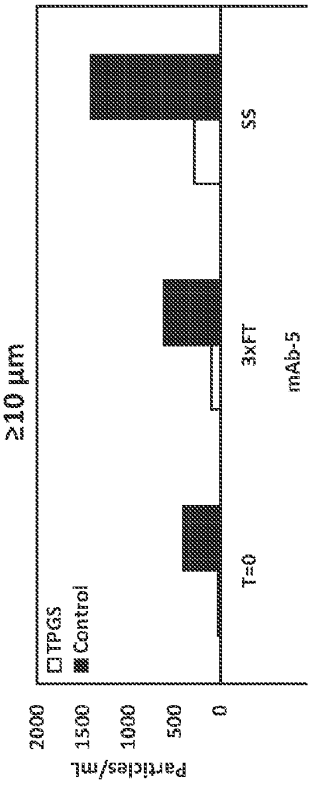


FIG.21D

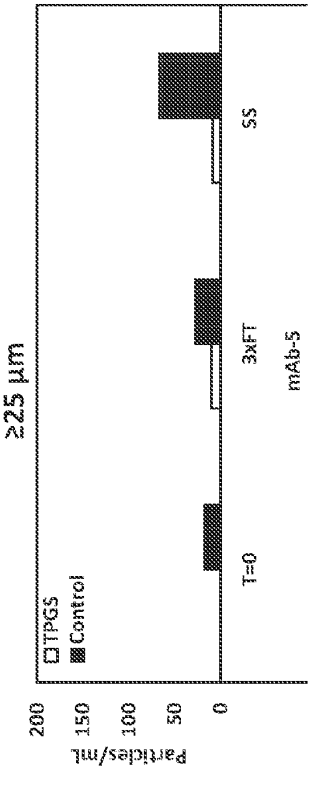


FIG. 22A

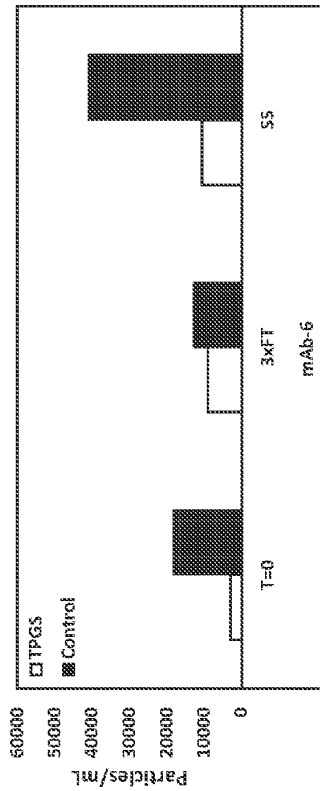


FIG. 22B

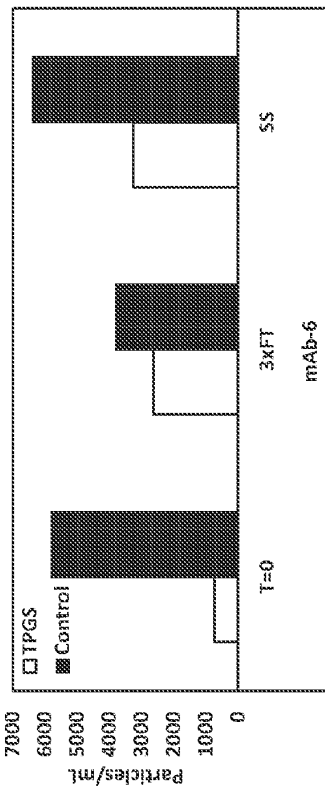


FIG. 22C

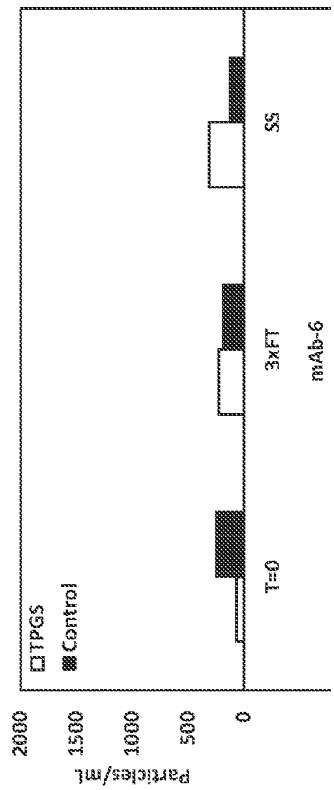


FIG. 22D

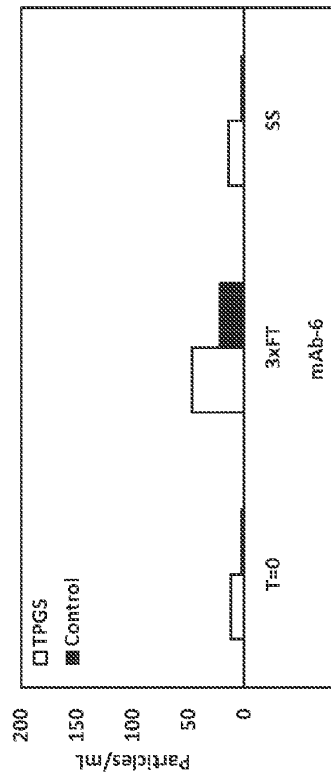


FIG.23

Stability of mAb-5 and mAb-6 Formulated in TPGS vs. Control (HPSEC)

		T0			3xFT			SS		
Molecule	Formulation	%Mon	% HMW	% Small Frag	%Mon	% HMW	% Small Frag	%Mon	% HMW	% Small Frag
mAb-6	TPGS	98.7	1.31	0.00	98.7	1.32	0.00	97.9	2.11	0.00
	Control	98.6	1.41	0.00	98.8	1.24	0.00	98.4	1.57	0.00
mAb-5	TPGS	99.1	0.82	0.07	99.2	0.80	0.00	99.0	0.90	0.09
	Control	99.1	0.84	0.07	99.1	0.83	0.07	99.0	0.93	0.08

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(54) Title: COMPOSITIONS FOR OPHTHALMIC CARE

(57) Abstract: A controlled release pharmaceutical composition for eye injection for ophthalmic care. The composition comprises at least one active pharmaceutical ingredient and at least one biocompatible polymer and at least one biocompatible solvent. The composition, which is capable of forming an in-situ implant composition, comprises an injectable solution, suspension, emulsion or dispersion. The formulation can be injected through a needle that is suitable in size for an eye injection. The present compositions can be used for treatment of ophthalmic conditions, including glaucoma, dry and wet age-related macular degeneration, diabetic retinopathy, dry eye syndrome, and uveitis.

Compositions for ophthalmic care

Field of invention

The present invention relates to injectable long-term controlled release pharmaceutical compositions for ophthalmic care, their composition materials, formulations, manufacturing methods, application methods, and usage.

Background

Glaucoma is a progressive multifactorial disease characterised by damage to the optic nerve and progressive visual loss that, if left untreated, can lead to blindness. It is a significant cause of visual morbidity, accounting for falls, road traffic accidents, loss of independence and 12% of blind registrations. Open angle glaucoma (OAG) is the most common form, with a prevalence of about 2% among adults older than 40 years; it is strongly associated with elevated intraocular pressure. Lowering intraocular pressure can slow progression of the disease and is the only treatment available. Raised intraocular pressure without optic nerve damage is termed ocular hypertension, which, in some patients, progresses to open angle glaucoma; lowering intraocular pressure reduces this risk.

The standard first-line treatment for OAG and ocular hypertension is eye drops that lower intraocular pressure, requiring multiple hospital visits for monitoring and treatment adjustment. Long-term and multiple topical medications are associated with multiple ocular and systemic side-effects, poor patient adherence, and are a risk factor for later surgical failure. Selective laser trabeculoplasty reduces intraocular pressure by increasing aqueous outflow through the trabecular meshwork with a single, painless outpatient laser procedure, minimal recovery time, and good safety profile. It was introduced in 1995 and received US FDA approval in 2001, yet is not routinely offered as first-line treatment. Selective laser trabeculoplasty superseded argon laser trabeculoplasty, with fewer adverse events, greater ease of use, and improved repeatability. The intraocular pressure-lowering effect is comparable to medical treatment and can delay or prevent the need for eye drops, avoiding the associated side-effects. The effect of selective laser

trabeculoplasty is not permanent, but it can be repeated. When successful, it reduces the risk of non-adherence, by removing or lessening the need for complex treatment regimes. (www.thelancet.com Vol 393 April 13, 2019) There is clear need to improve the outcome of the current medical OAG treatments as well as to avoid repeatable laser trabeculoplasty, i.e., an optimal patient friendly OAG treatment does not exist in the market.

Age-related macular degeneration (AMD) is one of the most common causes of irreversible blindness worldwide. In 2015 it affected 6.2 million people globally. In 2013 it was the fourth most common cause of blindness after cataracts, preterm birth, and glaucoma. It most commonly occurs in people over the age of fifty and in the United States is the most common cause of vision loss in this age group.

A minority of patients with early (dry) AMD can progress to the vision-threatening forms of AMD called late AMD. The commonest form of late AMD is “exudative” or “wet” AMD. Wet AMD occurs when abnormal blood vessels grow underneath the retina. These unhealthy vessels leak blood and fluid, which can prevent the retina from working properly. Eventually the bleeding and scarring can lead to severe permanent loss of central vision, but the eye is not usually at risk of losing all vision (going 'blind') as the ability to see in the periphery remains. There is a rarer form of late AMD called geographic atrophy, where vision is lost through severe thinning or even loss of the macula tissue without any leaking blood vessels.

Wet AMD is typically treated with intravitreal injections (injections into the eye) using a medicine called ranibizumab (also known as Lucentis, the brand name). Ranibizumab is one of a group of anti-VEGF medicines which, when injected into the eye on a regular basis, can stop the abnormal blood vessels growing, leaking and bleeding under the retina. Most people with wet AMD need to have these injections several times a year. Laser treatment is also available for AMD but is not effective for most cases. There is currently no treatment for dry AMD. Clearly existing AMD products are not optimal and there is a need for them to be improved upon with better clinical outcomes.

Uveitis is the inflammation of the uvea, the pigmented layer that lies between the inner retina and the outer fibrous layer composed of the sclera and cornea. Uveitis usually affects people aged 20 to 59, but it can occur at any age, including in children. Men and women are affected equally. It is estimated that two to five in every 10,000 people will be affected by uveitis in the UK every year. Despite being an uncommon eye condition, uveitis is a leading cause of visual impairment in the UK. It is further estimated that the more serious types of uveitis are responsible for one in every 10 cases of visual impairment in the UK.

The main treatment of uveitis is steroid medication (corticosteroids) which can reduce inflammation inside the eye. Several different types of steroid medication may be used, depending on the type of uveitis you have. Eye drops are often used for uveitis affecting the front of the eye, whereas injections, tablets and capsules are more often used to treat uveitis affecting the middle and back of the eye. In some cases, other treatments may also be needed in addition to corticosteroids. These include eye drops to relieve pain or widen (dilate) the pupil, a type of medication called an immunosuppressant, and, rarely, surgery.

Clearly existing uveitis products are not optimal and there is a need for them to be improved upon with better clinical outcomes.

Controlled release pharmaceutical compositions are known and are understood to be compositions designed to maintain the therapeutic level of an active pharmaceutical ingredient (API) over a specified (and at times a particularly extended) period of time.

DURYSTA™ (bimatoprost intracameral, solid biodegradable sustained-release implant) is indicated for the reduction of intraocular pressure (IOP) in patients with open angle glaucoma (OAG) or ocular hypertension (OHT), however it is known to have challenging implantation site at anterior chamber and short 12 weeks release time. Similar issues have been seen with other marketed solid biodegradable products, such as OZURDEX®, which utilizes NOVADUR®technology.

Other examples of existing controlled release pharmaceutical compositions are those that function as an in-situ forming implant (ISFI). One such prior art drug delivery system is the ATRIGEL® system, which includes a bioresorbable polyester, and can be used for both parenteral and site-specific drug delivery (Pandya. Y. et al., International Journal of Biopharmaceutics. 2014; 5(3): 208-213.). In the ATRIGEL® drug delivery system, the polymer component and the API are dissolved in a biocompatible solvent. This solution of polymer and API is then administered by injection, after which it solidifies *in vivo* upon contact with aqueous bodily fluids and forms a solid implant.

Such in-situ forming implants may result in targeted drug delivery to a particular area and achieve some level of sustained release. However, drug delivery systems such as these still suffer from a high initial burst effect. Another in-situ forming implant including a bioresorbable polyester where the burst effect may be improved is disclosed in WO2014001904, where the in-situ forming implant is administered by subcutaneous injection. In WO2019097262 (A1) it was discovered that a polyester that has a reduced metal content in comparison to the prior art achieves improved controlled release when the polyester is included in a pharmaceutical composition. it was thought that the residual metal catalyst triggers degradation of the polyester, and of the API of the pharmaceutical formulation, where the polyester/API degradation adversely impacts the release profile of the eventual controlled release formulation. It was shown that the particularly low metal catalyst content of the provides a reduction in the burst effect, reduced lag time, improved API stability, and thus prolonged sustained and steady release, when these polyesters are included in a controlled release pharmaceutical composition.

However, none of the above mentioned ISFI were designed for eye injections due to the technical issues mentioned above, very small injection size, long release time and unsolved toxicity issues of ISFI due to the sensitivity of the eye.

Summary of the Invention

It is an aim of the present invention to provide a controlled release pharmaceutical composition for eye injection for ophthalmic care.

The composition comprises at least one active pharmaceutical ingredient and at least one biocompatible polymer and at least one biocompatible solvent, wherein

i) the composition is in the form of an injectable solution, suspension, emulsion or dispersion,

ii) the composition is in the form of an in-situ forming implant composition, and

iii) the biocompatible polymer is bioresorbable.

It is another aim of the invention to provide a method of treating a subject in need thereof with a controlled release pharmaceutical composition for eye injection for ophthalmic care, comprising administering to said subject an injection of a composition of the present kind.

More specifically, the present invention is mainly characterized by what is stated in the characterizing parts of the independent claims.

Considerable advantages are obtained by the invention.

Surprisingly, the formulation can be injected through a needle that is suitable in size for an eye injection through reducing the viscosity of the polymer/API formulation with a biocompatible solvent. Additionally, it was found that this solvent may be chosen so that it has no adverse toxicological effects on rabbit eye which is known to be more sensitive than the human eye. Thus, it is also assumed that no adverse effects will happen in human eye.

It has also been found that even from the very small injections such as 10 µL, or even smaller, such as from 2 up to 10 µL, very long sustained release of active ingredients was achieved. The smaller the volume of vitreous injection the smaller is the acute pressure elevation inside the eye right after the injection, which is beneficial. Also, the smaller the injection, the smaller is the risk of having something in vitreous blocking the sight or pathway to retina.

With the present compositions sustained release over 6 months can be reached even from injection volumes of no more than 10 to 20 µL

In preferred embodiments, the compositions comprise polyesters which have a reduced metal content. Typically they also have controlled structure and very low residual monomer content. Such compositions provide improved controlled release and multi-month release time when the polyester is included in a controlled release pharmaceutical composition for eye injection.

The present compositions are in particular endotoxin free, non-toxic formulations providing a reduction in the burst effect, reduced lag time, improved API stability, and thus prolonged sustained and steady release, when these polyesters are included in a controlled release pharmaceutical composition for eye injection.

Surprisingly, the present formulations can be injected through a needle that is feasible in size for an eye injection through reducing the viscosity of the polymer/API formulation with a biocompatible solvent. Additionally, it was found that this solvent may be chosen so that it has no adverse toxicological effects.

Surprisingly it was also found that even from the very small injections such as 10 µL, or smaller, very long sustained release of active ingredients was achieved. The smaller the volume of vitreous injection the smaller is the acute pressure elevation inside the eye right after the injection, which is beneficial.

The present compositions can be used for treatment of ophthalmic conditions, including glaucoma, dry and wet age-related macular degeneration, diabetic retinopathy, dry eye syndrome, and uveitis.

By the present invention, the total consumption of active pharmaceutical ingredient is reduced; using conventional dropwise administration a considerable part is lost and a significant portion thereof ends up in the systemic blood circulation.

In addition, examples of eye diseases that can be treated with the present sustained drug delivery methods include the following: Anophthalmia and Microphthalmia,

Bietti's Crystalline Dystrophy, Behçet's Disease, Chataract, Coloboma, Idiopathic Intracranial Hypertension, Retinitis Pigmentosa, Retinoblastoma, Stargardt Disease, and Usher Syndrome.

Brief Description of the Drawings

Figure 1 shows the results of injection force measurements for plasebo PLGA5050 (13 kDa) 40%/DMSO 60%;

Figures 2a and 2b show schematically *in vivo* implant formation: Figure 2a depicts Subcutaneous ISFI SiSu® injection in a rat SC tissue, and Figure 2b depicts intravitreal ISFI SiSu® injection in rabbit vitreous;

Figures 3a and 3b are photographs showing implant formation *in vitro* by 10 µl injection of formulation into PP pouch immersed in PBS buffer;

Figure 4 is a simplified drawing of premixed formulation

Figure 5 is a simplified drawing of the formation of a formulation using preparative syringe mixing;

Figure 6 is a simplified drawing of the formation of a formulation using an injection pen;

Figures 7a and 7b show the long-term release of latanoprost from an *in vitro* implant as a function of time, Figure 7a depicting the cumulative release and Figure 7b the daily release;

Figures 8a, 8b and 8c and 8d show a second and a third example of the release of latanoprost from an *in vitro* implant as a function of time, Figure 8a and 8b depicting the release from implants with no additive and Figure 8c and 8d the release of a composition with triethyl citrate as an additive;

Figure 9 shows the burst reduction and daily release rate increase of latanoprost from a composition containing benzyl benzoate as an additive (20 and 10 µL injections);

Figure 10 shows graphically hydrolysis of latanoprost in porcine vitreous adjusted to pH 7.4 and with non-adjusted pH;

Figures 11a and 11b show the release of regorafenib from an *in vivo* implant as a function of time, Figure 11a depicting the cumulative release and Figure 11b the daily release;

Figures 12a and 12b show the release, as a function of time, of regorafenib from *in vitro* PLGA90:10-implants containing different amounts of benzyl benzoate, Figure 12a depicting the cumulative release and Figure 12b the daily release;

Figures 13a and 13b show the release, as a function of time, of regorafenib from an *in vitro* PLGA60:40-implant containing benzylalcohol, Figure 13a depicting the cumulative release and Figure 13b the daily release;

Figures 14a and 14b show the release of sunitinib from an *in vivo* implant as a function of time, Figure 14a depicting the cumulative release with 55% of DMSO in formulation and Figure 14b 65 % of DMSO;

Figures 15a and 15b; figures 15a and b shows how triacetin amount has an effect on the duration and level of released drug, with higher triacetin amounts the duration is shorter, and release higher

Figures 16a and 16b show the effect of benzyl benzoate on the release of sunitinib from an *in vitro* implant as a function of time, Figure 16a depicting the cumulative release and Figure 16b the daily release;

Figures 17a and 17b shows that with 6% benzyl benzoate the 1 d burst of sunitinib malate can be totally avoided from an *in vitro* implant, Figure 17a depicting the cumulative release and Figure 17b the daily release;

Figures 18a and 18b show the release of dexamethasone (200 µg loading) from an *in vitro* implant as a function of time, Figure 18a depicting the cumulative release and Figure 18b the daily release; Figure 18c and Figure 18d results with 1200 µg dexamethasone loading and changing GA amounts in PLGA polymer

Figures 19a and 19b show the effect of BB (benzyl benzoate) and BA (benzyl alcohol) on the release of dexamethasone from an *in vitro* implant as a function of time, Figure 19a depicting the cumulative release with BB additive and Figure 19b the cumulative release with BA;

Figure 20 shows the effect of SAIB (sucrose acetate isobutyrate) on the cumulative release of dexamethasone from an *in vitro* implant as a function of time;

Figure 21 shows the effect of solvent choice (NMP/DMSO) and BB amount on the release of dexamethasone from an *in vitro* implant as a function of time;

Figure 22 shows the release of exenatide (a peptide) formulated with triacetin as solvent and additive from an *in vitro* implant as a function of time;

Figures 23 show the release of bovine serum albumin (BSA) from an *in vitro* implant comprising PLGA at ratio of 50:50 as a function of time; Figure 23a cumulative release and Figure 23b the daily release

Figure 24 shows the release of insulin from an *in vivo* implant as a function of time;

Figure 25 shows the release of risperidone (molecular weight 410 g/mol) from an implant formulation of two different injection volumes as a function of time;

Figure 26 shows graphically the release of exenatide from two different polymer matrices the formulations otherwise being the same;

Figure 27 comprises a series of photos showing opened *ex vivo* porcine eyes with injected implant using various amounts of biocompatible solvent;

Figure 28 shows cumulative release of latanoprost in PBS and in porcine vitreous with same composition as a function of time. The latanoprost released in vitreous is quantified as latanoprost acid, as quite immediate latanoprost hydrolysis occurs in vitreous;

Figures 29a and 29b show a PLGA8515 42% / DMSO 58% placebo implant at 5 months' time point inside rabbit eye pictured after sacrifice; and

Figure 30 shows the dynamic viscosity of a composition of PGLA polymer (60 % by weight) in DMSO (40 % by weight).

Description of Embodiments

Definitions

1. Intravitreal injection
   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 viscous fluid called the vitreous humor.

2. Intracameral injection
   is an injection of a substance into the eye anterior chamber.

3. *In-situ* forming implant
   In situ forming implant drug delivery systems provide a means by which a controlled release depot can be physically inserted into a target site without the use of surgery. ISFI avoid the use of large needles or microsurgery and

they are injected as low viscous solutions and transform in the body to a gel or solid depot. Different triggers can be used to stimulate this transformation: (1) in situ cross-linking, (2) in situ solidifying organogels, and (3) in situ phase separation.

4. Biocompatible polymer

   Polymer that does not produce toxic or harmful products and stimulate an immune response in biological systems. This is essential so that during the treatment, the material does not induce a rejection response

5. Biodegrabable & (Bio)resorbable polymer & polyester

   As used herein, the term "bioresorbable polymer" refers to a polymer that is capable of removal by cellular activity and/or dissolution in a biological system such as the human body or *in vivo*. Typically, the present bioresorbable polyesters degrade *in vivo* by hydrolysis of their ester backbone into non-toxic products

6. Monomer

   As used herein, the term "monomer" takes its usual definition in the art and so refers to a molecular compound that may chemically bind to another monomer to form a polymer. The one or more monomers that form the polyester polymer disclosed herein are understood to include all enantiomers, diastereomers, racemates and mixtures thereof of the monomers in question.

7. As used herein, the term "metal catalyst" refers to a metal substance that accelerates and/or initiates the polymerisation reaction and has been added to the polymerisation system by purpose. As such, this term covers elemental metals, inorganic metal compounds, metal salts, oxides, halides, hydroxides, carboxylates, organometallic compounds, metal complexes metallocenes.

8. Placebo

   As used herein placebo means drug product without active ingredient.

9. Viscosity

As used herein, viscosity stands for dynamic viscosity measured at 25 °C using a rheometer and extrapolating shear rate to 0 s^{-1}.

Unless otherwise stated herein or clear from the context, any percentages referred to herein are expressed as percent by weight based on a total weight of the respective composition.

Unless otherwise stated, properties that have been experimentally measured or determined herein have been measured or determined at room temperature. Unless otherwise indicated, room temperature is 25°C.

Unless otherwise stated, properties that have been experimentally measured or determined herein have been measured or determined at atmospheric pressure.

It must also be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless otherwise specified.

As used herein, the term "about" refers to a value which is ± 5% of the stated value.

As used herein, unless otherwise indicated, the term "high molecular weight", in particular when used with reference to proteins, stands for molecules having a molecular weight of more than 1 kDa, in particular more than 2 kDa, such as more than 3 kDa or more than 4 kDa. A high molecular weight protein may have generally a molecular weight of 1 to 200 kDa, for example 2 to 100 kDa.

As used herein, unless otherwise indicated, the term "average molecular weight" refers to a number average molecular weight (also abbreviated "Mn").

As used herein, the molecular weight has been measured by gel-permeation chromatography using polystyrene standards.

The skilled person in the art will appreciate that the polyester is an example of the suitable polymer for the bioresorbable controlled drug delivery composition for eye injection and is not excluding other type of bioresorbable polyesters i.e. polyester can be manufactured from other monomers and polymers than lactides, glycolide or caprolactone, if different properties are desired in the biodegradable controlled drug delivery formulation, which will be apparent to those of skill in the art.

According to preferred embodiment of the present invention the following bioresorbable polymers, copolymers and terpolymers may be used for controlled drug delivery composition: Polylactide (i.e. poly(lactic acid), PLA), polyglycolide (PGA) and poly(∈-caprolactone) (PCL), polydioxanone (PDO), polytrimethylenecarbonate (PTC), polylactides (PLA), poly-L-lactide (PLLA), poly-DL-lactide(PDLLA), PolyL-DL-lactide (PLDL), stereocopolymers; copolymers of glycolide, glycolide/trimethylene carbonate copolymers (PGA/TMC); other copolymers of PLA, such as lactide/tetramethylglycolide copolymers, lactide/trimethylene carbonate copolymers, lactide/d-valerolactone copolymers, lactide/∈-caprolactone copolymers, L-lactide/DL-lactide copolymers, glycolide/L-lactide copolymers (PGA/PLLA), polylactide-co-glycolide; terpolymers of PLA, such as lactide/glycolide/trimethylene carbonate terpolymers, lactide/glycolide/∈-caprolactone terpolymers, PLA/polyethylene oxide copolymers; polydepsipeptides; unsymmetrically (3,6-substituted)-poly-1,4-dioxane-2,5-diones; polyhydroxy-alkanoates, such as polyhydroxybutyrates (PHB); PHB/b-hydroxyvalerate copolymers (PHB/PHV); poly-b-hydroxypropionate (PHPA); poly-p-dioxanone (PDS); poly-d-valerolactone-poly-∈-caprolactone, poly(∈-caprolactone-DL-lactide) copolymers; methylmethacrylate-N-vinyl pyrrolidone copolymers; polyesteramides; polyesters of oxalic acid; polydihydropyrans; polyalkyl-2-cyanoacrylates; polyurethanes (PU); polyvinylalcohol (PVA); polypeptides; poly-b-malic acid (PMLA); poly-b-alkanoic acids; polycarbonates; polyorthoesters; polyphosphates; poly(ester anhydrides); and mixtures thereof; and natural polymers, such as sugars, starch, cellulose and cellulose derivatives, polysaccharides, collagen, chitosan, fibrin, hyaluronic acid, polypeptides and proteins.

In one embodiment, the polyester is formed from by polymerisation of lactide, glycolide, caprolactone, or combinations thereof. Such monomers are cyclic and

are often used to form polymers by way of ring opening polymerisation. Preferably, the polyester is polylactic acid, polyglycolic acid, polycaprolactone, poly(lactide-co-glycolide), poly(lactide-co-caprolactone), or poly(glycolide-co-caprolactone), poly(lactide-co-glycolide-co-caprolactone, inclusive of all possible stereoisomers of these polymers.

Alternatively, the polyester can be formed by polymerisation lactic acid, glycolic acid and caprolactone or combinations thereof by condensation polymersation.

The polyester may be capped with polyethylene glycol (PEG), polypropylene glycol (PPG), and/or polyvinyl alcohol (PVA), at the terminus of one or more polymer chains. The polyester may also be a block co-polymer, comprising (in addition to one or more polyester blocks) at least one block of polyethylene glycol (PEG), polypropylene glycol (PPG), or polyvinyl alcohol (PVA).

The polyester can be linear or non-linear, such as branched (including star-shaped, hyperbranched and dendrimeric polymers).

In one embodiment, the polyester is non-linear, in particular branched, such as star-shaped. It has been found that using non-linear, in particular branched, such as star-shaped polyesters release of API is better controlled – in particular, the release rate of the API is more even.

Generally, in the present context, the term "branched polymer" stands for a polymer which has at least 3 terminal groups. In one embodiment, the branched polyester has 4 or more terminal groups, for example 4 or 6 terminal groups.

In embodiments, the use of branched polyesters can reduce viscosity of the injectable composition and, as a result, thin needles can be used for the injections.

The number average molecular weight of the polyester typically varies in the range of 1,000 g/mol to 250,000 g/mol, depending on the configuration and shape of the polyester molecule.

In some embodiments, the molecular weight of polylactides or lactide and glycolide copolymers is about 2,000 to 100,000 g/mol, such as 5,000 to 50,000 g/mol.

In one embodiment, the composition is formulated for use with an injection needle having a diameter corresponding to any of classes G20 to G31, for example G26 to G31.

The metal content of the purified polyester is > 0 ppm and < 40ppm, and the total residual monomer content is preferably ≤ 0.2wt%, in particular ≤ 0.1wt%. Residual monomer content is measured using and GC or GC-MS against suitable monomer standards, which is a method familiar to those skilled in the art. Preferably, the total residual monomer content of the purified polyester may be <0.05wt% or <0.01wt%. The metal content of the purified polyester may be < 35ppm, < 30ppm, < 25 ppm, < 20 ppm, < 15 ppm, < 10 ppm, < 5 ppm, or < 2 ppm.

The ppm (i.e. parts per million) refers to the mass of elemental metal present in relation to the total mass of the polyester, e.g. a value of 1 ppm refers to 1 × 10-6 grams of elemental metal per 1 gram of polyester.

The metal content refers to the total amount of metal present in the polyester, inclusive of free metal, metal weakly coordinated to the polymer, plus metal strongly coordinated or bonded to the polymer. For example, the metal is selected from tin, zinc, iron, aluminium, titanium, platinum, bismuth, manganese, antimony, nickel, calcium, magnesium, sodium, lithium, yttrium, lanthanum, samarium, zirconium, ruthenium or combinations thereof but not excluding other typical polyester catalyst known in the art.

Polymers of the above kind are found to be especially suitable for controlled release pharmaceutical ISFI composition for eye injection:

Without wishing to be bound by theory, it is thought that these residual metal catalysts and residual monomers trigger degradation of the polyester, and also degradation of the API of the pharmaceutical formulation, where this polyester/API

degradation adversely impacts product shelf life time, the release profile and time of the eventual controlled release formulation.

It is thought that the particularly low metal catalyst and residual monomer content of the polyesters disclosed herein provides a reduction in the burst effect, reduced lag time, improved API and polymer stability, and thus prolonged sustained and steady release, when these polyesters are included in a controlled release pharmaceutical composition for eye injection.

In an embodiment, the polyester is purified by the following method: i) the polyester is dissolved in an organic solvent wherein the organic solvent comprises at least one heteroatom selected from oxygen, nitrogen, sulphur, chlorine and phosphorus; ii) the metals are coordinated with organic solvent and other impurities such that residual monomers are separated from the polyester during the precipitation step and iii) in separation and washing step. It was surprisingly found that this results in a greater reduction in the metal content and residual monomer content in comparison to prior art purification methods.

Disclosed herein is a controlled release pharmaceutical composition for eye care injection comprising at least one active pharmaceutical ingredient, at least one biocompatible solvent, optionally one or more excipient(s)/additive(s) and at least one of the purified polyester disclosed herein.

The one or more active pharmaceutical ingredients comprised by the compositions disclosed herein are understood to include all enantiomers, diastereomers, racemates and mixtures thereof. The drug can also be a salt, a solvate, a hydrate, a pro-drug, a co-crystal, a derivative, in free base form, or a mixture thereof.

The pharmaceutical composition may also additionally include other safe excipients and ingredients for improving its properties/usability, such as formulation or API stability, drug release profile, and injectability.

Examples of excipients include biocompatible excipient, in particular selected from vanillin, triacetin, benzyl benzoate, benzyl alcohol, triethyl citrate, acetyl triethyl

citrate, anisole, SAIB, hydroxypropylmethylcellulose, HP-b-CD, cyclodextrins, MCT (medium chain triglycerides), polycaprolactone diols, dextrans, sucrose, crown ethers, chitosan, mannitol, trehalose, or combinations thereof.

The active pharmaceutical ingredient comprised by the controlled release pharmaceutical compositions for eye injections disclosed herein may include one or more anti-VEGFs, VEGFs, Tyrosine kinase inhibitors, antiparasites, H2 receptor antagonists, antimuscarinics, prostaglandins and prostaglandin analogues, non-steroidal anti-inflammatory agents, proton pump inhibitors, aminosalycilates, corticosteroids, chelating agents, cardiac glycosides, phosphodiesterase inhibitors, thiazide, diuretics, anesthetic agents, carbonic anhydrase inhibitors, antihypertensives, anti-cancers, anti-depressants, calcium channel blockers, analgesics, opioid antagonists, antiplatels, anticoagulants, fibrinolytics, statins, adrenoceptor agonists, beta blockers, antihistamines, respiratory stimulants, micolytics, expectorants, barbiturates, anxiolytics, central nervous system agents, tricyclic antidepressants, 5HT1 antagonists, opiates, 5HT1 agonists, antiemetics, antiepileptics, dopaminergics, antibiotics, antifungals, anthelmintics, antivirals, antiprotozoals, antidiabetics, insulin and its derivatives, GLP-1 receptor agonists, thyrotoxins, female sex hormones, male sex hormones, antioestrogens, hypothalamics, pituitary hormones, posterior pituitary hormone antagonists, peptide drugs, protein drugs, protein kinases, antigens, antidiuretic hormone antagonists, bisphosphonates, dopamine receptor stimulants, androgens, steroid reductase inhibitors, non-steroidal anti-inflammatories, immunosuppressants, local anaesthetic, sedatives, anti-psoriatics, silver salts, topical antibacterials, vaccines, or vaccine antigens.

Preferably, the active pharmaceutical ingredient includes one or more tyrosine kinase inhibitors: 3-[4-(1-formylpiperazin-4-yl)-benzylidenyl]-2-indolinone, Acalabrutinib, Afatinib, Alectinib, Axitinib, Axotinib, Bosutinib, Brigatinib, Cabozantinib, Canertinib, Capmatinib, Cediranib, Ceritinib, Crenolanib, Crizotinib, Dabrafenib, Dacomitinib, Dasatinib, Dovitinib, Entrectinib, Erlotinib, Fedratinib, Flumatinib, Foretinib, Fostamatinib, Gefitinib, Geldanamycin, Genistein, Gilteritinib, Glesatinib, Ibrutinib, Icotinib, Imatinib mesylate, Lapatinib, Larotrectinib, Lestaurtinib, Lorlatinib, Lenvatinib, Midostaurin, Motesanib, Neratinib, Nilotinib,

Nintedanib, Osimertinib, Pacritinib, Pazopanib, PD173955, Pexidartinib, Piceatannol, Ponatinib, Radicicol, Radotinib, Regorafenib, Ruxolitinib, Selpercatinib, Saracatinib, Savolitinib, Selumetinib, Sorafenib, Sunitinib, Tandutinib, Tesevatinib, Trametinib, Tucatinib, Vandertanib, Vatalanib, Vemurafenib.

More preferably, the active pharmaceutical ingredient includes one or more prostaglandins: Naturally occurring prostaglandins, Alprostadil, Bimatoprost, Carboprost, Cloprostenol, Dinoprostone, Enprostil, Fenprostalene, Fluprostenol, Iloprost, Latanoprost, Latanoprostene bunod, Luprostiol, Misoprostol, Netarsudil, Prostalene, Tafluprost, Travoprost, Unoprostone, Other preferred active pharmaceutical ingredients include dexamethasone and cyclosporine.

Most preferably, the active pharmaceutical ingredient includes one or more anti-VEGFs: Adalimumab, Aflibercept, Anecortave, Bevacizumab, Brolucizumab, Etanercept, Infliximab, Pegaptanib, Ranibizumab, Verteporfin and their biosimilars.

In one embodiment, the loading of the active pharmaceutical ingredient varies between 0.1wt% and 90wt% of the total weight of the polymer-solvent mixture, preferably 0.2wt% to 50wt%, more preferably 0.5wt% to 30wt%, most preferably from 1wt% to 10wt%.

Preferably, the controlled release pharmaceutical composition for eye injection is in the form of an in-situ forming implant composition. When the controlled release pharmaceutical composition for eye injection is in the form of an in-situ forming implant composition, the composition comprises at least one purified polyester disclosed herein and at least one active pharmaceutical ingredient and at least one biocompatible solvent and possibly additional excipients.

The term “biocompatible solvent” takes its usual definition in the art and so refers to a solvent that is not harmful or toxic to living tissue.

In one embodiment, the “biocompatible solvent” is miscible with water, in particular it is miscible with water in all proportions.

In one embodiment, the "biocompatible solvent" is further capable of dissolving, partially or wholly, the biodegradable polymer.

In one embodiment, the biocompatible solvent is a liquid capable of dissolving at least 1 mg/ml of the purified polyester at 35 to 37 °C, and capable of dissolving, dispersing or suspending at least one active pharmaceutical ingredient.

In one embodiment, the biocompatible solvent is selected from N-methyl-2-pyrrolidone, triacetin, dimethylsulfoxide, anisole, benzyl benzoate, benzyl alcohol, acetone, butyl acetate, propyl acetate, ethyl acetate, methyl acetate, ethyl formate, isopropyl acetate, glycofurol or combinations thereof.

In one embodiment, the biocompatible solvent is selected from dimethylsulfoxide, optionally in combination with benzyl benzoate.

Typically, the weight ratio between biocompatible polymer and biocompatible solvent varies in the range from 1 : 99 to 99 :1, preferably from 10 : 90 to 90 : 10, more preferably from 20 : 80 to 80 : 20, most preferably from 40 : 60 to 60 : 40.

In one embodiment, the injection volume varies between 1 microliter and 1000 microliter, preferably between 2 to 500 microliter. For example the injection volume can be in the range from 3 microliter to 100 microliter, more preferably from 5 microliter to 50 microliter, most preferably from 10 microliter to 30 microliter. For an intravitreally given injections volumes in the range from 5 to 25 µl are preferred, for subjconjunctivally given injections volumes in the range of 2 to 15 µl are preferred.

In one embodiment, the dynamic viscosity, at 25 °C, of an injectable composition is between 10 and 100,000 mPas. Preferably the viscosity is 100 to 3000 mPas and more preferably 200 to 2000 mPas.

In one embodiment, the dynamic viscosity, at 25 °C, of an injectable composition is about 800 to 1200 mPas.

An embodiment comprises a method of treating a subject in need thereof with a controlled release pharmaceutical composition for eye injection, in any part of the eye, for ophthalmic care, or in therapy of ophthalmic conditions, comprising administering to said subject an injection of a composition of the present kind. In one embodiment, the injection is given to the eye intravitreally, intracamerally, periocularly, subchoroidally, subconjunctivally, subretinal, or under eye mucosal layer or utilizing pre-injected watery bleb under the surface of the eye. enable injection outside the sclera. Such a bleb will enhance the solidifying of the composition.

In one embodment, an injection having a volume of 1 to 50 µl, in particular 2 to 25 µl, is given intravitreally or subconjunctivally

In one embodiment, the injection is given to the eye as needed or regularly between once a week and annually, in particular once each 3rd to 6th month.

The compositions of the invention are particularly useful in reducing intraocular pressure, especially intraocular pressure which is associated with glaucoma. The injectable ophthalmic compositions of the invention provide a steady release of an amount of API or combination of APIs which is effective in treating elevated intraocular pressure. The daily dosage and drug release duration is controlled by formulation composition and injection volume.

In embodiments of the present technology, drug release from the *in situ* implant will be effected through a succession of phases involving skin formation, swelling, shrinking, and degradation. In one embodiment, comprising an active ingredient (API), such as latanoprost, mixed with dimethyl sulfoxide (DMSO) as a primary solvent and benzyl benzoate (BB) as a cosolvent, a composition injected will, within minutes of injection, form a liquid implant surrounded by a thin skin, which will form a thicker shell, due to penetration of water into, and release of liquid components and API from, the injected composition. In a following stage, lasting up to a few weeks, further penetration of water and release, although now at a slower rate, of DMSO, BB and active ingredient, will give cause to swelling of the implant, solidification of the polymer and formation of a porous matrix. After about 2 weeks

and lasting for the next 2 to 6 months or even longer, a solid implant, having a porous matrix, will release the active ingredient diffusing in the water phase present inside the porous solid implant from which it migrates into the biological surrounding. Gradually, the initially formed implant will degrade and the implant will start to shrink until it disappears due to degradation and bioresorption of the matrix. In one embodiment, the cosolvent is benzyl benzoate will be released at a slower rate than the active ingredient thus ensuring the beneficial effect of benzyl benzoate to the end of implant existence.

The following non-limiting examples illustrate the invention.

Examples

Polymers

Biodegradable polyester polymers for ISFI formulations were made and purified using the methods described in patent application WO2019097262 (A1).

The polymers comprised polylactides (PLA) optionally with glycolic acid comonomers.

The ratio between the L- and D-isomers of the lactide monomers varied from 0 to100 mole parts of L to 100 to 0 mole parts of D, typically a majority of the lactide monomers were L isomers, a polymer structure unit molar ratio of 100:0 to 70:30 (L-to-D) being particularly preferred.

The preferred polylactide homo- or copolymers (with glycolic acid) were branched and in one embodiment exhibited 4 terminal groups.

The following polylactides were employed:

PLGA5050, PLGA6040, PLGA4060, PLGA7030, PLGA8020, PLGA9010, PLA100, PLGA8515,

Preparation and dissolution ISFI formulations

Formulation preparation and dissolution study

In one embodiment, the biodegradable polymer which is in powder form is dissolved in the biocompatible solvent in suitable size laboratory mixer. After the fluid is homogenous and free of particulates the desired active ingredient is added and the formulation is mixed again thus resulting in a homogenous formulation, either one phase clear fluid or dispersion or suspension. Sterile filtering can be done either before or after addition of active ingredient. If done after the addition of active ingredient, the formulation has to be free of solid active ingredient particles.

In one embodiment, the above mentioned formulation is loaded to a 100 µL glass syringe through suction from a vial when some air bubbles may arise to the syringe, then this loading is promptly emptied back to the formulation container, thus achieving bubble free syringe tip. Then the glass syringe is filled to 70 µL limit from formulation container, this way bubble free loading is achieved. The injection needle is attached to the syringe whilst pointing the syringe tip upwards, continuing with filling the needle until a drop can be seen at the end of the needle. The product is ready for injection.

In one embodiment, for dissolution study, the formulation is injected to the stainless steel or polypropylene mesh pouch (eye e.g. 210 µm). The pouch is settled in dissolution vial containing dissolution media (e.g. phosphate buffered saline at 35.5 °C) volume enough to remain sink conditions during dissolution study. At the wanted time points the pouch with the implant is transferred to a new dissolution vial with dissolution media and from the one remained the drug concentration is analysed through HPLC analysis. Thus cumulative and daily drug release are easily calculated.

Thumb force

Thumb force should be measured to show the feasibility of the target product. The syringeability of the ISFI formulations were tested by practical thumb testing and Injection force measurements.

An example of a test is shown in Figure 1 In the test setup, with 200 N cell, polyethylene 1 ml syringe, rubber seal in plunger, 21 G needle, L16 mm Water and air were used as references.

The results shown in the figure are at good thumb force level < 10 N.

Implant formation
thinner needles

Formation *in vivo* and *in vitro* of implant formulations were examined.

Different formulations were injected subcutaneously and intravitreally as illustrated in Figures 2a and 2b. In Figure 2a, the reference numerals 1 to 3 are used for the epidermis 1, SC Fatty tissue 2 and the muscle 3. An *in vivo* implant 4 was formed by injection carried out with a syringe 5, 5a into an eye 6.

Figures 2a and 2b shows examples of *in vivo* implant formation by a) subcutaneous ISFI SiSu® injection 4a in a rat and b) intravitreal ISFI SiSu® injection 4b in rabbit.

Figures 3a and 3b shows examples of implant formation *in vitro* by 10 µl injection of formulation into PP pouch in PBS (pH 7.2) after 2 h incubation at 35.5 °C at 60 rpm using 26G needle, length 10mm. The volume of the syringe used was 100µl.

Syringes

To illustrate the use of the final product, i.e. controlled release pharmaceutical composition for eye injection forming implant *in situ*, opening, mixing the product, needle attachment and injecting the product intravitreally, three examples are given, viz. Case 1: premixed formulation; Case 2: using preparative syringe mixing; Case 3: using pen.

These cases are also shown the attached drawings: Figure 4 illustrates Case 1, Figure 5 illustrates Case 2, and Figure 6 illustrates Case 3.

Case 1: Product must be at room temperature prior to injection. Final product is provided in the form of single sterile syringe 13 containing formulation.

Formulation comprises the mixture of drug, purified bioresorbable polyester and solvent in a fluid form. The syringe is capped. The cap 11 is removed from the syringe and user inserts hypodermic needle 12 to the tip. Prior to administration, syringe is purged in upright position from any large air bubbles through the needle. The dosage is injected to the desired place in the eye.

Case 2: This product must be at room temperature prior to injection. The final product is provided in the form of sterile part A and B in two syringes 19.

Part A comprises the weighed drug in the powder or liquid form in the tip of syringe A, 14 which is capped. Part B comprises the excipient purified bioresorbable polyester and solvent in a fluid form in another syringe B 15 which is also capped. The caps are removed from the syringes and then they are combined with small sterile coupler 16 (one option is using syringes that can be coupled straight, e.g. syringes with male and female luer-lock). Mixing is carried out by compressing the fluid from syringe B through the coupler to the syringe A that has drug inside 19. Then the formed mixture of drug and fluid is compressed again from syringe A to the syringe B. This reciprocal movement is repeated 20 times and drug suspension, or solution is left to syringe A. The drug is then homogenously suspended or dissolved in the fluid in the syringe A 14. The syringe A is detached from coupler and a hypodermic needle18 is inserted into the tip. Prior to administration, purge in upright position any large air bubbles from the needle. The dosage is then injected e.g. intravitreally to the desired place.

After injection, the needle is kept in its place approximately for few seconds before removing it from tissue.

Case 3: This product must be at room temperature prior to injection (cf. Figure 6). The final product is provided in the form of pen that comprises two syringe pistons 22, 23, mandrel 21, septum 24, needle 25 with inner thread and transparent cylinder with side runner channel(s) holding pistons inside as depicted below.

The drug powder or liquid drug is stored in compartment A and purified bioresorbable polyester and solvent in a fluid form in compartment B separated by the pistons 23. Before administration following steps are carried out. The needle 25 is tightened to the septum 24 while the inner part of needle punctures the septum. Then the plunger 21 is pushed forward in upright position which makes the pistons 22 and 23 move with the fluid. Piston 23 stops moving when it reaches the position at the side runner channel(s) while piston 1 continues moving pushing the fluid from compartment B to compartment A through the side channel(s). Air from compartment A escapes from the needle. Mandrel is pushed forward until piston 22 reaches piston 23, when it stops. Now the drug and fluid are combined in compartment A. Next the drug is mixed with fluid by tapping it against other hand holding from cylinder until it is homogenous, tapping time depends on the tapping rate/speed.

Prior to administration, turn the mandrel clockwise 90° when further movement of the mandrel is allowed. Purge in upright position any air from the needle. Inject the dosage e.g intravitreally to the desired place in the eye. After injection keep the needle in its place approximately for few seconds before removing it from tissue.

***In vitro* release (examples)**

A number of active pharmaceutical ingredients (APIs), including latanoprost, regorafenib, sunitinib, dexamethasone, exenatide, BSA, insulin and risperidone, have been tested *in vitro* implant formulations for *in vivo* use.

Example 1A

Latanoprost

Latanoprost was tested for long-term drug release. The results are illustrated in Figures 7 to 9, depicting the long-term drug release on daily basis and cumulatively.

In vitro example of long term latanoprost release (35.5 °C, PBS pH 7.2) containing 68% DMSO (LAF98) is depicted in Figures 7a and 7b.

Figures 8a, 8b and 8c, 8d show cumulative and daily release curves with two dosages. The effect of additives and excipients on the release of latanoprost was also tested (Figure 8c and d).

The effect of additive (benzyl benzoate, BB) shown in Figure 9. As will appear, 2 % of BB reduces the burst to 36 % of the original value of formulation without the additive. The BB addition seems to reduce burst.

Example 1B
Hydrolysis of latanoprost to latanoprost acid in porcine vitreous (cf. also dissolution tests within the vitreous)

Porcine vitreous was mixed with Hanks' balanced salt solution containing 50 mM Hepes (HBSS-buffer, pH 7.4) at and vitreous mix (200 µl) was pipetted to 96-well plates. The plates were incubated at +37°C and 5% CO_2 with mild shaking for one hour to equilibrate the pH of vitreous into neutral range (pH 7.3–7.5). After incubation, 50 µl of 50 µg/mL latanoprost was added to plates and mixed. The final vitreous/buffer ratio was 1:1 and latanoprost concentration 10 µg/mL Latanoprost was incubated in vitreous at +37°C and 5% CO_2 with mild shaking and samples were collected at time points (0, 1, 2, 4, 6, 24, 29, 48 hours). At each time point the 150 µl of the sample was mixed by vortexing with the 150 µl acetonitrile. The samples were stored at -20°C until HPLC analysis.

Chemical stability of latanoprost was studied simultaneously in HBSS-buffer, pH 7.4, using the same protocol but replacing the vitreous with the buffer.

Hydrolysis of latanoprost was evaluated in buffer and in vitreous with pH adjusted to 7.4. No latanoprost acid was formed in mere buffer whereas in vitreous the amount of latanoprost acid was steadily increased during 48 h experiment (Fig. 10). This indicates that the hydrolysis of latanoprost in vitreous was mediated by enzymatic hydrolysis. Latanoprost acid formation in vitreous seemed to follow zero-order kinetics with the rate constant of 0.111 µg/hr.

Example 2

Regorafenib

Regorafenib was used as an example API for tyrosine kinase inhibitor release from ISFI implant. Different formulations regorafenib ISFIs made using different PGLA polymers, solvents and additives using the methods described above chapters: All the ingredients were mixed together, and this homogenous mixture was loaded into the syringe with needle and injected to dissolution pouch. The release of the regorafenib was studied by taking samples from dissolution media and using UPLC for release calculations.

Solubility of regorafenib in water and PBS is negligible. Thus, Tween80 was added in PBS to increase solubility in the dissolution media. Regorafenib *in vitro* release studies from PGLA/DMSO ISFI were carried out in sink conditions, 2–5 ml PBS pH 7.2 (+Tween80 0.05 %) in plastic and glass vessels at 35.5 °C (estimated at 10 mm depth in eye) and 60 rpm, orbit 61 mm in dark.

An example of effect of injection volume (10 and 20 µl) for regorafenib cumulative and daily release is shown in Figures 11a and 11b for 4 months study.

An example of the effect of an additive (benzyl benzoate, BB) on regorafenib release is shown in Figures 12a to 12d.

BA (benzyl alcohol) additive (0…10 %) impact on regorafenib release. The results are shown in Figures 13a and 13b. These all are only examples and does not exclude other possible release rates.

Example 3

Sunitinib

Sunitinib maleate and free base were used as example APIs for tyrosine kinase inhibitor release from ISFI implant. Different formulations sunitinib ISFIs made using different PGLA polymers, solvents and additives using the methods described above chapters: All the ingredients were mixed together, and this homogenous

mixture was loaded into the syringe with needle and injected to dissolution pouch. The release of the sunitinib was studied by taking samples and using UPLC.

Sunitinib *in vitro* release studies from PGLA ISFI were carried out using sink conditions, 2-5ml PBS pH 7.2 in plastic and glass vessels at 35.5 °C (estimated at 10 mm depth in eye) and 60 rpm, orbit 61 mm in dark. An example of effect of polymer composition (amount of glycolide in the polymer) for sunitinib free base cumulative and daily release is shown below where the formulation has been diluted with 55 or 65 % of DMSO. There is a clear effect on both cases by which polymer grade have been used.

Release of sunitinib free base is shown in Figures 14a and 14b.

The effect of triacetin on the release rate of sunitinib malate is shown in Figures 15a and 15b.

An example of effect of additives on sunitinib release rate is shown in Figures 16a and 16b. In particular, the figures show the effect of BB (benzyl benzoate) addition in amounts of 0, 2, 4 and 8% on sunitinib release.

By use of additives, the burst can be eliminated totally. This was shown in one example employing 6 % of BB as an additive and depicted in Figures 17a and 17b.

Example 4

Dexamethasone

Dexamethasone was used as an example API for corticosteroid release from ISFI implant.

Different formulations for Dexamethasone ISFIs were made using different PGLA polymers, solvents and additives using the methods described above. Thus, all the ingredients were mixed together, and this homogenous mixture was loaded into the syringe with needle and injected to dissolution pouch.

The release of the Dexamethasone was studied by taking samples and analysing the samples with HPLC.

The effect of the glycolide portion in the PLGA polymer on the properties of the formulation are illustrated in Figures 18a and 18b, also in 18c and 18d with higher drug loading. It can be seen from the figures that the loading of the drug may have an effect on the burst behavior.

Dexamethasone *in vitro* release studies from PGLA ISFI were carried out, 2–5 ml PBS pH 7.2 in plastic and glass vessels at 35.5 °C (estimated at 10 mm depth in eye) and 60 rpm, orbit 61 mm in dark. An example of the effect of additive (benzyl benzoate, BB, and benzyl alcohol, BA) in formulation composition for Dexamethasone cumulative and daily release is shown in Figures 19a and 19b.

The effect of the of addition of SAIB to a formulation including PLGA which facilitates the solid implant formation is illustrated in Figure 19 showing the cumulative effect as a function of time.

SAIB seems to reduce the burst for this formulation composition, where amount of polymer is constant.

The effect of solvents (e.g. DMSO vs. NMP) and solvent mixtures were also studied. The example illustrated in Figure 21 shows that NMP as a solvent gives higher burst for dexamethasone formulations with PLGA, which may also facilitate the drug release control through solvent selection or solvent blending.

Example 5

Exenatide

Exenatide is an example of high molecular weight peptide. As an example for peptide (Exenatide) release below, the effect of additive/solvent (DMSO/triacetin) ratio for release profile, instead of pure solvents also peptide compatible additives may be applied if different drug release profiles are needed. The results are shown in Figure 22.

Example 6

BSA

BSA (bovine serum albumin) was tested as a model protein.

This example demonstrates the release of high molecular weight protein release from the ISFI formulation.

Purified PLGA5050 polymer was used for preparation of following formulation. PLGA5050 was dissolved in DMSO (70 %) and subsequently powder bovine serum albumin (BSA, 10 mg/ml) was suspended in the polymer solution. The drug release rate was measured in phosphate buffered saline (PBS, pH 7.4) by injecting 0.25 ml of suspension in a stainless steel mesh screen pouch, and dropping the pouch in a PBS (12 ml) containing glass vessel, after which precipitation of the formulation started. The glass vessels were placed in a 37 °C tempered incubator with 60 rpm revolving speed (orbit 2 inches). The *in vitro* analysis of the BSA release was carried out by sampling the PBS and analysing the intact BSA in ultrahigh performance liquid chromatography equipment (UHPLC). After every sampling the whole PBS volume was replaced with fresh PBS. The cumulative and daily release profiles from the drug release experiment are shown in Figures 23a and 23b.

Example 7

Insulin

The results for an example of insulin cumulative and daily releases are given in Figures 24a and 24b, respectively.

Example 8

Risperidone

The effect of injection volume and thus also dose on small molecule release (risperidone, Formula C23H27FN4O2, molar mass 410 Daltons) are shown in Figures 25a and 25b; Figure 25a depicting cumulative release and Figure 25b

depicting daily release.

Example 9

The influence of the polymer shape (linear vs. branched) of the resorbable biopolymer on the release of API is illustrated using a copolymer of polylactide and glycolic acid (PL:GA mole ratio of 50:50) in Figure 26.

As will appear, with a branched polymer a steadier and longer drug release is achieved.

Example 10

The dynamic viscosity at 25 °C of an injectable composition comprising PLGA (at a molar ratio of 80:20) in a solvent of DMSO is depicted as a function of spindle speed is shown in Figure 30.

As will appear, the viscosity of this composition was in the range of about 900 to 1200 mPas.

***Ex-Vivo* and *In-Vivo* studies**

Endotoxin test for the formulation, polymer and solvent

Endotoxin tests were carried out for formulation: PLAG8515 42 wt.% and 58 wt.% DMSO. Polymer was dissolved in DMSO (Gaylord, Procipient – USP, Ph. Eur) and then formulation was filter sterilized using 70% isopropanol and sterilized 100 µL (Luer Lock) syringes and 0.2 µm PTFE syringe filters inside disinfected (hydrogen peroxide + 70% IPA wiping + UV 18 h) Class 5 isolator.

The microbiological bioburden and endotoxin tests for placebo formulation and drug (Latanoprost) containing formulation were carried out using LAL endotoxin kit.

Endotoxin tests showed that the studied formulation gave below 0.25 EU/ml values, which fulfills the criteria for eye applications.

Injection of ISFI into isolated pig eye

The aim of this study was to clarify how different placebo formulations at different viscosities form an implant in porcine vitreous *ex vivo*.

Six Hamilton 100 µl LL syringes prefilled with placebo formulations at different DMSO (Gaylord, Procipient – USP, Ph.Eur) concentrations (50%, 55%, 57.5%, 60%, 65% and 70%), used polymer was PLGA8020 . Six fresh porcine eyes (two months old female pigs; a` ca 40 kg). 26G 10 mm needles. More details about the formulations in Table 1.

Table 1.

Formulation	Polymer	DMSO wt.%	Vol/ µL	Injections	Depth of injection/mm
REB50%	PLGA8020	50	100	6x10 µL	2, 3, 4, 5, 6, 7
REB55%	PLGA8020	55	100	6x10 µL	2, 4, 7
REB57.5%	PLGA8020	57.5	100	6x10 µL	2, 4, 7
REB60%	PLGA8020	60	100	6x10 µL	2, 4, 7
REB65%	PLGA8020	65	100	6x10 µL	2, 4, 7
REB70%	PLGA8020	70	100	6x10 µL	2, 4, 7

Injections: The needle in the syringe was inserted about 4.5 mm out of limbus at a 90° angle, through the sclera into the vitreous and the vehicle was slowly (*ca.* 5 s) injected into the vitreous.

The first injections were performed six times with 50 % formulation at different depths into one eye (Table 1). The other five eyes were injected three times; 2, 4 and 7 mm. The depths were marked in the needles with a permanent marker.

Five minutes after the last injection, the cornea, iris and lens were removed from the eyeball to see vitreous and implants.

Results: There were no problems in the preparation of syringes and needles for the injections. Three depths (2 mm, 4 mm and 7 mm) were used after the first injection and formulation. The injection depths of between 4 and 5 mm were the best ones, as it is far enough from the retina and may not disturb an animal's sight. The 57.5% DMSO containing formulation showed to be the most suitable for intravitreal injections in porcine due to the implant shape and suitable thumb force needed for the injection. It is clear that the viscosity and thus implant formation is also effected by the molecular weight and composition of the polymer and the solvent type.

Figure 27 shows photos of *ex vivo* porcine eyes with injected implants and with used solvent (DMSO) amount.

Dissolution tests within the vitreous

These tests were carried out to ensure that the latanoprost released from the implant would go through hydrolysis to latanoprost acid, which is the active form of the molecule. Purpose of this study was also study the conversion rate of latanoprost to latanoprost acid. In addition, the release of latanoprost from ReBio test formulations was evaluated in vitreous vs. in PBS.

First hydrolysis of latanoprost was evaluated in buffer and in vitreous with pH adjusted to 7.4 and non pH adjusted. No latanoprost acid was formed in mere buffer whereas in vitreous the amount of latanoprost acid was steadily increased during 48 h experiment (Figure 28).

This indicates that the hydrolysis of latanoprost in vitreous was mediated by enzymatic hydrolysis. Latanoprost acid formation in vitreous seemed to follow zero-order kinetics with the rate constant of 0.111 µg/hr.

Next the drug release from implant was also tested in porcine vitreous vs. PBS (= phosphate buffered saline). It was noticed as expected that the latanoprost did not change to its acid form in the PBS, thus below the cumulative drug release data is measured for latanoprost (LA) acid form in vitreous and ester form in PBS media. The release curves resemble each other, but the release is a bit higher in the

vitreous, this is most probably due to the higher water solubility of the hydrolysed acid form of the latanoprost, and thus faster distribution in the media.

As discussed above, Figure 10 shows the cumulative release of latanoprost and latanoprost acid from injected composition as a function of time in vitreous and PBS media at 35.5 °C. Formulation composition in this example was 35% PLGA9010/ 60% DMSO/ 4% b-HPCD - 180µg Latanoprost.

Tolerability studies in rats and rabbits

In order to test the tolerability of DMSO in eye, DMSO (USP, Ph. Eur.) was injected to the rat eyes intravitreally using 34 G needle The injected volume was 3 µl and three concentrations were injected; 60%, 1/10 dilution, 1/100 dilution. Used Ph. Eur. grade DMSO was 0.2 µm Filter sterilized in Class 5 isolator before the injection.

The rats were monitored for two weeks using the following endpoints:

- fundus imaging, including blood vessels
- cloudiness and other symptoms in the anterior part of the eye
- optic nerve head
- state of the vasculature in the posterior eye segment (FAG)
- ERG
- intraocular pressure
- retinal thickness with OCT
- histology (in the end)

Results: No adverse effect were noticed that could be related to DMSO in this study.

To study tolerability of placebo formulations, they were injected to the living rabbits' eyes (5 Rabbits, 10 eyes) intravitreally using 26G needle. Tested formulation was filter sterilized by filtering the formulation with 0.2 µm PTFE syringe filter before loading in the isolator to 100 µL syringes. The injected volume was 10 µL The formulation consisted of PLGA8515 42% and DMSO 58%, forming homogenous clear fluid.

The rabbits were monitored for 5 months using the following endpoints:

- Biomicroscopy (slit lamp), including possible blood leakages
- Endothelial swelling
- Cloudiness and other symptoms in the anterior part of the eye
- Lens opacity
- Location/mobility of the implant and its possible fragmentation, photo of implant inside living eye
- ERG (electroretinography), 2…3 times per study period
- Intraocular pressure
- Histology (in the end)

Results: There were no adverse effects caused by the implant compared to normal eye. Example picture of remaining implant in the Rabbit eye vitreous is depicted in the Figure 29.

The following embodiments are typical of the present technology:

1. A composition comprising at least one active pharmaceutical ingredient, at least one biocompatible polymer and at least one biocompatible solvent, wherein

- the composition is in the form of an injectable solution, suspension, emulsion or dispersion,
- the composition is in the form of an *in-situ* forming implant composition, and
- the biocompatible polymer is bioresorbable.

2. The composition of embodiment 1, wherein the composition is suitable for treatment of glaucoma, intraocular pressure, wet Age-Related Macular Degeneration, dry Age-Related Macular Degeneration, diabetic retinopathy, dry eye syndrome, cataract, uveitis.

3, The composition of embodiment 1 or 2, wherein the active pharmaceutical ingredient is selected from active pharmaceutical ingredients for ophthalmic care.

4. The composition of any of embodiments 1 to 3, wherein the active pharmaceutical ingredient is selected from anti-VEGFs, and VEGFs (and their biosimilars), Tyrosine

kinase inhibitors, antiparasites, H2 receptor antagonists, antimuscarinics, prostaglandins and its analogues, non-steroidal anti-inflammatory agents, proton pump inhibitors, aminosalycilates, corticosteroids, chelating agents, cardiac glycosides, phosphodiesterase inhibitors, thiazide, diuretics, anesthetic agents, carbonic anhydrase inhibitors, antihypertensives, anti-cancers, anti-depressants, calcium channel blockers, analgesics, opioid antagonists, antiplatels, anticoagulants, fibrinolytics, statins, adrenoceptor agonists, beta blockers, antihistamines, respiratory stimulants, micolytics, expectorants, barbiturates, anxiolytics, central nervous system agents, tricyclic antidepressants, 5HT1 antagonists, opiates, 5HT1 agonists, antiemetics, antiepileptics, dopaminergics, antibiotics, antifungals, anthelmintics, antivirals, antiprotozoals, antidiabetics, insulin and its derivatives, GLP-1 receptor agonists, thyrotoxins, antioestrogens, hypothalamics, pituitary hormones, posterior pituitary hormone antagonists, peptide drugs, protein drugs, protein kinases, antigens, antidiuretic hormone antagonists, bisphosphonates, dopamine receptor stimulants, androgens, steroid reductase inhibitors, non-steroidal anti-inflammatories, immunosuppressants, local anaesthetic, sedatives, anti-psoriatics, silver salts, topical antibacterials, vaccines, and vaccine antigens and combinations thereof.

5. The composition according to any of the preceding embodiment, wherein the active pharmaceutical ingredient is selected from Latanoprost, Tafluprost, Travoprost, Bimatoprost, Bevacizumab, Ranibizumab, Aflibercept, Adalimumab, Sunitinib, Axitinib, Regorafenib, Pazopanib, Dexamethasone, Fluocinolone, Cyclosporine, triamcinolone acetonide and combinations thereof.

6. The composition according to any of the preceding embodiment, wherein the bioresorbable polymer is selected from Polylactide (i.e. poly(lactic acid), PLA), polyglycolide (PGA) and poly(∈-caprolactone) (PCL), polydioxanone (PDO), polytrimethylenecarbonate (PTC), polylactides (PLA), poly-L-lactide (PLLA), poly-DL-lactide(PDLLA), PolyL-DL-lactide (PLDL), stereocopolymers; polyglycolide (PGA); copolymers of glycolide, glycolide/trimethylene carbonate copolymers (PGA/TMC); other copolymers of PLA, such as lactide/tetramethylglycolide copolymers, lactide/trimethylene carbonate copolymers, lactide/d-valerolactone copolymers, lactide/∈-caprolactone copolymers, L-lactide/DL-lactide copolymers,

glycolide/L-lactide copolymers (PGA/PLLA), polylactide-co-glycolide; terpolymers of PLA, such as lactide/glycolide/trimethylene carbonate terpolymers, lactide/glycolide/∈-caprolactone terpolymers, PLA/polyethylene oxide copolymers; polydepsipeptides; unsymmetrically3,6-substitutedpoly-1,4-dioxane-2,5-diones; polyhydroxyalkanoates, such as polyhydroxybutyrates (PHB); PHB/b-hydroxyvalerate copolymers (PHB/PHV); poly-b-hydroxypropionate (PHPA); poly-p-dioxanone (PDS); poly-d-valerolactone-poly-∈-caprolactone, poly(∈-caprolactone-DL-lactide) copolymers; methylmethacrylate-N-vinyl pyrrolidone copolymers; polyesteramides; polyesters of oxalic acid; polydihydropyrans; polyalkyl-2-cyanoacrylates; polyurethanes (PU); polyvinylalcohol (PVA); polypeptides; poly-b-malic acid (PMLA); poly-b-alkanoic acids; polycarbonates; polyorthoesters; polyphosphates; poly(ester anhydrides); and mixtures thereof; and natural polymers, such as sugars, starch, cellulose and cellulose derivatives, polysaccharides, collagen, chitosan, fibrin, hyaluronic acid, polypeptides and proteins or blends and combinations thereof.

7. The composition according to any of the preceding embodiments, wherein the polyester is non-linear, such as branched (including star-shaped or hyperbranched or dendrimers).

8. The composition according to any of the preceding embodiments, wherein the bioresorbable polymer is a purified polyester, wherein the metal content of the polyester is < 40ppm and the metal selected from tin, zinc, iron, aluminium, titanium, platinum, bismuth, manganese, antimony, nickel, calcium, magnesium, sodium, lithium, yttrium, lanthanum, samarium, zirconium, ruthenium and combinations thereof.

9. The composition according to any of the preceding embodiments, wherein the dynamic viscosity, at 25 °C, of the composition is between 10 and 100,000 mPas, preferably 100 to 3000 mPas and more preferably 200 to 2000 mPas, measured with a rheometer and extrapolating the shear rate to 0 s^{-1}.

10. The composition according to any of the preceding embodiments, which composition is formulated for use with an injection needle having a diameter corresponding to any of classes G20 to G31 or thinner, typically G26 to G31.

11. A controlled release pharmaceutical composition for eye injection comprising a composition according to any of embodiments 1 to 10 for use in therapy of ophthalmic conditions.

12. The pharmaceutical composition for use according to embodiment 11, wherein the injection is given to any part of the eye comprising intravitreally, intracamerally, periocularly, subchoroidally, subconjunctivally, subretinal, or under eye mucosal layer or utilizing pre-injected watery bleb under the surface of the eye.

13. The pharmaceutical composition for use according to embodiment 12 or 13, wherein the injection is given to the eye as needed or regularly between once a week and annually.

Reference Numerals

1	Epidermis
2	SC fatty tissue
3	Muscle
4, 4a, 4b	ISFI
5, 5a	Syringe
6	Eye
11	Cap
12	Needle
13	Syringe
14	Syringe A
15	Syringe B
16	Coupler
17	Cap
18	Needle
19	Mixing

20	Ready-to-use syringe
21	Plunger
22	Piston 1
23	Piston 2
24	Septum
25	Needle

Claims

1. A controlled release pharmaceutical composition for eye injection for ophthalmic care, comprising at least one active pharmaceutical ingredient and at least one biocompatible polymer and at least one biocompatible solvent, wherein

i) the composition is in the form of an injectable solution, suspension, emulsion or dispersion,

ii) the composition is in the form of an *in-situ* forming implant composition, and

iii) the biocompatible polymer is bioresorbable.

2. The controlled release pharmaceutical composition for eye injection of claim 1, wherein the composition is for treatment of glaucoma, intraocular pressure, wet Age-Related Macular Degeneration, dry Age-Related Macular Degeneration, diabetic retinopathy, dry eye syndrome, cataract, uveitis.

3. The controlled release pharmaceutical composition for eye injection according to claims 1 or 2, wherein the active pharmaceutical ingredient is selected from anti-VEGFs, and VEGFs (and their biosimilars), Tyrosine kinase inhibitors, antiparasites, H2 receptor antagonists, antimuscarinics, prostaglandins and its analogues, non-steroidal anti-inflammatory agents, proton pump inhibitors, aminosalycilates, corticosteroids, chelating agents, cardiac glycosides, phosphodiesterase inhibitors, thiazide, diuretics, anesthetic agents, carbonic anhydrase inhibitors, antihypertensives, anti-cancers, anti-depressants, calcium channel blockers, analgesics, opioid antagonists, antiplatels, anticoagulants, fibrinolytics, statins, adrenoceptor agonists, beta blockers, antihistamines, respiratory stimulants, micolytics, expectorants, barbiturates, anxiolytics, central nervous system agents, tricyclic antidepressants, 5HT1 antagonists, opiates, 5HT1 agonists, antiemetics, antiepileptics, dopaminergics, antibiotics, antifungals, anthelmintics, antivirals, antiprotozoals, antidiabetics, insulin and its derivatives, GLP-1 receptor agonists, thyrotoxins, antioestrogens, hypothalamics, pituitary hormones, posterior pituitary hormone antagonists, peptide drugs, protein drugs, protein kinases, antigens, antidiuretic hormone antagonists, bisphosphonates, dopamine receptor stimulants, androgens, steroid reductase inhibitors, non-steroidal anti-inflammatories,

immunosuppressants, local anaesthetic, sedatives, anti-psoriatics, silver salts, topical antibacterials, vaccines, and vaccine antigens and combinations thereof.

4. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the active pharmaceutical ingredient is selected from Latanoprost, Tafluprost, Travoprost, Bimatoprost, Bevacizumab, Ranibizumab, Aflibercept, Adalimumab, Sunitinib, Axitinib, Regorafenib, Pazopanib, Dexamethasone, Fluocinolone, Cyclosporine, triamcinolone acetonide and combinations thereof.

5. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the bioresorbable polymer is selected from Polylactide (i.e. poly(lactic acid), PLA), polyglycolide (PGA) and poly(∈-caprolactone) (PCL), polydioxanone (PDO), polytrimethylene (PTC) carbonate, polylactides (PLA), poly-L-lactide (PLLA), poly-DL-lactide(PDLLA), PolyL-DL-lactide (PLDL), stereocopolymers; polyglycolide (PGA); copolymers of glycolide, glycolide/trimethylene carbonate copolymers (PGA/TMC); other copolymers of PLA, such as lactide/tetramethylglycolide copolymers, lactide/trimethylene carbonate copolymers, lactide/d-valerolactone copolymers, lactide/∈-caprolactone copolymers, L-lactide/DL-lactide copolymers, glycolide/L-lactide copolymers (PGA/PLLA), polylactide-co-glycolide; terpolymers of PLA, such as lactide/glycolide/trimethylene carbonate terpolymers, lactide/glycolide/∈-caprolactone terpolymers, PLA/polyethylene oxide copolymers; polydepsipeptides; unsymmetrically 3,6-substituted poly-1,4-dioxane-2,5-diones; polyhydroxy-alkanoates, such as polyhydroxybutyrates (PHB); PHB/b-hydroxyvalerate copolymers (PHB/PHV); poly-b-hydroxypropionate (PHPA); poly-p-dioxanone (PDS); poly-d-valerolactone-poly-∈-caprolactone, poly(∈-caprolactone-DL-lactide) copolymers; methylmethacrylate-N-vinyl pyrrolidone copolymers; polyesteramides; polyesters of oxalic acid; polydihydropyrans; polyalkyl-2-cyanoacrylates; polyurethanes (PU); polyvinylalcohol (PVA); polypeptides; poly-b-malic acid (PMLA); poly-b-alkanoic acids; polycarbonates; polyorthoesters; polyphosphates; poly(ester anhydrides); and mixtures thereof; and natural polymers, such as sugars, starch, cellulose and cellulose derivatives, polysaccharides, collagen, chitosan,

fibrin, hyaluronic acid, polypeptides and proteins or blends and combinations thereof.

6. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the bioresorbable polymer is a purified polyester, wherein the metal content of the polyester is < 40 ppm, in particular the metal content of the polyester is > 0 ppm and < 40ppm, and the metal selected from tin, zinc, iron, aluminium, titanium, platinum, bismuth, manganese, antimony, nickel, calcium, magnesium, sodium, lithium, yttrium, lanthanum, samarium, zirconium, ruthenium and combinations thereof.

7. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the purification comprises the steps of:

i) dissolving the polyester in an organic solvent to form a polyester-solvent solution, wherein the organic solvent comprises at least one heteroatom selected from oxygen, nitrogen, sulphur, chlorine and phosphorus;

ii) precipitating the polyester from the polyester-solvent solution by combining the polyester-solvent solution with an organic non-solvent, said non-solvent being an alcohol; and

(iii) separating the precipitated polyester from the solvent and non-solvent to obtain a purified polyester.

8. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the total amount of residual monomers is less than 0.2wt%, such as less than 0.1wt%.

9. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the polyester is formed from the polymerisation of lactide, glycolide, caprolactone, or combinations thereof.

10. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the polyester is polylactic acid, polyglycolic acid, polycaprolactone, poly(lactide-co-glycolide), poly(lactide-co-caprolactone), poly(glycolide-co-caprolactone), or poly(lactide-co-glycolide-co-caprolactone).

11. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the polyester is non-linear, such as branched (including star-shaped or hyperbranched or dendrimers).

12. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the composition comprises at least one biocompatible liquid capable of dissolving at least 1 mg/ml of the purified polyester at 35–37 °C, and capable of dissolving, dispersing or suspending at least one active pharmaceutical ingredient.

13. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the composition comprises at least one biocompatible solvent or additive selected from N-methyl-2-pyrrolidone, triacetin, dimethylsulfoxide, benzyl benzoate, benzyl alcohol, triethyl citrate, triethyl acetyl citrate, ethyl acetate,anisole, glycofurol, PEG (polyethylene glycol), PPG (polypropylene glycol), polycaprolactones and combinations thereof, in particular the composition comprises dimethylsulfoxide as a solvent and optionally benzyl benzoate as a cosolvent.

14. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the composition comprises a biocompatible excipient, in particular selected from vanillin, SAIB, polycaprolactonediol, hydroxypropylmethylcellulose, HP-b-CD, cyclodextrins, MCT, dextrans, sucrose, crown ethers, chitosan, mannitol, trehalose, or combinations thereof.

15. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the weight ratio between biocompatible polymer and biocompatible solvent varies in the range from 1 : 99 to 99 :1, preferably from 10 : 90 to 90 : 10, more preferably from 20 : 80 to 80 : 20, most preferably from 40 : 60 to 60 : 40.

16. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the injection volume varies between 1 microliter and 1000 microliter, preferably 2 microliter to 500 microliter, for example

between 3 microliter and 100 microliter, more preferably from 5 microliter to 50 microliter, most preferably from 10 microliter to 30 microliter or 2 to 15 microliter.

17. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the API loading varies between 0.1wt% and 90wt% of the total weight of the polymer-solvent mixture, preferably 0.2wt% to 50wt%, more preferably 0.5wt% to 30wt%, most preferably from 1wt% to 10wt%.

18. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the daily dosage and drug release duration is controlled by formulation composition and injection volume.

19. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, wherein the dynamic viscosity, at 25 °C, of the injectable composition is between 10 and 100,000 mPas, preferably 100 to 3000 mPas and more preferably 200 to 2000 mPas, measured with a rheometer and extrapolating shear rate to 0 s^{-1}.

20. The controlled release pharmaceutical composition for eye injection according to any of the preceding claims, formulated for use with an injection needle having a diameter corresponding to any of classes G20 to G31.

21. A drug product for injecting a subject with a controlled release pharmaceutical composition for eye injection comprising,

i) a closed syringe comprising the formulation and a needle for injection or
ii) a closed vial or ampule containing the formulation and empty syringe to be filled from vial or ampule containing the formulation and needle for injection or
iii) a closed syringe comprising the formulation and empty syringe to be filled from the first syringe and a needle for injection.

22. A drug product for injection a subject with a controlled release pharmaceutical composition for eye injection comprising

- a kit containing a first syringe with placebo formulation and a second syringe with active ingredient, the syringes being adapted to be connected to each other and the contents mixed with reciprocal action between the syringes, and
- a needle to be attached for injection to the syringe where the mixed formulation is left.

23. The drug product according to claim 21 or 22, wherein the pharmaceutical composition is according to any of claims 1 to 20.

24. A method of treating a subject in need thereof with a controlled release pharmaceutical composition for eye injection for ophthalmic care, comprising administering to said subject an injection of a composition according to any of claims 1 to 18.

25. The method according to claim 24, wherein the injection is given to any part of the eye comprising intravitreally, intracamerally, periocularly, subchoroidally, subconjunctivally, subretinal, or under eye mucosal layer or utilizing pre-injected watery bleb under the surface of the eye.

26. The method according to claim 24 or 25, wherein an injection having a volume of 1 to 50 µl, in particular 2 to 25 µl, is given intravitreally or subconjunctivally

27. The method according to any of claims 24 to 26, wherein the injection is given to the eye as needed or regularly between once a week and annually, in particular once each 3rd to 6th month.

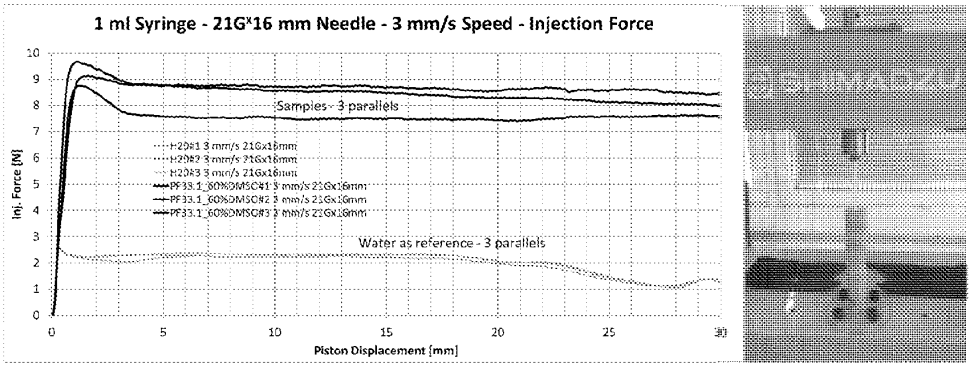


Fig. 1

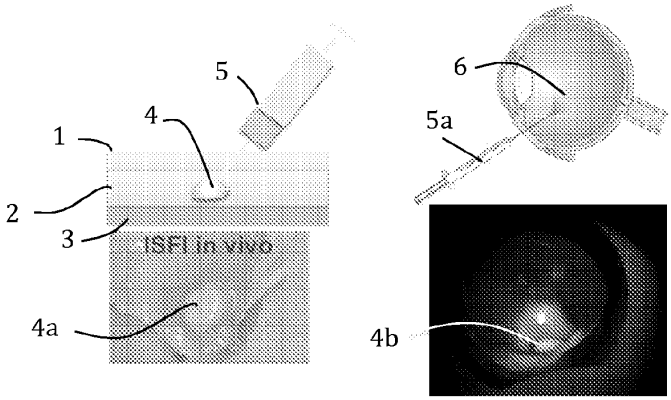


Fig. 2a

Fig. 2b

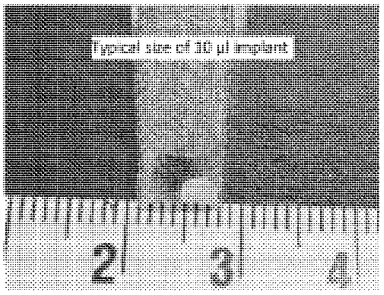


Fig. 3a

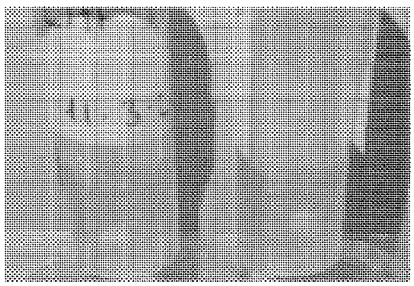


Fig. 3b

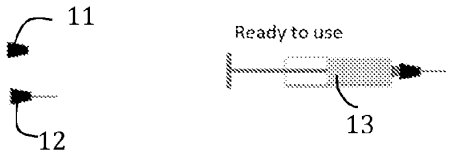


Fig. 4

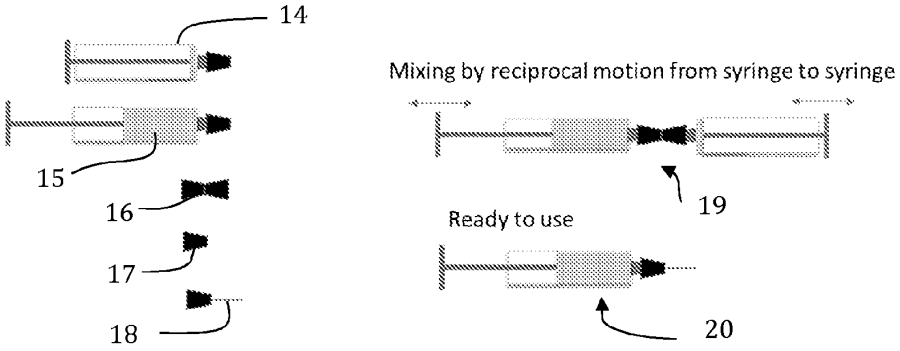


Fig. 5

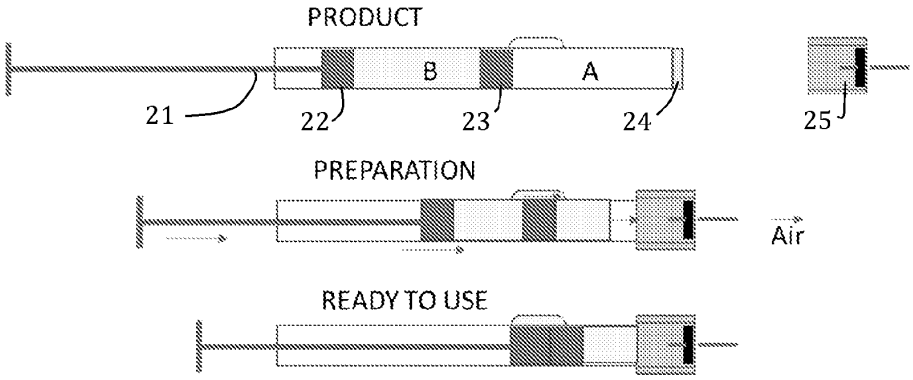


Fig. 6

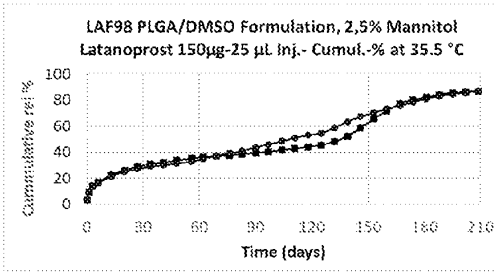


Fig. 7a

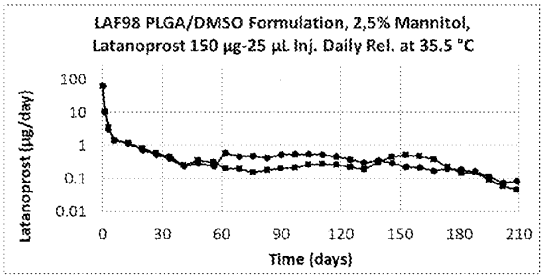


Fig. 7b

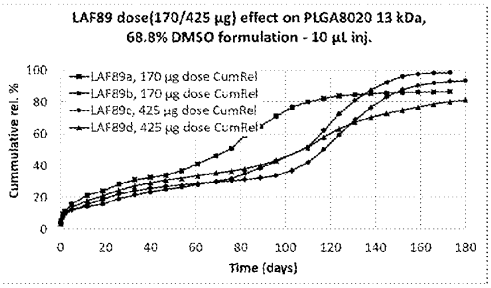


Fig. 8a

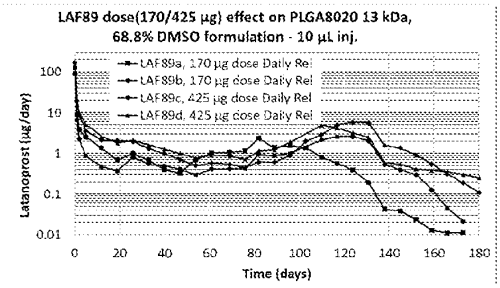


Fig. 8b

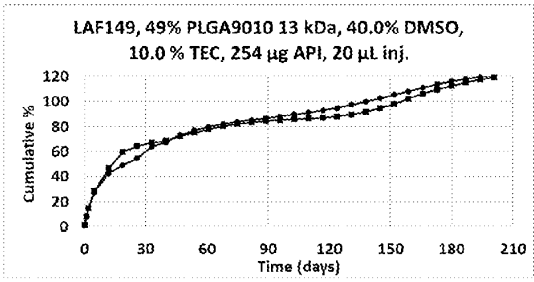


Fig. 8c

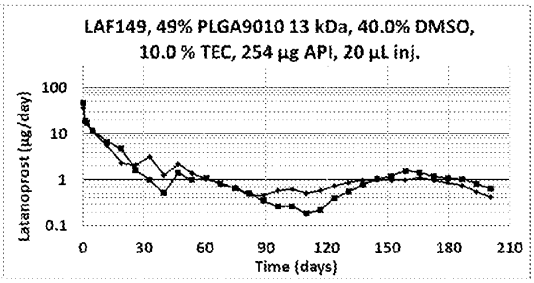


Fig. 8d

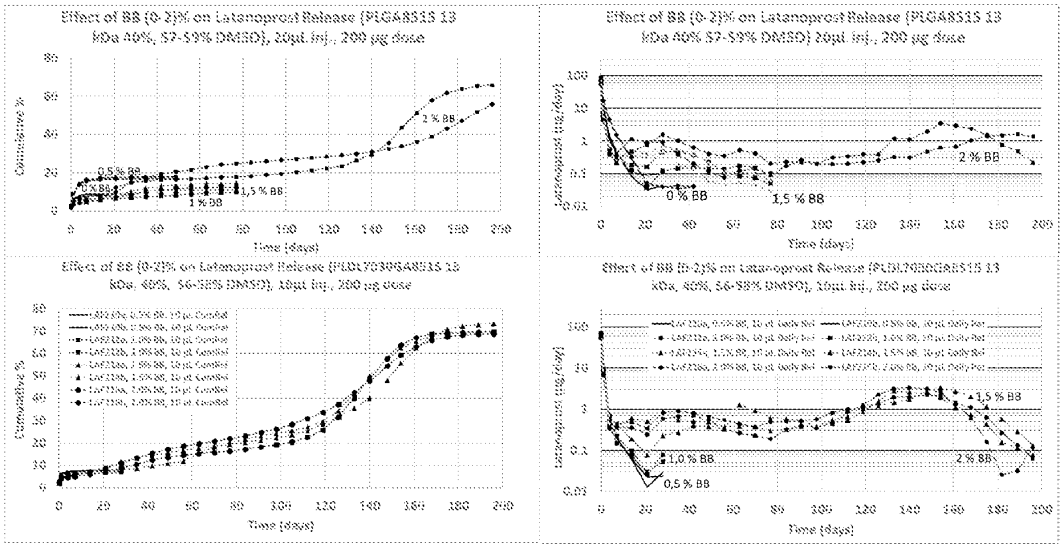


Fig. 9a to 9d

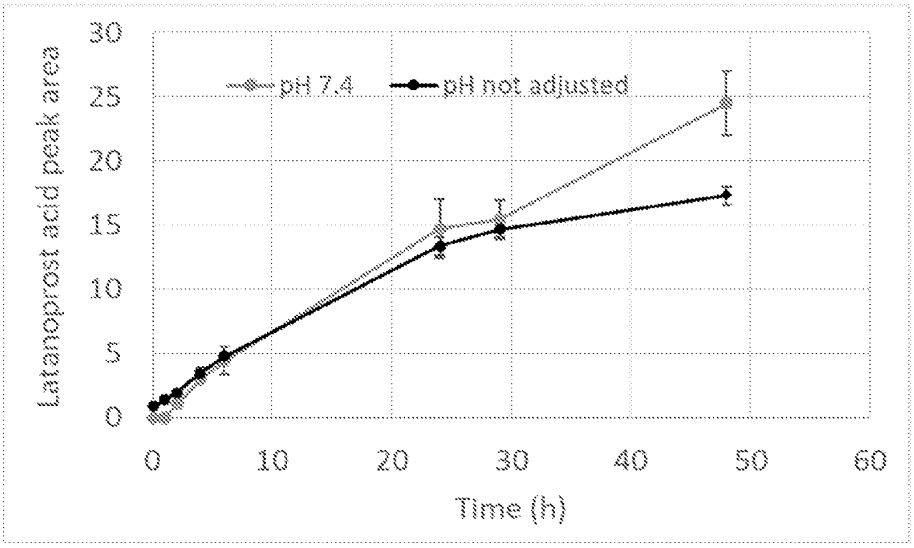


Fig. 10

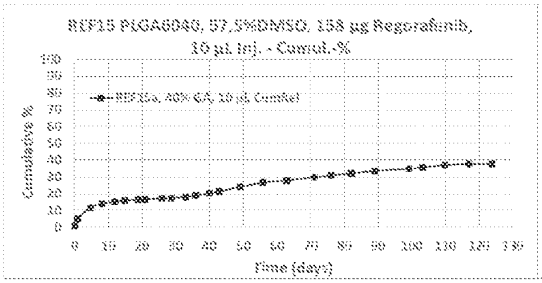


Fig. 11a

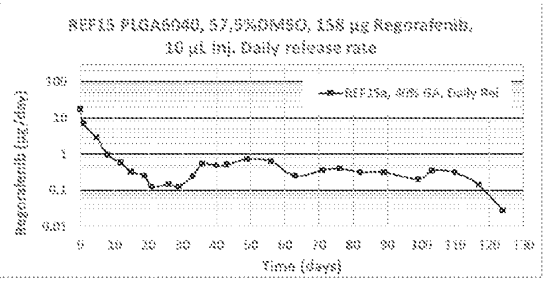


Fig. 11b

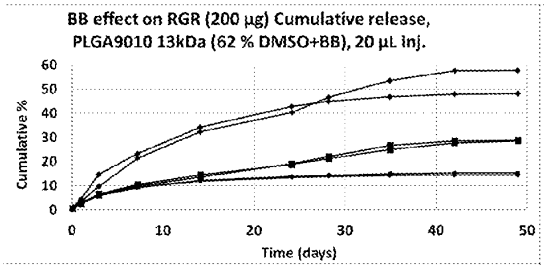


Fig. 12a

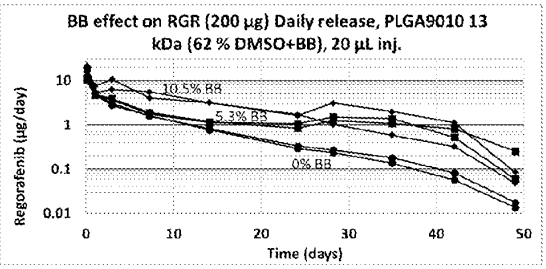


Fig. 12b

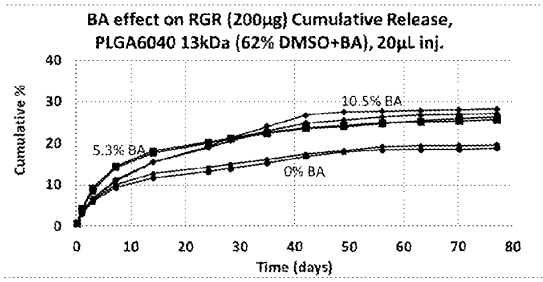


Fig. 13a

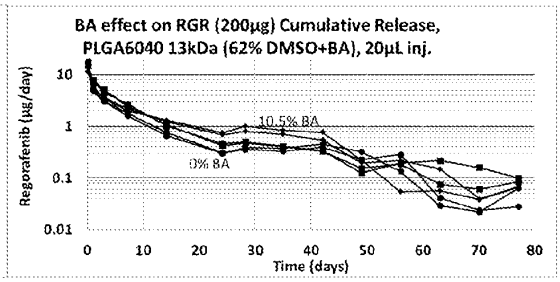


Fig. 13b

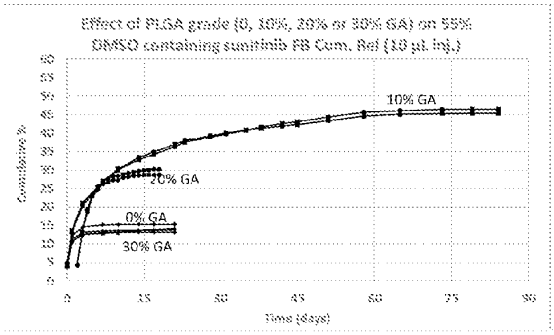


Fig. 14a

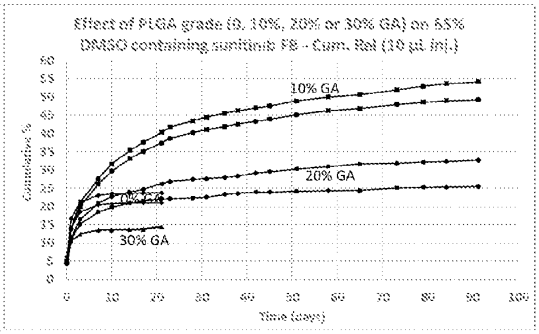


Fig. 14b

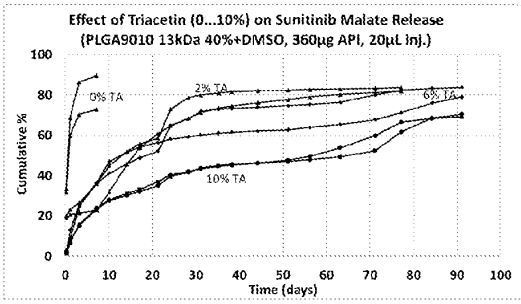


Fig. 15a

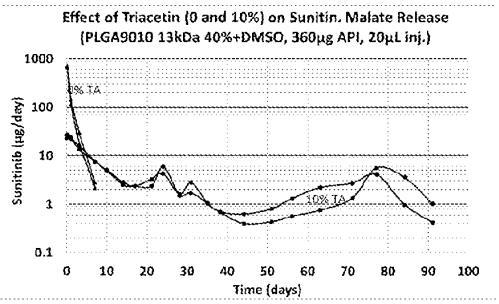


Fig. 15b

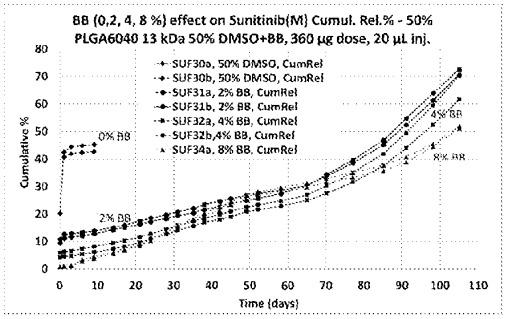


Fig. 16a

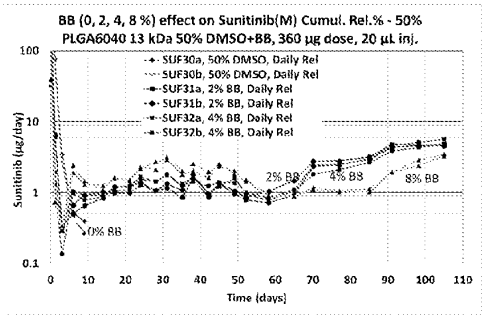


Fig. 16b

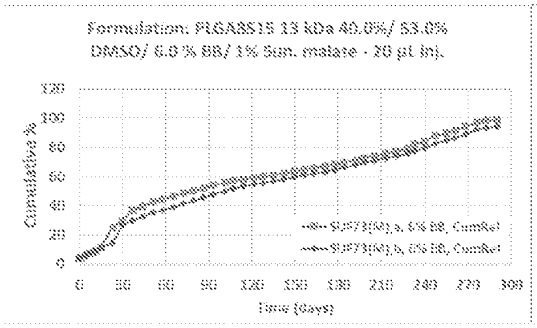


Fig. 17a

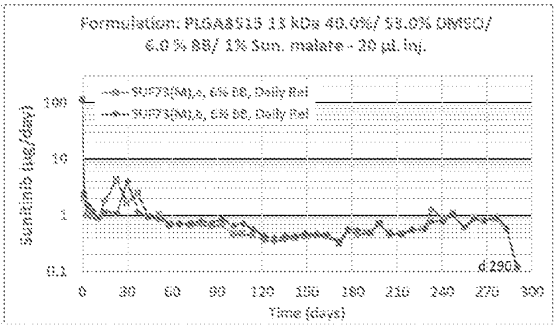


Fig. 17b

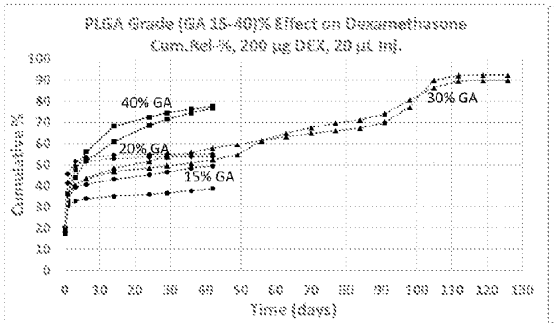


Fig. 18a

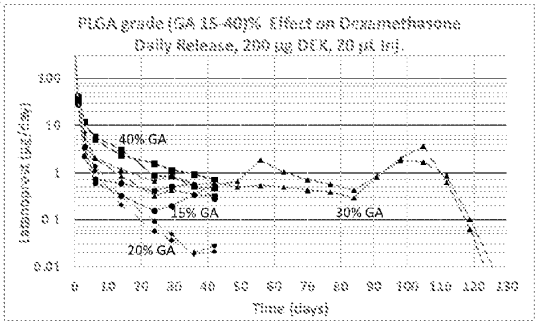


Fig. 18b

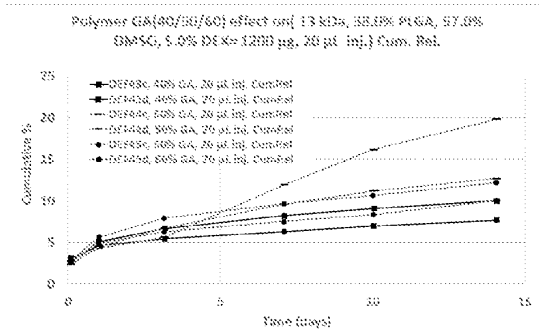


Fig. 18c

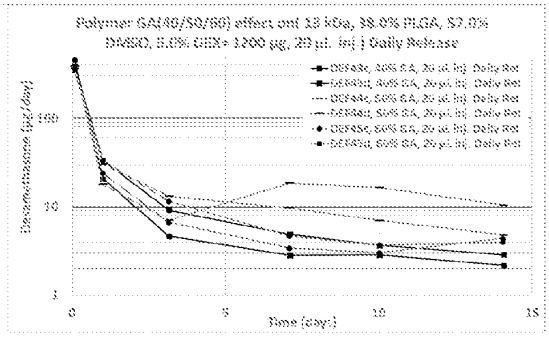


Fig. 18d

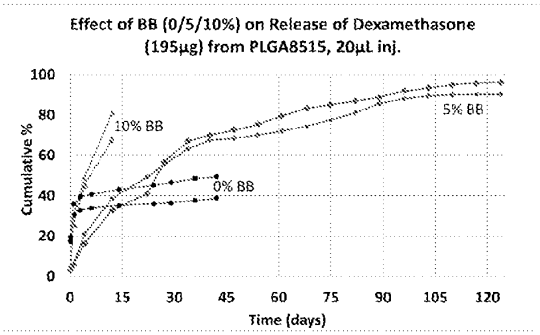


Fig. 19a

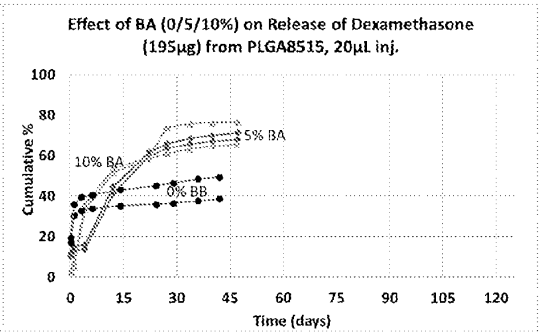


Fig. 19b

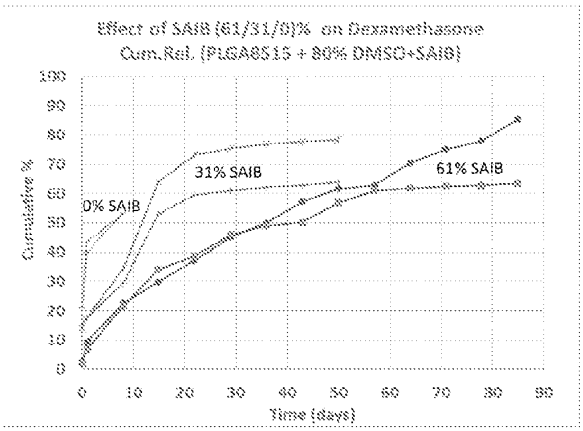


Fig. 20

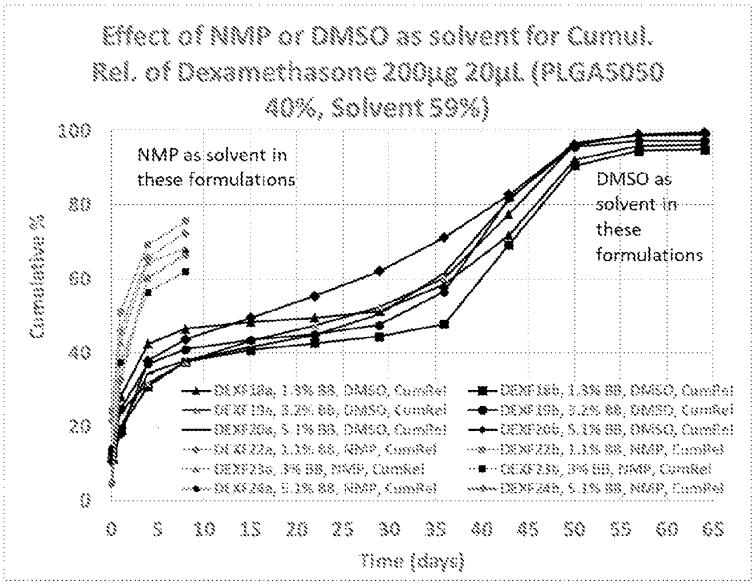

Effect of NMP or DMSO as solvent for Cumul. Rel. of Dexamethasone 200µg 20µL (PLGA5050 40%, Solvent 59%)
NMP as solvent in these formulations
DMSO as solvent in these formulations
Cumulative %
Time (days)

Fig. 21

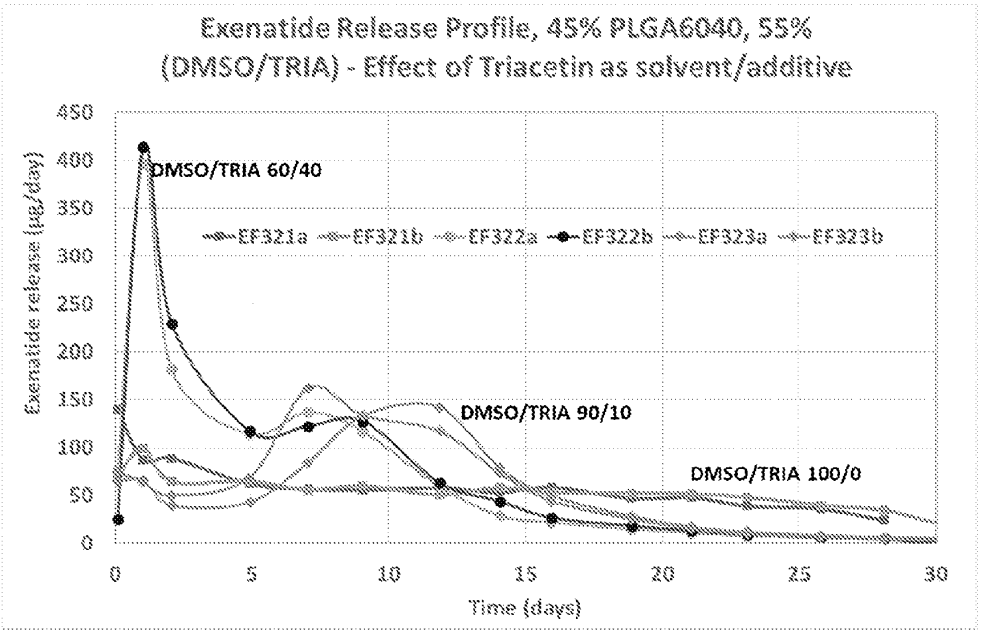

Exenatide Release Profile, 45% PLGA6040, 55% (DMSO/TRIA) - Effect of Triacetin as solvent/additive
DMSO/TRIA 60/40
DMSO/TRIA 90/10
DMSO/TRIA 100/0
EF321a EF321b EF322a EF322b EF323a EF323b
Exenatide release (µg/day)
Time (days)

Fig. 22

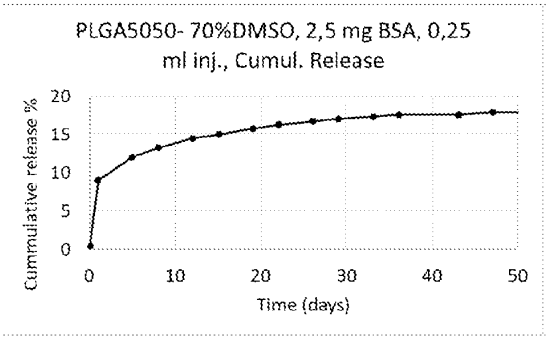


Fig. 23a

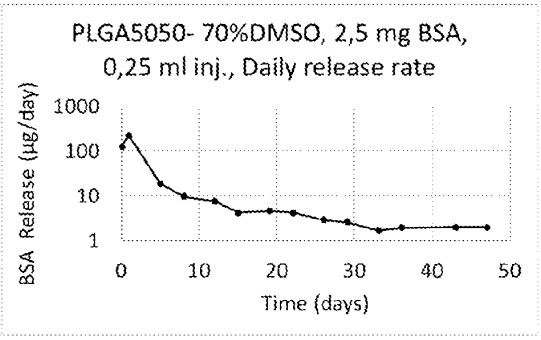


Fig. 23b

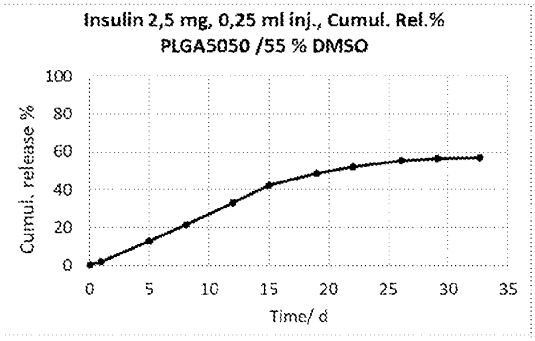


Fig. 24a

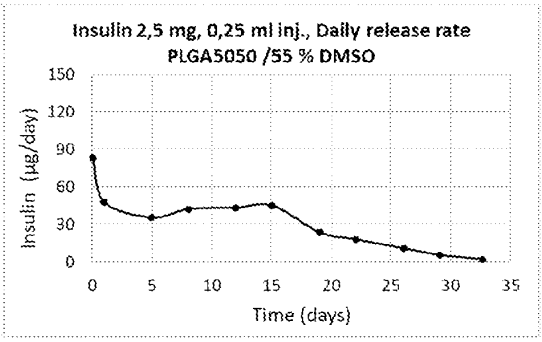


Fig. 24b

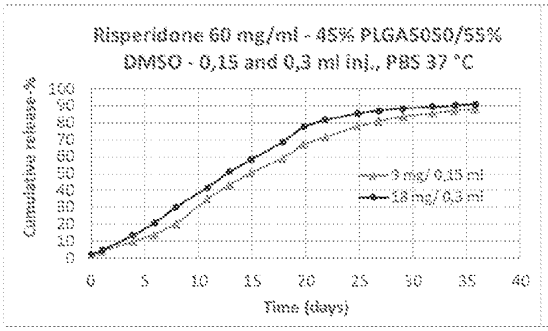


Fig. 25a

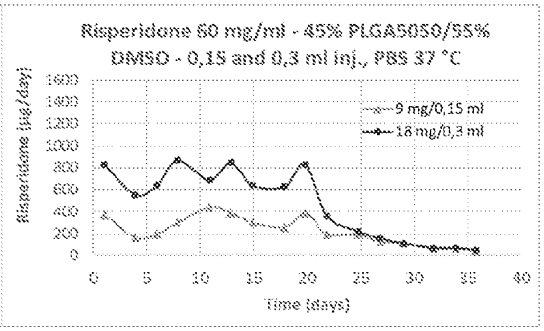


Fig. 25b

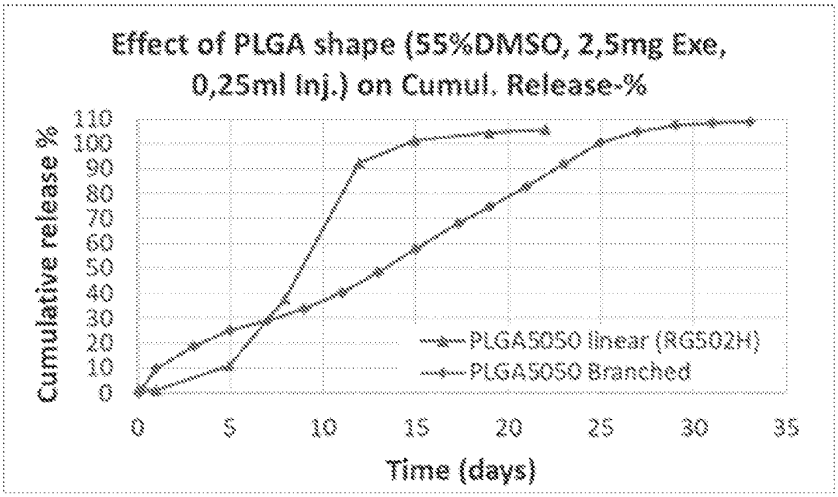

Effect of PLGA shape (55%DMSO, 2,5mg Exe, 0,25ml Inj.) on Cumul. Release-%
Cumulative release %
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
Time (days)
PLGA50S0 linear (RG502H)
PLGA50S0 Branched

Fig. 26

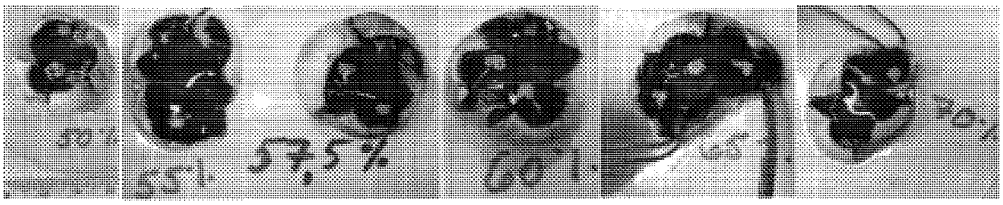

55%
57,5%
60%

Fig. 27

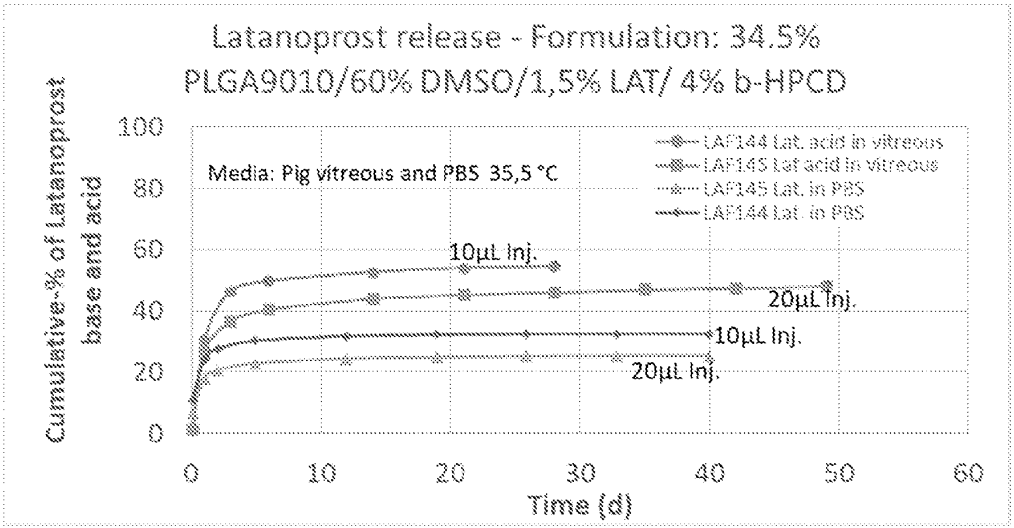


Fig. 28

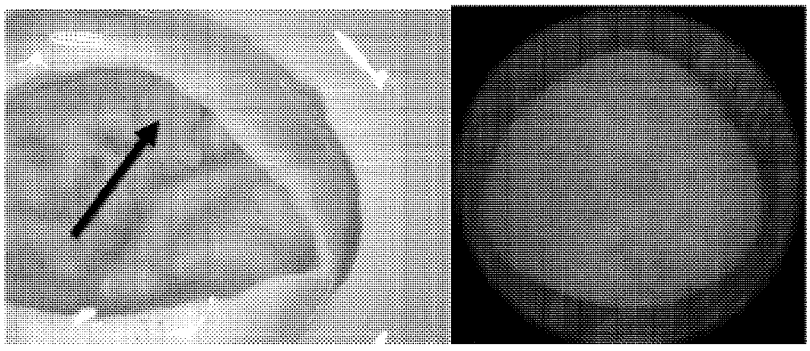


Fig. 29a Fig. 29b

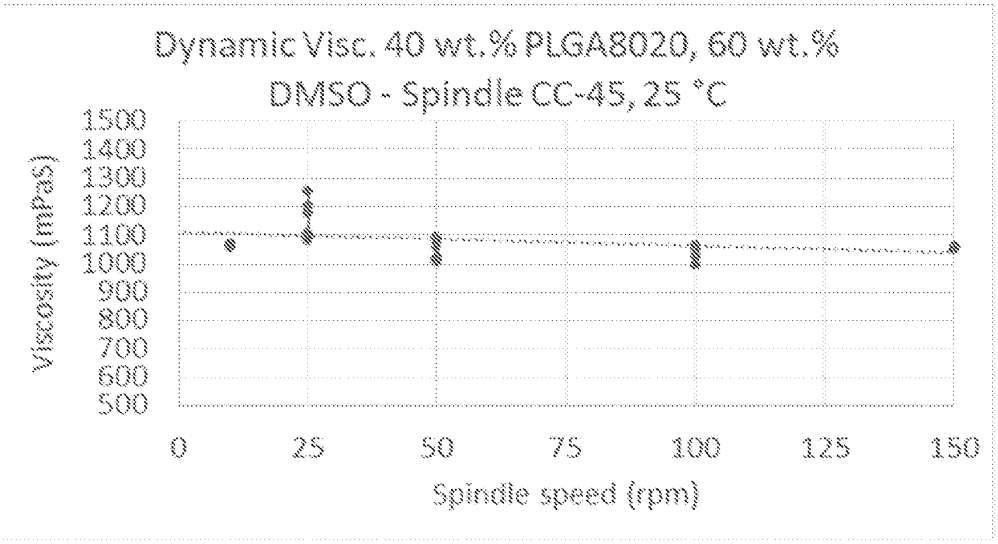

Dynamic Visc. 40 wt.% PLGA8020, 60 wt.%
DMSO - Spindle CC-45, 25 °C
Viscosity (mPas)
1500
1400
1300
1200
1100
1000
900
800
700
600
500
0
25
50
75
100
125
150
Spindle speed (rpm)

Fig. 30

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(54) Title: NOVEL FORMULATION OF FUSION PROTEIN

(57) Abstract: The present invention relates to novel liquid formulations comprising pharmacologically active fusion protein. The present invention discloses the use of histidine buffer in combination with other excipients to stabilize the fusion protein by lowering the product related impurities. In another aspects invention provides a formulation of fusion protein with low viscosity.

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TITLE: NOVEL FORMULATION OF FUSION PROTEIN

FIELD OF THE INVENTION

The present invention relates to novel liquid formulations comprising pharmacologically active fusion protein. The present invention discloses the use of histidine buffer in combination with other excipients to stabilize the fusion protein by lowering the product related impurities. In another aspects invention provides a formulation of fusion protein with low viscosity.

BACKGROUND OF THE INVENTION

In earlier days antibody formulations were stabilised with lyophilisation of protein and were reconstituted prior to use with appropriate solvent system. However, with time requirement of high concentrated and stable liquid formulation increased. As fusion proteins are not very stable, high concentration of fusion protein in liquid formulation often results in precipitation of fusion protein and degradation of the same. Further it has been also noted that solution preparation of antibodies or fusion protein often turns very viscous due to intermolecular interaction.

Based on the increased demand in the concentrated liquid formulation comprising fusion proteins, there is always needed to prepare stable liquid formulations comprising high concentrated pharmacologically fusion protein.

It is observed that many of fusion protein such as Etanercept, Abatacept, Belatacept, Aflibercept are formulated with phosphate buffer. However, the present invention provides an improved formulation capable to stabilize high concentration fusion protein by using histidine as buffer, stabilizer and optionally amino acid.

The formulations of the invention are useful for subcutaneous delivery to a patient in need thereof. In order to deliver maximum therapeutic benefits to patients, it is desirable that formulations for subcutaneous (SC) delivery comprise a high antibody concentration. However, high concentration of antibody, or antigen-binding fragment thereof, may contribute to other properties of the product which would be undesirable, e.g., low injectability due to increased viscosity and higher than physiological osmolality and increased aggregation.

For an example, commercial formulation of Abatacept has very high amount of sucrose around 170mg/ml which makes the formulation very viscous. Therefore, it is preferred that an antibody product intended for SC administration balances the effects of concentration while maintaining a level of drug that will provide the highest therapeutic benefit.

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An ideal product comprises a high protein concentration, low viscosity, an osmolality similar to physiological conditions, and a low level of aggregation under typical storage conditions. Increased viscosity at high protein concentration may not only make it difficult to extract the product from its container with a syringe, but also to inject the necessary dose into a patient from the syringe (syringeability). Advantageously, embodiments of the invention provide formulations that comprise a high concentration fusion protein thereof, and a viscosity level that is acceptable for subcutaneous delivery. Additionally, the formulations of the invention do not lead to high levels of aggregation.

SUMMARY OF THE INVENTION

The present invention relates to stable liquid formulations comprising high concentrated pharmacologically active fusion protein and process for preparation of the same.

The present invention discloses a stable pharmaceutical formulation of high concentration of fusion protein molecule comprising histidine buffer, amino acid, stabilizer and surfactant, wherein the fusion protein comprises the receptor fused with constant region of Immunoglobulin.

The present invention discloses a stable pharmaceutical formulation of high concentration of fusion protein molecule comprising histidine buffer, amino acid, stabilizer and surfactant, wherein the fusion protein is a CTLA4-Ig molecule.

The present invention discloses a stable pharmaceutical formulation of high concentration of fusion protein molecule where the concentration of fusion protein is above 100mg/ml.

The present invention discloses a stable pharmaceutical formulation of high concentration of a fusion protein molecule comprising histidine buffer, amino acid, surfactant and stabilizer, wherein the fusion protein is a CTLA4-Ig molecule; wherein the stabilizer is sugar which is present in lower amount than the CTLA4-Ig molecule.

In certain embodiment, the invention provides a stable formulation comprises high concentration of fusion protein and at least one amino acid and suitable excipients wherein amino acid is selected from arginine, lysine, proline, glycine, phenylalanine, aspartic acid, methionine, glutamic acid, and asparagine.

In certain embodiment, the formulations of the present invention are stable and avoid problem of precipitation of pharmacologically active fusion protein.

In an embodiment, the stable formulation of the present invention possesses advantageous properties such as low viscosity, low aggregation, and low osmolality.

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In certain embodiment, the invention provides can be supplied in pre-filled syringe or auto-injector.

In an embodiment, the present invention provides a pharmaceutical formulation comprising;

a. high concentration of pharmacologically active fusion protein;

b. suitable buffer comprising L-histidine and L-histidine HCl;

c. suitable amino acid selected from arginine, lysine, glycine, proline, methionine and combination thereof;

d. suitable stabilizer;

e. suitable surfactant selected from polysorbate 20, polysorbate 80 or Poloxamer 188 and;

f. pH selected from about from 6.5 to about 7.5.

In an embodiment, the present invention provides a pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4-Ig fusion protein;

b. suitable buffer comprising L-histidine and L-histidine HCl;

c. suitable amino acid selected from arginine, lysine, glycine, proline, methionine and combination thereof;

d. suitable stabilizer;

e. at least one surfactant selected from polysorbate 20, polysorbate 80 or Poloxamer 188 and;

f. pH selected from about from 6.5 to about 7.5.

In an embodiment, the present invention provides a pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4-Ig fusion protein;

b. suitable buffer comprising L-histidine and L-histidine HCl;

c. suitable amino acid selected from arginine, lysine, glycine, proline, methionine and combination thereof;

d. suitable stabilizer is selected from sucrose or cyclodextrin;

e. suitable surfactant selected from polysorbate 20, polysorbate 80 or Poloxamer 188 and;

f. pH selected from about from 6.5 to about 7.5.

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In an embodiment, the present invention provides a pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4-Ig fusion protein;

b. suitable buffer comprising L-histidine and L-histidine HCl;

c. suitable amino acid is arginine or arginine HCl;

d. sucrose;

e. surfactant is poloxamer 188 and;

f. pH 7.3 ± 0.2;

In an embodiment, the present invention a pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4-Ig fusion protein;

b. suitable buffer comprising L-histidine and L-histidine HCl;

c. suitable amino acid is lysine or lysine HCl;

d. sucrose;

e. surfactant is poloxamer 188 and;

f. pH 7.3 ± 0.2

In certain embodiment the sugar concentration is lower than concentration of fusion protein CTLA4-Ig. In certain embodiment the fusion protein and sugar ratio is selected from not more than 1:0.9, 1:0.8, 1:0.7, 1:0.6, or 1:0.5.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4-Ig fusion protein;

b. suitable buffer comprising L-histidine and L-histidine HCl;

c. suitable amino acid is arginine or arginine HCl;

d. suitable amino acid is lysine or lysine HCl;

e. sucrose;

f. surfactant is poloxamer 188 and;

g. pH 7.3 ± 0.2.

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In another embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4-Ig fusion protein;

b. suitable buffer comprising L-histidine and L-histidine HCl;

c. sucrose;

d. surfactant is Poloxamer 188 and;

e. pH 7.3 ± 0.2.

wherein the concentration of histidine buffer is at least 150mM.

In certain embodiment, the stable pharmaceutical formulation has kinematic viscosity of at least about 5 cPs. In certain embodiment, the formulation has viscosity of about 5 cPs to about 10 cPs. In certain embodiment, the formulation has kinematic viscosity of about 7 cPs to about 10 cPs. In certain embodiment, the formulation has kinematic viscosity of about 8 cPs to about 10 cPs.

In preferred embodiment, the stable pharmaceutical formulation has kinematic viscosity of about 10 cPs or less.

In another preferred embodiment, the stable pharmaceutical formulation has kinematic viscosity of about 7 cPs to about 9 cPs.

In certain embodiment, the stable pharmaceutical formulation has osmolality selected from about 700 to about 900 mOsm/kg. In certain embodiment, the stable pharmaceutical formulation has osmolality selected from about 730 to about 900 mOsm/kg. In certain embodiment, the stable pharmaceutical formulation has osmolality selected from about 750 to about 900 mOsm/kg. In certain embodiment, the stable pharmaceutical formulation has osmolality selected from about 770 to about 850 mOsm/kg.

In preferred embodiment, the stable pharmaceutical formulation has osmolality of about 800±50 mOsm/kg.

In another preferred embodiment, the stable pharmaceutical formulation has osmolality of about 780 to about 825 mOsm/kg.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The terms used in this specification generally have their ordinary meanings in the art, within the context of the invention, and in the specific context where each term is used. Certain terms that

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are used to describe the invention are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner regarding the description of the invention. Synonyms for certain terms are provided. A recital of one or more synonyms does not exclude the use of other synonyms. The use of examples anywhere in this specification including examples of any terms discussed herein is illustrative only, and in no way limits the scope and meaning of the invention or of any exemplified term. The invention is not limited to the various embodiments given in this specification. Unless otherwise defined, 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 pertains. In the case of conflict, the present document, including definitions will control.

The term "fusion protein" refers to proteins created through the joining of two or more genes that originally coded for separate proteins. The fusion proteins are made using recombinant DNA techniques. Fusion protein consisting of receptor including but not limited to selected from CTLA4, TNFR, VEGF, HER-2, PCSK9 fused with constant region of immunoglobulin selected from IgG1, IgG2, IgG3 and IgG4. In addition, any modification is performed in natural amino acid to obtain desired biological activity.

The terms "CTLA4-Ig" or "CTLA4-Ig molecule" or "CTLA4Ig molecule" are used interchangeably and refer to a protein molecule that comprises at least a polypeptide having a CTLA4 extracellular domain or portion thereof and an immunoglobulin constant region or portion thereof. The extracellular domain and the immunoglobulin constant region can be wild-type, or mutant or modified, and mammalian, including human or mouse. The polypeptide can further comprise additional protein domains. A CTLA4-Ig molecule can also refer to multimer forms of the polypeptide, such as dimers, tetramers, and hexamers. A CTLA4-Ig molecule is also capable of binding to CD80 and/or CD86. In certain embodiment, the CTLA4-Ig is Abatacept.

The term "B7-1" refers to CD80; the term "B7-2" refers CD86; and the term "B7" refers to both B7-1 and B7-2 (CD80 and CD86). The term "B7-1-Ig" or "B7-1Ig" refers to CD80-Ig; the term "B7-2-Ig" or "B7-2Ig" refers CD86-Ig.

As used throughout the specification and in the appended claims, the singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise.

The phrase "consists essentially of," or variations such as "consist essentially of" or "consisting essentially of," as used throughout the specification and claims, indicate the inclusion of any recited elements or group of elements, and the optional inclusion of other elements, of similar or different nature than the recited elements, that do not materially change the basic or novel properties of the specified dosage regimen, method, or formulation. As a non-limiting example, a

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binding compound that consists essentially of a recited amino acid sequence may also include one or more amino acids, including substitutions of one or more amino acid residues, that do not materially affect the properties of the binding compound.

"Comprising" or variations such as "comprise", "comprises" or "comprised of" are used throughout the specification and claims in an inclusive sense, i.e., to specify the presence of the stated features but not to preclude the presence or addition of further features that may materially enhance the operation or utility of any of the embodiments of the invention, unless the context requires otherwise due to express language or necessary implication.

The term "around," "about" or "approximately" shall generally mean within 20 percent, within 10 percent, within 5, 4, 3, 2 or 1 percent of a given value or range. Numerical quantities given are approximate, meaning that the term "around," "about" or "approximately" can be inferred if not expressly stated.

The term used "Size variants" refers to LMW, HMW or aggregates.

The term used "Low molecular weight (LMW) species" which is a protein backbone-truncated fragments & considered as product-related impurities that contribute to the size heterogeneity of antibody. LMW species often have low or substantially reduced activity relative to the monomeric form of the antibody and can lead to immunogenicity or potentially impact pharmacokinetic properties in vivo. As a result, LMW species are considered critical quality attributes that are routinely monitored during drug development and as part of release testing of purified drug product during manufacturing.

The term used "high molecular weight or HMW "is product-related impurities that contribute to the size heterogeneity of antibody products. The formation of HMW species within a therapeutic antibody drug product as a result of protein aggregation can potentially compromise both drug efficacy and safety (e.g., eliciting unwanted immunogenic response). HMW is considered critical quality attributes that are routinely monitored during drug development and as part of release testing of purified drug product during manufacturing.

The term used "aggregates" are classified based on types of interactions and solubility. Soluble aggregates are invisible particles and cannot be removed with a filter. Insoluble aggregates can be removed by filtration and are often visible to the human eye. Both types of aggregates cause problems in biopharma development. Covalent aggregates arise from the formation of a covalent bond between multiple monomers of a given peptide. Disulfide bond formation of free thiols is a common mechanism for covalent aggregation. Oxidation of tyrosine residues can lead to formation

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of bityrosine which often results in aggregation. Reversible protein aggregation typically results from weaker protein interactions they include dimers, trimers, multimers among others.

A "surfactant" is a surface-active molecule containing both a hydrophobic portion (e.g., alkyl chain) and a hydrophilic portion (e.g., carboxyl and carboxylate groups). Surfactant may be added to the formulations of the invention. Surfactants suitable for use in the formulations of the present invention include, but are not limited to, polysorbates (e.g., polysorbates 20 or 80); poloxamers (e.g., poloxamer 188).

The term "pharmaceutical" as used herein with reference to a composition, e.g., an aqueous formulation, that it is useful for treating a disease or disorder.

The term "pharmaceutical formulation" refers to preparations which are in such a form as to permit the biological activity of the active ingredients to be effective, and therefore may be administered to a subject for therapeutic use.

A "stable" formulation is one in which the protein therein essentially retains its physical stability and/or chemical stability and/or biological activity upon storage. Various analytical techniques for measuring protein stability are available in the art for example. In one embodiment, the stability of the protein is determined according to the percentage of monomer protein in the solution, with a low percentage of degraded (e.g., fragmented) and/or aggregated protein.

The term "stabilizer" refers to excipients that are used to maintain the desirable properties of the product until it is consumed by the patient. The stabilizers may be sugars, polymers, salts etc. Stabilizer can stabilize the fusion protein to for at least 2 weeks, at least 4 weeks, at least 1 month, at least 2 months, at least 3 months, at least 6 months, at least 8 months, at least 12 months based on the selection of temperature and sugar.

The term "sugar" refers to monosaccharides, disaccharides, oligosaccharides and polysaccharides. Examples include sucrose, trehalose, dextrose, maltose, sorbitol, mannitol, cyclodextrin and others. In most preferred embodiment, the sugar is sucrose.

The term "viscosity" refers to the resistance of a liquid formulation to flow, such as when injected through a syringe needle during administration to a patient. Viscosity of a protein solution depends on the nature of the individual and protein-protein interaction (PPI). Both of the individual characteristics, such as particle size and shape, as well as the pair interactions can be influenced by components in the formulation. As a result, it is often desirable to reduce viscosity values so that formulations are suitable for a particular application or process (i.e.: injection). Increased viscosity at high protein concentration may not only make it difficult to extract the product from

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its container with a syringe, but also to inject the necessary dose into a patient from the syringe (syringeability parameters). Increased viscosity also leads to more challenging in handling product during manufacturing (such as filtration and filling).

Hence, it is desirable to keep viscosity low in formulation as it increases glide force and break loose force of syringe. Furthermore, it is known that high viscosity causes severe pain to patient during injection. In addition, high viscosity causes loss of product due to its tendency to bind to container closure.

It is noted that commercial formulation of CTLA4-IgG1 (Abatacept) contains high sucrose concentration i.e., 170mg/ml which makes the formulation very viscous.

It is understood that the value of viscosity is dependent on the conditions under which the measurement was taken, such as temperature, the rate of shear and the shear stress employed. The apparent viscosity is defined as the ratio of the shear stress to the rate of shear applied. There are number of alternative methods for measuring apparent viscosity. For example, viscosity can be tested by a suitable cone and plate, parallel plate or other type of viscometer or rheometer.

The term "osmolality" refers to a measure of solute concentration, defined as the number of mmole of solute per kg of solution. A desired level of osmolality can be achieved by the addition of one or more stabilizer such as a sugar or polyol including sorbitol, mannitol, dextrose, trehalose, maltose, mannose and/or sucrose.

The present invention provides stable and improved liquid formulations which can optionally be lyophilized, comprising of high concentration of therapeutic protein(s), preferably fusion protein, in suitable buffer(s), one or more suitable amino acid, and other excipients which are optionally selected from suitable surfactants and stabilizer or tonicity agents. The stable formulation provides desirable viscosity & prevents formation of aggregates of protein.

In certain embodiment, the novel formulations of the present invention are suitable for subcutaneous administration. The novel formulations of the present invention are stable and avoid precipitation of pharmacologically active fusion protein.

In certain embodiment, the present invention is stable at room temperature. In certain embodiment, the novel formulations of the present invention are stable at 2°C to 8°C.

In certain embodiment, the formulation provides stability of fusion protein for at least two weeks. In an embodiment, the formulation is stable for at least 1 month, 3 months, 6 months, 10 months, 1 year or 2 years.

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In certain embodiment, the novel formulations of the present invention are stable at 40°C.

In certain embodiment, the formulation provides stability of fusion protein for at least two weeks to one month.

In certain embodiment, the novel formulations of the present invention comprise high concentration of pharmacologically active fusion protein, histidine buffer, amino acid, sugar and surfactant.

In certain embodiment, the pharmacology active fusion protein binds with CD80/86 to block T cell activation for the treatment of arthritis. In certain embodiment, the pharmacology fusion protein is CTLA4-IgG1 (Abatacept).

In an embodiment, the novel formulations of the present invention are mainly useful for the treatment of, but not limited to rheumatoid arthritis (RA), Juvenile Idiopathic Arthritis (pJIA), graft versus host disease (GVHD) and psoriatic arthritis (PsA).

In embodiment, the pharmacological active fusion protein is present in high concentration at least 100 mg/ml. In certain embodiment, the pharmacological fusion protein is present in high concentration selected from about 100 mg/ml to about 200 mg/ml. In certain embodiment, the pharmacological fusion protein is present in high concentration of about 125 mg/ml. The skilled person can adjust the concentration of protein for an example 87.5 mg/0.7 mL and 50 mg/0.4 mL.

In certain embodiment, the pharmacological active fusion protein is present in high concentration selected from 125 mg/ml.

In certain embodiment, the pharmacological active fusion protein is present in high concentration selected from 87.5 mg/0.7 mL.

In certain embodiment, the pharmacological active fusion protein is present in high concentration selected from 50 mg/0.4 mL. In certain embodiment, the invention provides a novel stable formulation comprises a suitable buffer selected from phosphate, citrate, phosphate-citrate, histidine and acetate and salt thereof. However, the present invention found the histidine is most suitable buffer for stabilizing fusion protein.

In certain embodiment, the invention provides a novel stable formulation comprises more than one buffer.

The present invention provides novel liquid formulations comprising high concentrated pharmacologically active fusion protein by using histidine as a buffer. The present applicant found

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that histidine has suitable buffering property for fusion protein and provides better stability in combination with sucrose and/or amino acid and surfactant.

In certain embodiment, the histidine buffer is prepared by L-histidine and L-histidine HCl.

In certain embodiment, histidine can also be used in its salt form such as histidine hydrochloride.

In certain embodiment, the buffer concentration is at least about 1 mg/ml. In certain embodiment, the buffer concentration is in the range of about 1 mg/ml to about 10 mg/ml. In certain embodiment, the buffer concentration is in the range of about 1 mg/ml to about 5 mg/ml. In certain embodiment, the buffer concentration is about 4 mg/ml. In certain embodiment, the buffer concentration is about 4.2 mg/ml.

In certain embodiment, the buffer is concentration is at least about 50 mM. In certain embodiment, the buffer is concentration is in the range of about 50 mM to about 200 mM.

In certain embodiment, the buffer is concentration is in the range of about 20mM, about 25mM, about 30mM, about 35mM, about 40mM, about 45mM, about 50mM to about 60mM, about 60mM to about 70mM, about 70mM to about 80mM, about 80mM to about 90mM, about 90mM to about 100mM, about 100mM to about 110mM, about 110mM to about 120mM, about 120mM to about 130mM, about 130mM to about 140mM, about 140mM to about 150mM, about 150mM to about 160mM, about 160mM to about 170mM, about 170mM to about 180mM, about 180mM to about 190mM, about 195mM, about 200mM, about 203mM and about 205mM.

In an embodiment, the concentration of histidine buffer is more than 100mM. In an embodiment, the concentration of histidine buffer is in the range of about 125mM, about 140mM, about 150mM, about 170mM, about 190mM, about 200mM, about 203mM and about 205mM.

In an embodiment, the concentration of histidine buffer is about 150mM.

In another embodiment, the concentration of histidine buffer is below about 100mM. in certain embodiment the concentration of histidine buffer is about 50mM, about 60mM, about 70mM, about 80mM and about 90mM.

In another embodiment, the formulation comprising the buffer concentration is about 200mM to about 205mM. In another preferred embodiment the buffer concentration is about 203mM. In such embodiment, the formulation reduced or does not contain amino acid the formulation doesn't comprise or have reduced amount of amino acid when buffer concentration is about 200mM or above.

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In certain embodiment, amino acids are selected from arginine, lysine, methionine, glycine, proline, asparagine, phenylalanine & suitable salt thereof. In certain embodiment, the lysine, and arginine can be used in its salt form such as lysine hydrochloride, or arginine hydrochloride.

In preferred embodiment, the formulation does not comprise histidine as amino acid while the present formulation only uses histidine as buffer. In another preferred embodiment the formulation comprising histidine buffer and amino acid selected from arginine, lysine, methionine, glycine, proline, asparagine, phenylalanine & suitable salt thereof along with suitable stabilizer and surfactant. The formulation is essentially free of histidine as amino acid.

In certain embodiment, the amino acid present in the concentration of at least about 1 mg/ml. In certain embodiment, the amino acid present in the range of about 1 mg/ml to about 75 mg/ml. In certain embodiment, the amino acid present in the range of about 5 mg/ml to about 50 mg/ml. In certain embodiment, the amino acid present in range of about 9 mg/ml to about 50 mg/ml. In certain embodiment, the amino acid present in range of about 9 mg/ml to about 20 mg/ml.

In certain embodiment, the amino acid arginine presents in the concentration from about 7mg/ml to about 25 mg/ml. In certain embodiment, the amino acid arginine presents in the concentration from about 15mg/ml to about 21mg/ml.

In another aspects, formulation is optimized with low concentration of arginine. In certain embodiment, the amino acid arginine presents in the concentration from about 7mg/ml to about 14mg/ml. In preferred embodiment, the arginine concentration is about 9mg/ml to about 11mg/ml. In another preferred embodiment, the arginine concentration is about 10.5 mg/ml to about 11mg/ml.

In certain embodiment, the amino acid lysine presents in the concentration from about 7mg/ml to about 20 mg/ml. In certain embodiment, the amino acid lysine presents in the concentration from about 11mg/ml to about 13.70 mg/ml.

In another aspects, formulation can be optimized with low concentration of lysine.

In certain embodiment, the amino acid lysine presents in the concentration from about 7mg/ml to about 13 mg/ml. In preferred embodiment, the lysine concentration is about 9mg/ml to about 11mg/ml. In another preferred embodiment, the lysine concentration is about 9.3 mg/ml to about 10mg/ml.

In another embodiment, the formulation comprises arginine or lysine or both.

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In certain embodiment, the amino acid is present in the concentration of at least about 50mM. In certain embodiment, the amino acid is present in the range of about 50mM to about 60mM, about 60mM to about 70mM, about 70mM to about 80mM, about 80mM to about 90mM, about 90mM to about 100mM, about 100mM to about 110mM, about 110mM to about 120mM, about 120mM to about 130mM, about 130mM to about 140mM, and about 140mM to about 150mM.

In certain embodiment, the amino acid arginine presents in the concentration more than about 50mM. In certain embodiment, the amino acid arginine presents in the concentration from about 50mM to about 150mM. In certain embodiment, the amino acid arginine presents in the concentration from about 80mM to about 125mM. In preferred embodiment, the amino acid arginine presents in the concentration from about 100mM to about 110mM.

In another aspects, formulation can be optimized with low concentration of arginine. In certain embodiment, the amino acid arginine presents in the concentration from about 25mM to about 50mM. In preferred embodiment, the arginine concentration is about 30mM to about 50mM. In another preferred embodiment, the arginine concentration is about 40mM to about 50mM.

In certain embodiment, the amino acid lysine presents in the concentration more than about 50mM. In certain embodiment, the amino acid lysine presents in the concentration from about 50mM to about 100mM. In preferred embodiment, the amino acid lysine presents in the concentration from about 75mM to about 80mM.

In another aspects, formulation can be optimized with low concentration of lysine. In certain embodiment the amino acid lysine presents in the concentration from about 25mM to about 50mM. In preferred embodiment, the lysine concentration is about 30mM to about 50mM. In another preferred embodiment, the lysine concentration is about 40mM to about 50mM. In certain embodiment, the stabilizers selected from sugars.

In certain embodiment, the stabilizer is selected from sucrose or cyclodextrin.

In preferred embodiment, the stabilizer is sucrose.

In certain embodiment, the sugar concentration is lower than concentration of fusion protein. In certain embodiment, the fusion protein and sugar ratio is selected from not more than 1:0.9, 1:0.8, 1:0.7, 1:0.6, or 1:0.5.

In certain embodiment, the sucrose concentration is lower than concentration of fusion protein. In certain embodiment, the fusion protein and sucrose ratio is selected from not more than 1:0.9, 1:0.8, 1:0.7, 1:0.6, or 1:0.5.

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In certain embodiment, the sucrose is in the concentration of at least about 50g/L. In certain embodiment, the sucrose is present in the range of about 50g/L to about 60g/L, about 60g/L to about 70g/L, about 70g/L to about 80g/L, about 80g/L to about 90g/L, about 90g/L to about 100g/L, and about 110g/L to about 120g/L.

In an embodiment, the sucrose is present in the concentration of about 100g/L, about 101g/L, about 102g/L, about 103g/L, about 104g/L, about 105g/L, about 106g/L, about 107g/L, about 108g/L, about 109g/L and about 110 g/L.

In certain embodiment, the surfactant is selected from polysorbate and poloxamer 188. In certain embodiment, the surfactant is selected from different grades of polysorbate such as but not limited to polysorbate 20, polysorbate 80, poloxamer 188 or mixture thereof can be used. In certain embodiment, the surfactant is poloxamer 188.

In certain embodiment, the surfactant is present in the concentration of at least about 1 mg/ml. In certain embodiment, the surfactant is present in amount of about 1 mg/ml, about 2 mg/ml, about 3 mg/ml, about 4 mg/ml, about 5 mg/ml, about 6 mg/ml, about 7 mg/ml, about 8 mg/ml, about 9 mg/ml, about 10 mg/ml, about 11 mg/ml, about 12 mg/ml, about 13 mg/ml, about 14 mg/ml, and about 15 mg/ml.

In preferred embodiment, the surfactant is present in concentration of about 8 mg/ml.

In certain embodiment, the novel formulations of the present invention have of about pH 6.0 to about pH 7.5. In certain embodiment, the novel formulations of the present invention have of about pH 6.5 to about pH 7.5. In an embodiment, the novel formulations of the present invention have of about pH 6.5, about pH 6.6, about pH 6.7, about pH 6.8, about pH 6.9, about pH 7.0, about pH 7.1, about pH 7.2, about pH 7.3, about pH 7.4, and about pH 7.5.

In preferred embodiment the pH is 7.3±0.2.

In certain embodiment, the novel formulations have viscosity of at least about 5 cps. In certain embodiment, the formulation has viscosity of about 5 cps to about 15 cps. In certain embodiment, the formulation has viscosity of about 7 cps to about 15 cps. In certain embodiment, the formulation has viscosity of about 9 cps to about 15 cps.

In one aspect of such embodiment, the viscosity of the novel formulation is about 5cps, about 6cps, about 7cps, about 8cps, about 9cps, about 10cps, about 11cps, about 12cps, about 13cps, about 14cps and about 15cps.

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In preferred embodiment, the novel formulation has kinematic viscosity of about 10 cPs or less.

In another preferred embodiment, the novel formulation has kinematic viscosity of about 7 cPs to about 9 cPs.

In certain embodiment, the present invention discloses a desirable viscosity about 7cps to about 9cps when formulated fusion protein CTLA4-Ig in histidine buffer with arginine or lysine in combination with sucrose and surfactant.

In another embodiment, the present invention discloses a desirable viscosity about 8cps to about 10cps when formulated fusion protein CTLA4-Ig in histidine buffer with arginine or lysine in combination with sucrose and surfactant.

In certain embodiment, the stable pharmaceutical novel liquid formulations have osmolality selected from about 700 to about 900 mOsm/kg. In certain embodiment, the stable pharmaceutical novel liquid formulations have osmolality selected from about 730 to about 900 mOsm/kg. In certain embodiment, the stable pharmaceutical novel liquid formulations have osmolality selected from about 750 to about 900 mOsm/kg. In certain embodiment, the stable pharmaceutical novel liquid formulations have osmolality selected from about 770 to about 900 mOsm/kg.

In one aspect of such embodiment, the osmolality of the novel formulation is about 700 mOsm/kg, about 710 mOsm/kg, about 720 mOsm/kg, about 730 mOsm/kg, about 740 mOsm/kg, about 750 mOsm/kg, about 760 mOsm/kg, about 770 mOsm/kg, about 780 mOsm/kg, about 790 mOsm/kg, about 800 mOsm/kg, about 810 mOsm/kg, about 820 mOsm/kg, about 830 mOsm/kg, about 840 mOsm/kg, about 850 mOsm/kg, about 860 mOsm/kg, about 870 mOsm/kg, about 890 mOsm/kg, and about 900 mOsm/kg.

In certain aspect, the present invention discloses a desirable viscosity about 750mOsm/Kg to about 850 mOsm/Kg when formulated fusion protein CTLA4-Ig in histidine buffer with arginine or lysine in combination with sucrose and surfactant.

In preferred embodiment, the novel formulation has osmolality of about 800±50 mOsm/kg.

In another preferred embodiment, the novel formulation has osmolality of about 780 to about 825 mOsm/kg

In another embodiment, the present invention discloses a desirable viscosity about 780mOsm/Kg to about 825 mOsm/Kg when formulated fusion protein CTLA4-Ig in histidine buffer with arginine or lysine in combination with sucrose and surfactant.

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In an embodiment, the present invention discloses a stable pharmaceutical formulation of a fusion protein molecule comprising histidine buffer, amino acid, stabilizer and surfactant, wherein the fusion protein comprises the receptor fused with constant region of Immunoglobulin.

In an embodiment, the present invention discloses a stable pharmaceutical formulation of a fusion protein molecule comprising histidine buffer, amino acid, stabilizer and surfactant, wherein the fusion protein is a CTLA4-Ig molecule.

In an embodiment, the present invention discloses a stable pharmaceutical formulation of a fusion protein molecule comprising buffer, amino acid, surfactant and stabilizer, wherein the fusion protein is a CTLA4-Ig molecule; wherein the stabilizer is sugar which is present in lower amount than the CTLA4-IgG.

In certain embodiment, the invention provides a novel stable formulation comprises high concentration of fusion protein and at least one suitable aggregation inhibitor and suitable excipients wherein the suitable aggregation inhibitor is amino acid selected from arginine, lysine, proline, glycine, phenylalanine, aspartic acid, methionine, glutamic acid, and asparagine.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active fusion protein;

b. suitable buffer L-histidine and L-histidine HCl;

c. suitable amino acid selected from arginine, lysine, glycine, proline, methionine and combination thereof;

d. suitable stabilizer;

e. suitable surfactant selected from polysorbate 20, polysorbate 80 or Poloxamer 188 and;

f. pH selected from about from 6.5 to about 7.5.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4-Ig fusion protein;

b. suitable buffer L-histidine and L-histidine HCl;

c. suitable amino acid selected from arginine, lysine, glycine, proline, methionine and combination thereof;

d. suitable stabilizer;

e. at least one surfactant selected from polysorbate 20, polysorbate 80 or Poloxamer 188 and;

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f. pH selected from about from 6.5 to about 7.5.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4-Ig fusion protein;

b. suitable buffer L-histidine and L-histidine HCl;

c. suitable amino acid selected from arginine, lysine, glycine, proline, methionine and combination thereof;

d. suitable stabilizer is sucrose;

e. suitable surfactant selected from polysorbate 20, polysorbate 80 or Poloxamer 188 and;

f. pH selected from about from 6.5 to about 7.5.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4-Ig fusion protein;

b. suitable buffer L-histidine and L-histidine HCl;

c. suitable amino acid selected from arginine, lysine, glycine, proline, methionine and combination thereof;

d. suitable stabilizer is cyclodextrin;

e. suitable surfactant selected from polysorbate 20, polysorbate 80 or Poloxamer 188 and;

f. pH selected from about from 6.5 to about 7.5.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4-Ig fusion protein;

b. suitable buffer L-histidine and L-histidine HCl;

c. suitable amino acid is arginine or arginine HCl;

d. sucrose;

e. poloxamer 188 and;

f. pH 7.3 ± 0.2;

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4-Ig fusion protein;

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b. suitable buffer comprising L-histidine and L-histidine HCl;

c. suitable amino acid is lysine or lysine HCl;

d. sucrose;

e. poloxamer 188 and;

f. pH 7.3 ± 0.2

In certain embodiment the sucrose concentration is lower than concentration of fusion protein. In certain embodiment the fusion protein and sucrose ratio is selected from not more than 1:0.9, 1:0.8, 1:0.7, 1:0.6, or 1:0.5.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4 Ig fusion protein;

b. suitable buffer L-histidine and L-histidine HCl;

c. suitable amino acid is arginine or arginine HCl;

d. suitable amino acid is lysine or lysine HCl;

e. sucrose;

f. poloxamer 188 and;

g. pH 7.3 ± 0.2

The pharmacologically active fusion protein which binds to CD80 and CD86 molecule is abatacept or belatacept.

In another aspect of such embodiment the formulation has high monomer and low aggregation or HMW % and LMW %.

In certain embodiment the formulation provides HMW % below 10% or 9% or 8% or 7% or 6% or 5% or 4% or 3% or 2% or 1% when stored at 25°C for 2 weeks analysed by SEC-HPLC or CE-SDS.

In certain embodiment the formulation provides LMW % below 10% or 9% or 8% or 7% or 6% or 5% or 4% or 3% or 2% or 1% when stored at 25°C for 2 weeks analysed by SEC-HPLC or CE-SDS.

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In certain embodiment the formulation provides HMW % below 10% or 9% or 8% or 7% or 6% or 5% or 4% or 3% or 2% or 1% when stored at 25°C for 4 weeks analysed by SEC-HPLC or CE-SDS.

In certain embodiment the formulation provides LMW % below 10% or 9% or 8% or 7% or 6% or 5% or 4% or 3% or 2% or 1% when stored at 25°C for 4 weeks analysed by SEC-HPLC or CE-SDS.

In certain embodiment the formulation provides monomer more than 90%. In certain embodiment the formulation provides monomer more than 95%, 96%, 97%, 98% analysed by SEC-HPLC or CE-SDS.

In certain embodiment the formulation is stable for at least 2 weeks at 25°C. In certain embodiment the formulation is stable for at least 4 weeks at 25°C. In certain embodiment the formulation is stable for at least 8 weeks at 25°C. In certain embodiment the formulation is stable for at least 12 weeks at 25°C. In certain embodiment the formulation is stable for at least 16 weeks at 25°C. In certain embodiment the formulation is stable for at least 20 weeks at 25 °C. In certain embodiment the formulation is stable for at least 24 weeks at 25°C.

In certain embodiment the formulation is stable for at least one weeks at 30°C. In certain embodiment the formulation is stable for at least 2 weeks at 30°C. In certain embodiment the formulation is stable for at least 8 weeks at 2°C to 8°C.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4-Ig fusion protein;

b. suitable buffer L-histidine and L-histidine HCl;

c. suitable amino acid is arginine or arginine HCl;

d. sucrose;

e. polysorbate 20 and;

f. pH 7.3 ± 0.2;

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4-Ig fusion protein;

b. suitable buffer L-histidine and L-histidine HCl;

c. suitable amino acid is lysine or lysine HCl;

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d. sucrose;

e. polysorbate 20 and;

f. pH 7.3 ± 0.2

The present invention discloses that use of amino acid can be reduced or avoided to prepare stable formulation when histidine buffer is used in high concentration more than about 150mM, about 160mM, about 170mM, about 180mM, about 190mM and about 200mM.

In another embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4 Ig fusion protein;

b. suitable buffer L-histidine and L-histidine HCl;

c. Sucrose ;

d. Optionally suitable aggregation inhibitor selected from arginine, glycine or lysine and salt thereof;

e. poloxamer 188 and;

f. pH selected from about from 7.1 to about 7.4;

wherein the histidine buffer concentration is about 150mM to about 200mM

In another embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4 Ig fusion protein;

b. suitable buffer L-histidine and L-histidine HCl;

c. suitable amino acid is arginine, or its salt selected from arginine HCl, arginine citrate, arginine succinate, arginine phosphate, arginine sulphate;

d. sucrose ;

e. poloxamer 188;

f. pH selected from about from 7.1 to about 7.4.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

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a. high concentration of pharmacologically active CTLA4 Ig fusion protein in an amount of 125 mg/ml;

b. buffer L-histidine and L-histidine HCl in an amount of about 25mM to 80 mM;

c. suitable amino acid is arginine or arginine HCl in an amount of about 50mM to 125mM;

d. sucrose in an amount of about 100g/L to 130g/L;

e. Poloxamer 188 in an amount of about 1g/L to 10g/L and;

f. pH 7.3 ± 0.2.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4 Ig fusion protein in an amount of 125 mg/ml;

b. buffer L-histidine and L-histidine HCl in an amount of about 25mM;

c. suitable amino acid is arginine or arginine HCl in an amount of about 125mM;

d. sucrose in an amount of about 110 g/L;

e. Poloxamer 188 in an amount of about 8g/L and;

f. pH 7.3 ± 0.2;

In such embodiment the viscosity is 8.41 cPs and osmolality is 856 mOsm/kg

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4 Ig fusion protein in an amount of 125 mg/ml;

b. buffer L-histidine and L-histidine HCl in an amount of about 100mM to 200 mM;

c. suitable amino acid is arginine or arginine HCl in an amount of about 10mM to 80mM;

d. sucrose in an amount of about 100g/L to 110g/L;

e. Poloxamer 188 in an amount of about 1g/L to 10g/L and;

f. pH 7.3 ± 0.2.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

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a. high concentration of pharmacologically active CTLA4 Ig fusion protein in an amount of 125 mg/ml;

b. buffer L-histidine and L-histidine HCl in an amount of 150mM;

c. suitable amino acid arginine or arginine HCl in an amount of 50mM;

d. sucrose in an amount of 110 g/L;

e. Poloxamer 188 in an amount of 8 g/L and;

f. pH 7.3 ± 0.2.

In such embodiment the viscosity is 8.09 cPs and osmolality is 785 mOsm/kg

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4 Ig fusion protein in an amount of 125 mg/ml;

b. buffer L-histidine and L-histidine HCl in an amount of about 100mM to 200 mM;

c. suitable amino acid is lysine or lysine HCl in an amount of about 10mM to 80mM;

d. sucrose in an amount of about 100g/L to 110g/L;

e. Poloxamer 188 in an amount of about 1g/L to 10g/L and;

f. pH 7.3 ± 0.2.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4 Ig fusion protein in an amount of 125 mg/ml;

b. buffer L-histidine and L-histidine HCl in an amount of 150mM;

c. suitable amino acid lysine or lysine HCl in an amount of 50mM;

d. sucrose in an amount of 110 g/L;

e. poloxamer 188 in an amount of 8 g/L and;

f. pH 7.3 ± 0.2.

In such embodiment the viscosity is 8.74 cPs and osmolality is 818 mOsm/kg

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

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a. high concentration of pharmacologically active CTLA4-Ig fusion protein;

b. suitable buffer L-histidine and L-histidine HCl;

c. suitable amino acid is arginine or arginine HCl;

d. suitable amino acid is lysine or lysine HCl;

e. sucrose;

f. poloxamer 188 and;

g. pH 7.3 ± 0.2.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4 Ig fusion protein in an amount of about 125 mg/ml;

b. buffer L-histidine and L-histidine HCl in an amount of about 10mM to 80 mM;

c. suitable amino acid is arginine or arginine HCl in an amount of about 50mM to 140mM;

d. suitable amino acid is lysine or lysine HCl in an amount of about 20mM to 100mM;

e. sucrose in an amount of about 100g/L to 110 g/L;

f. poloxamer 188 in an amount of about 1g/L to 10g/L and;

g. pH 7.3 ± 0.2.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4 Ig fusion protein in an amount of 125 mg/ml;

b. buffer L-histidine and L-histidine HCl in an amount of 25mM;

c. suitable amino acid is arginine or arginine HCl in an amount of 100mM;

d. suitable amino acid is lysine or lysine HCl in an amount of 75mM;

e. sucrose in an amount of 100g/L;

f. poloxamer 188 in an amount of 8g/L and;

g. pH 7.3 ± 0.2;

In such embodiment the viscosity is 7.51 cPs and osmolality is 846 mOsm/kg.

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In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4 Ig fusion protein in an amount of = 125 mg/ml;

b. buffer L-histidine and L-histidine HCl in an amount of about 10mM to 100 mM;

c. suitable amino acid is arginine or arginine HCl in an amount of about 50mM to 100mM;

d. suitable amino acid is lysine or lysine HCl in an amount of about 50mM to 100mM;

e. sucrose in an amount of about 100g/L to 110 g/L;

f. poloxamer 188 in an amount of about 1g/L to 10g/L and;

g. pH 7.3 ± 0.2.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4 Ig fusion protein in an amount of 125 mg/ml;

b. buffer L-histidine and L-histidine HCl in an amount of 100 mM;

c. suitable amino acid is arginine or arginine HCl in an amount of 50mM;

d suitable amino acid is lysine or lysine HCl in an amount of 50mM;

e. sucrose in an amount of 110g/L;

f. poloxamer 188 in an amount of 8g/L and;

g. pH 7.3 ± 0.2.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4 Ig fusion protein;

b. suitable buffer L-histidine and L-histidine HCl;

c. sucrose;

d. poloxamer 188 and;

e. pH 7.3 ± 0.2.

In one aspect such embodiment, the formulation is free of amino acid or suitable salt thereof.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

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a. high concentration of pharmacologically active CTLA4 Ig fusion protein in an amount of about 125 mg/ml;

b. buffer L-histidine and L-histidine HCl in amount of about 150mM to about 200mM;

c. sucrose in an amount of about 100g/L to 110g/L;

d. poloxamer 188 in an amount of about 8g/L and;

e. pH 7.3 ± 0.2.

In an embodiment, the present invention provides a stable pharmaceutical formulation comprising;

a. high concentration of pharmacologically active CTLA4 Ig fusion protein in an amount of 125 mg/ml;

b. buffer L-histidine and L-histidine HCl in amount of 200mM;

c. sucrose in an amount of 110g/L;

d. poloxamer 188 in an amount of 8g/L and;

e. pH 7.3 ± 0.2;

In such embodiment the viscosity is 7.84 cPs and osmolality is 736 mOsm/kg.

In an embodiment the present formulation with histidine buffer comprises reduced amount of low molecular weight (LMW) impurities compared to fusion protein formulated with phosphate buffer.

The present invention provides examples below for illustrative purpose and scope of invention is not limited with illustrative examples.

All below examples are performed at 25°C.

Example 1

CTLA-4 IgG1 fusion protein injectable formulation was formulated with 125 mg/mL concentration for subcutaneous administration.

Formulation (F1) was prepared with addition of 20 mM of Phosphate buffer, 110 g/L Sucrose, 150 mM Arginine HCl and Poloxamer (8 mg/mL) at pH 7.3 ± 0.2. The DS solution was filtrated with 0.2µm filter to get the filtrated solution and filled 1 mL of filtered solution in 1 mL glass PFS. The composition of formulation (F1) is given below:

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Compositions	mg / mL
CTLA-4 IgG1 fusion protein	125
Disodium monophosphate Heptahydrate	2.14
monobasic sodium phosphate monohydrate	0.28
Sucrose	110
L-Arginine HCl (150mM)	31.6
Poloxamer 188	8

Analytical tests	Results	
	0 Day	14 Day
% High molecular weight (HMW) species by SEC	0.43	2.38
% Low molecular weight (LMW) species by SEC	0.40	1.17

F1 shows highest HMW % & LMW % on Day 14 in comparison to other formulations F3 to F7 (Histidine buffer). It is also important to note that F1 formulation shows high HMW % and LMW % even on day 0 in comparison to other formulations F3 to F7 (Histidine buffer).

Example 2

CTLA-4 IgG1 fusion protein injectable formulation was formulated with 125 mg/mL concentration for subcutaneous administration.

Formulation (F2) was prepared with addition of 20 mM of Phosphate buffer, 110 g/L Sucrose, 150 mM Lysine HCl and Poloxamer (8 mg/mL) at pH 7.3 ± 0.2. The DS solution was filtrated with 0.2µm filter to get the filtrated solution and filled 1 mL of filtered solution in 1 mL glass PFS. The composition of formulation (F2) is given below:

Compositions	mg / mL
CTLA-4 IgG1 fusion protein	125
Disodium monophosphate Heptahydrate	2.14
monobasic sodium phosphate monohydrate	0.28

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Sucrose	110
L-Lysine HCl (150mM)	27.4
Poloxamer 188	8

Analytical tests	**Results**	
	0 Day	14 Day
% High molecular weight (HMW) species by SEC	0.39	1.74
% Low molecular weight (LMW) species by SEC	0.36	1.38

F2 shows highest HMW % & LMW % on Day 14 in comparison to other formulations F3 to F7 (Histidine buffer). It is also important to note that F2 formulation shows high HMW % and LMW % even on day 0 in comparison to other formulations F3 to F7 (Histidine buffer).

Example 3

CTLA-4 IgG1 fusion protein injectable formulation was formulated with 125 mg/mL concentration for subcutaneous administration.

Formulation (F3) was prepared with addition of 25 mM of Histidine buffer, 125 g/L of Sucrose, 125 mM of Arginine HCl, and Poloxamer (8 mg/mL) at pH 7.3 ± 0.2. The bulk solution was filtrated with 0.2µm filter to get the filtrated solution and filled 1 mL of filtered solution in 1 mL glass PFS. The composition of formulation (F3) is given below:

Compositions	**mg / mL**
CTLA-4 IgG1 fusion protein	125
L - Histidine (22 mM)	3.5
L - Histidine HCl monohydrate (3 mM)	0.7
Sucrose (110 g/L)	110
Arginine HCl (125 mM)	26.33
Poloxamer 188	8

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Analytical tests	Results		
	0 Day	14 Day	28 Day
% High molecular weight (HMW) species by SEC	0.19	1.61	2.96
% Low molecular weight (LMW) species by SEC	0.40	0.8	1.5

Comparison with S01 (INN PFS) where drug substance CTLA-4 IgG1 is added in formulation excipients as follow:

Compositions	mg / mL
Abatacept (CTLA-4 IgG1)	125
Dibasic sodium phosphate anhydrous (6mM)	0.838
monobasic sodium phosphate monohydrate (2 mM)	0.28
Sucrose (500mM)	170
Poloxamer 188	8

Analytical tests	Results		
	0 Day	14 Day	28 Day
% High molecular weight (HMW) species by SEC	0.37	1.38	1.91
% Low molecular weight (LMW) species by SEC	0.38	4.05	8.95

F3 has shown that HMW % need to be controlled but still able to control LMW % effectively compared to F1, F2 and S01 formulation. Further S01 shows very high LMW % on day 28. Hence it shows that histidine controls LMW % effectively.

F3 viscosity is 8.41 cPs and osmolality is 856 mOsm/kg.

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Example 4

CTLA-4 IgG1 fusion protein injectable formulation was formulated with 125 mg/mL concentration for subcutaneous administration.

Formulation (F4) was prepared with addition of 25 mM of Histidine buffer, 100 g/L of Sucrose, 75 mM of Lysine HCl, 100 mM of Arginine HCl and Poloxamer (8 mg/mL) at pH 7.3 ± 0.2. The bulk solution was filtrated with 0.2µm filter to get the filtrated solution and filled 1 mL of filtered solution in 1 mL glass PFS. The composition of formulation (F4) is given below:

Compositions	**mg / mL**
CTLA-4 IgG1 fusion protein	125
L - Histidine (22 mM)	3.5
L - Histidine HCl monohydrate (3 mM)	0.7
Sucrose (300 mM)	100
L-Lysine (75 mM)	13.70
L-Arginine (100 mM)	21.07
Poloxamer 188	8

Analytical tests	**Results**		
	0 Day	14 Day	28 Day
% High molecular weight (HMW) species by SEC	0.13	1.89	3.54
% Low molecular weight (LMW) species by CE-SDS	0.34	0.79	1.39

Comparison with S01 (INN PFS) where drug substance CTLA-4 IgG1 is added in formulation excipients as follow:

Compositions	**mg / mL**
Abatacept (CTLA-4 IgG1)	125
Dibasic sodium phosphate anhydrous (6mM)	0.838
monobasic sodium phosphate monohydrate (2 mM)	0.28
Sucrose (500mM)	170

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Poloxamer 188	8

Analytical tests	Results		
	0 Day	14 Day	28 Day
% High molecular weight (HMW) species by SEC	0.37	1.38	1.91
% Low molecular weight (LMW) species by SEC	0.38	4.05	8.95

F4 has shown that HMW % need to be controlled but still able to control LMW % effectively compared to F1, F2 and S01 formulation. Further S01 shows very high LMW % on day 28. Hence it shows that histidine buffer is very effective controls LMW % effectively.

F4 viscosity is 7.51 cPs and osmolality is 846 mOsm/kg.

Example 5

CTLA-4 IgG1 fusion protein injectable formulation was formulated with 125 mg/mL concentration for subcutaneous administration.

Formulation was prepared (F5) with addition of 200 mM of Histidine buffer, 110 g/L of Sucrose and Poloxamer (8 mg/mL) at pH 7.3 ± 0.2. The bulk solution was filtrated with 0.2µm filter to get the filtrated solution and filled 1 mL of filtered solution in 1 mL glass PFS. The composition of formulation (F5) is given below:

Compositions	**mg / mL**
CTLA-4 IgG1 fusion protein	125
L - Histidine (200 mM)	28
L - Histidine HCl monohydrate (3 mM)	5.6
Sucrose	110
Poloxamer 188	8

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Analytical tests	Results		
	0 Day	14 Day	28Day
% High molecular weight (HMW) specie by SEC	0.18	0.93	1.69
% Low molecular weight (LMW) species by SEC	0.35	1.09	1.88

Comparison with S01 (INN PFS) where drug substance CTLA-4 IgG1 is added in formulation excipients as follow:

Compositions	mg / mL
Abatacept (CTLA-4 IgG1)	125
Dibasic sodium phosphate anhydrous (6mM)	0.838
monobasic sodium phosphate monohydrate (2 mM)	0.28
Sucrose (500mM)	170
Poloxamer 188	8

Analytical tests	Results		
	0 Day	14 Day	28 Day
% High molecular weight (HMW) species by SEC	0.37	1.38	1.91
% Low molecular weight (LMW) species by SEC	0.38	4.05	8.95

F5 shows that high concentration of histidine buffer without amino acid is able to control HMW % and LMW % in comparison to formulation F1, F2 and S01.

F5 viscosity is 7.84 cPs and osmolality is 736 mOsm/kg.

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Example 6

CTLA-4 IgG1 fusion protein injectable formulation is formulated with 125 mg/mL concentration for subcutaneous administration.

Prepared the formulation (F6) with addition of 150 mM of Histidine buffer, 110 g/L of Sucrose, 50 mM of Arginine HCl and Poloxamer (8 mg/mL) at pH 7.3 ± 0.2. The bulk solution was filtered with 0.2µm filter and filled 1 mL of filtered solution in 1 mL glass PFS. The composition of formulation (F6) is given below:

Compositions	**mg / mL**
CTLA-4 IgG1 fusion protein	125
L - Histidine (150 mM)	21
L - Histidine HCl monohydrate (3 mM)	4.2
Sucrose	110
L-Arginine (50 mM)	10.53
Poloxamer 188	8

Analytical tests	**Results**		
	0 Day	14 Day	28 Day
% High molecular weight (HMW) species by SEC	0.12	1.13	*N.A.
% Low molecular weight (LMW) species by CE-SDS	0.38	0.77	1.37

*N.A. data not available due to experimental error

Comparison with S01 (INN PFS) where drug substance CTLA-4 IgG1 is added in formulation excipients as follow:

Compositions	**mg / mL**
Abatacept (CTLA-4 IgG1)	125
Dibasic sodium phosphate anhydrous (6mM)	0.838
monobasic sodium phosphate monohydrate (2 mM)	0.28

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Sucrose (500mM)	170
Poloxamer 188	8

Analytical tests	**Results**		
	0 Day	14 Day	28 Day
% High molecular weight (HMW) species by SEC	0.37	1.38	1.91
% Low molecular weight (LMW) species by SEC	0.38	4.05	8.95

F6 shows that high concentration of histidine 150mM in combination with sucrose and amino acid controls effectively HMW % and LMW % in comparison to formulation F1, F2 and S01. It is also noted that LMW % is significantly high in S01 phosphate buffer while F6 shows very low LMW % and thereby improves stability.

F6 viscosity is 8.09 cPs and osmolality is 785 mOsm/kg.

Example 7

CTLA-4 IgG1 fusion protein injectable formulation is formulated with 125 mg/mL concentration for subcutaneous administration.

Prepare the formulation (F7) with addition of 150 mM of Histidine buffer, 110 g/L of Sucrose, 50 mM of Lysine HCl and Poloxamer (8 mg/mL) at pH 7.3 ± 0.2. The bulk solution was filtered with 0.2µm filter and filled in 1 mL glass PFS. The composition of formulation (F7) is given below:

Compositions	**mg / mL**
CTLA-4 IgG1 fusion protein	125
L - Histidine	21
L - Histidine HCl monohydrate	4.2
Sucrose	110
L-Lysine (50 mM)	9.13
Poloxamer 188	8

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Analytical tests	Results		
	0 Day	14 day	28 Day
% High molecular weight (HMW) species by SEC	0.14	0.88	1.7
% Low molecular weight (LMW) species by CE-SDS	0.37	0.85	1.46

Comparison with S01 (INN PFS) where drug substance CTLA-4 IgG1 is added in formulation excipients as follow:

Compositions	mg / mL
Abatacept (CTLA-4 IgG1)	125
Dibasic sodium phosphate anhydrous (6mM)	0.838
monobasic sodium phosphate monohydrate (2 mM)	0.28
Sucrose (500mM)	170
Poloxamer 188	8

Analytical tests	Results		
	0 Day	14 Day	28 Day
% High molecular weight (HMW) species by SEC	0.37	1.38	1.91
% Low molecular weight (LMW) species by SEC	0.38	4.05	8.95

F7 shows that high concentration of histidine 150mM in combination with sucrose and amino acid controls effectively HMW % and LMW % in comparison to formulation F1, F2 and S01. It is also noted that LMW % is significantly high in S01 phosphate buffer while F6 shows very low LMW % and thereby improves stability.

F7 viscosity is 8.74 cPs and osmolality is 818 mOsm/kg.

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Example 8

CTLA-4 IgG1 fusion protein injectable formulation is formulated with 125 mg/mL concentration for subcutaneous administration.

Prepared the formulation (F8) with addition of 100 mM of Histidine buffer, 110 g/L of Sucrose, 50mM of arginine, 50mM of lysine and Poloxamer (8 mg/mL) at pH 7.3 ± 0.2. The bulk solution was filtered with 0.2µm filter and filled in 1 mL glass PFS. The composition of formulation (F8) is given below:

Compositions	**mg / mL**
CTLA-4 IgG1 fusion protein	125
L - Histidine	15.05
L - Histidine HCl monohydrate	0.63
Sucrose	110
Arginine HCl	10.53
L-Lysine	9.13
Poloxamer 188	8

Analytical tests	**Results**	
	0 Day	7 Day
% High molecular weight (HMW) species by SEC	0.13	0.49
% Low molecular weight (LMW) species by CE-SDS	0.27	0.54

Comparison with S01 (INN PFS) where drug substance CTLA-4 IgG1 is added in formulation excipients as follow:

Compositions	**mg / mL**
Abatacept (CTLA-4 IgG1)	125
Dibasic sodium phosphate anhydrous (6mM)	0.838
monobasic sodium phosphate monohydrate (2 mM)	0.28
Sucrose (500mM)	170

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Poloxamer 188	8

Analytical tests	**Results**	
	0 Day	7 Day
% High molecular weight (HMW) species by SEC	0.37	0.99
% Low molecular weight (LMW) species by SEC	0.38	4.05

It is evident that S01 formulation has very high HMW % and LMW % comparison to formulation (F7).

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Claims:

1. A pharmaceutical liquid formulation comprising:
   a. high concentration of pharmacologically active fusion protein;
   b. histidine buffer;
   c. suitable amino acid;
   d. stabilizer;
   e. surfactant and;
   f. pH selected from about 6.5 to about 7.5;
2. The pharmaceutical formulation according to claim 1, wherein the concentration of the fusion protein is at least 100mg/ml.
3. The pharmaceutical formulation according to claim 2, wherein the concentration of pharmacologically active fusion protein is selected from about 50 mg/0.4ml, 87.5 mg/0.7 ml, about 125 mg/ml.
4. The pharmaceutical formulation according to claim 3, wherein the pharmacologically active fusion protein is present from at least about 125 mg/ml.
5. The pharmaceutical formulation according to claim 1, wherein the histidine buffer is present from about 25 mM to about 100 mM.
6. The pharmaceutical formulation according to claim 1, wherein the histidine buffer is present from about 100 mM to about 200 mM.
7. The pharmaceutical formulation according to claim 6, wherein the histidine buffer is selected from about 100 mM, about 150 mM or about 200 mM.
8. The pharmaceutical formulation according to claim 1, wherein the suitable amino acids are selected from selected from arginine, lysine, glycine or proline and suitable salt thereof.
9. The pharmaceutical formulation according to claim 8, wherein the concentration of suitable amino acids from about 10 mM to about 200 mM.
10. The pharmaceutical formulation according to claim 8, wherein the arginine is present from about 10 mM to about 100 mM.
11. The pharmaceutical formulation according to claim 10, wherein the arginine is about 50 mM.
12. The pharmaceutical formulation according to claim 8, wherein the lysine is present from about 10 mM to about 100 mM.
13. The pharmaceutical formulation according to claim 12, wherein the lysine is about 50 mM.
14. The pharmaceutical formulation according to claim 1, wherein the stabilizer selected from sugar or cyclodextrin.

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15. The pharmaceutical formulation according to claim 14, wherein the sugar concentration is lower than concentration of fusion protein.
16. The pharmaceutical formulation according to claim 14, wherein the sugar is sucrose.
17. The pharmaceutical formulation according to claim 16, wherein the fusion protein and sucrose ratio is selected from not more than 1:0.9, 1:0.8, 1:0.7, 1:0.6, 1:0.5.
18. The pharmaceutical formulation according to claim 14, wherein the sucrose is present from about 100 mg to about 110 mg.
19. The pharmaceutical formulation according to claim 14, wherein the sucrose is present from about 100 mg to about 105 mg.
20. The pharmaceutical formulation according to claim 1, wherein the surfactant is selected from polysorbate or poloxamer 188.
21. The pharmaceutical formulation according to claim 20, wherein the surfactant is poloxamer 188.
22. The pharmaceutical formulation according to claim 21, wherein the Poloxamer 188 as surfactant is present from about 1 mg/mL to 10 mg/mL.
23. The pharmaceutical formulation according to claim 21, wherein the Poloxamer 188 as surfactant is present from about 5 mg/mL to 8 mg/mL.
24. The pharmaceutical formulation according to claim 1, wherein the pH is about 7.3±0.2.
25. The pharmaceutical formulation according to claim 1, comprising a fusion protein consisting of receptor fused with constant region of immunoglobulin selected from IgG1, IgG2, IgG3 and IgG4.
26. The pharmaceutical formulation according to claim 25, comprising a receptor selected from CTLA4, TNFR, VEGF, HER-2, PCSK9 fused with constant region of IgG1.
27. The pharmaceutical formulation according to claim 25, wherein the fusion protein is CTLA4-Ig fusion protein.
28. A stable pharmaceutical liquid formulation according to claim 1 comprises:
    a. high concentration of pharmacologically active fusion protein;
    b. histidine buffer;
    c. arginine;
    d. sucrose;
    e. poloxamer 188 and;
    f. pH about 7.3±0.2;
29. A stable pharmaceutical liquid formulation according to claim 1 comprises:
    a. high concentration of pharmacologically active fusion protein;
    b. histidine buffer;

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c. lysine;

d. sucrose;

e. poloxamer 188 and;

f. pH about 7.3±0.2;

30. A stable pharmaceutical liquid formulation according to claim 1 comprises:

a. high concentration of pharmacologically active fusion protein;

b. histidine buffer;

c. arginine and lysine;

d. sucrose;

e. poloxamer 188 and;

f. pH about 7.3±0.2;

31. A stable pharmaceutical liquid formulation of fusion protein comprising:

a. high concentration of pharmacologically active fusion protein;

b. histidine buffer;

c. optionally suitable amino acid;

d. sucrose;

e. poloxamer 188 and;

f. pH about 7.3±0.2;

32. A drug delivery device comprising a pharmaceutical liquid formulation according to claim 1.

33. The pharmaceutical liquid formulation according to claim 1, comprises a kinematic viscosity of about 10 cPs or less.

34. The pharmaceutical liquid formulation according to claim 33, comprises a kinematic viscosity of about 7 to about 9 cPs.

35. The pharmaceutical liquid formulation according to claim 1, comprises osmolality of about 800±50 mOsm/kg.

36. The pharmaceutical liquid formulation according to claim 35, comprises osmolality of about 780 to about 825 mOsm/kg.

37. The pharmaceutical liquid formulation according to claim 1 comprises reduced amount of low molecular weight (LMW) impurities compared to fusion protein formulated with phosphate buffer.

38. The pharmaceutical formulation according to claim 1 essentially free of histidine amino acid.

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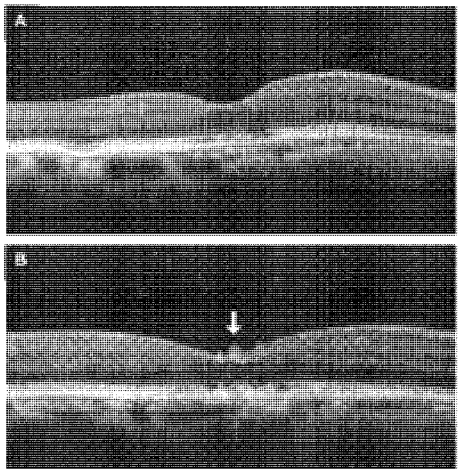


Figure 1

(57) Abstract: The invention relates to methods for treating ocular disease with a VEGF antagonist. In particular, the invention relates to methods for treating ocular disease, the method comprising selectively administering a VEGF antagonist to a subject in need thereof on the basis of said subject not having a preretinal or vitreous hyperreflective foci formation. The invention further relates to methods for determining whether a subject with ocular disease should be treated with a VEGF antagonist, methods of predicting the likelihood that a subject having ocular disease will develop intraocular inflammation in response to a treatment with a VEGF antagonist, and methods for producing a transmittable form of information for determining whether a subject with ocular disease should be treated with a VEGF antagonist or for predicting whether a subject with ocular disease may develop intraocular inflammation in response to a treatment with a VEGF antagonist.

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METHODS FOR TREATING OCULAR DISEASES

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on March 22, 2021, is named PAT059060_SEQ_LISTING_ST25.txt and is 8 KB in size.

FIELD

The invention relates to methods for treating ocular disease with a VEGF antagonist.

BACKGROUND

Age-related macular degeneration (AMD) is the leading cause of severe vision loss in people affecting 10%-13% of individuals over the age of 65 in North America, Europe, and Australia (Kawasaki 2010, Rein et al., Arch Ophthalmol. 2009;127:533-40, Smith 2001). Genetic, environmental and health factors play an important role in the pathogenesis of the disease. AMD is classified into 2 clinical subtypes: the non-neovascular (atrophic) or dry form and the neovascular (exudative) or wet form (Ferris et al., Arch Ophthalmol. 1984;102:1640-2, Lim et al., Lancet. 2012;379:1728-38, Miller et al., Am J Ophthalmol. 2013;155:1-35). Neovascular AMD (nAMD) is characterized by the growth of abnormal new blood vessels (neovascularization) under the retinal pigment epithelium (RPE) or subretinal space from the subjacent choroid, termed choroidal neovascularization (CNV) (Ferris et al., Arch Ophthalmol. 1984;102:1640-2). These newly formed vessels have an increased likelihood to leak blood and serum, damaging the retina by stimulating inflammation and scar tissue formation. This damage to the retina results in progressive, severe, and irreversible vision loss (Shah et al., Am J Ophthalmol. 2007;143:83-89, Shah et al., Am J Ophthalmol. 2009;116:1901-07). Without treatment, most affected eyes will have poor central vision (20/200) within 12 months (TAP 2003). Although the neovascular form of the disease is only present in about 10% of all AMD cases, it accounted for approximately 90% of the severe vision loss from AMD prior to the introduction of anti-vascular endothelial growth factor

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(VEGF) treatments (Ferris et al., Am J Ophthalmol. 1983;118:132-51, Sommer et al., N Engl J Med. 1991;14:1412-17, Wong et al., Ophthalmology. 2008;115:116-26).

VEGF has been shown to be elevated in patients with nAMD and is thought to play a key role in the neovascularization process (Spilsbury et al., Am J Pathol. 2000;157:135-44). The use of intravitreal (IVT) pharmacotherapy targeting VEGF has significantly improved visual outcomes in patients with nAMD (Bloch et al., Am J Ophthalmol. 2012;153:209-13, Campbell et al., Arch Ophthalmol. 2012;130:794-5). Anti-VEGF treatments, such as ranibizumab (LUCENTIS®), aflibercept (EYLEA®), and brolucizumab (Beovu®), inhibit VEGF signaling pathways and have been shown to halt the growth of neovascular lesions and resolve retinal edema.

One of the main ocular adverse events (AEs) associated with these agents is intraocular inflammation (IOI) (Tolentino M., Surv Ophthalmol, 2011;56:95-113; Mones et al., American Academy of Ophthalmology, 2020). In the VIEW studies, for example, in which patients with nAMD were treated with intravitreal ranibizumab or aflibercept, IOI was a predefined AE of interest (Schmidt-Erfurth et al., Ophtalmology, 2014;121:193-201). Between baseline and 96 weeks, this event was reported in 1.5% of patients receiving ranibizumab and 0.5% to 1.1% of patients in the three aflibercept arms (Schmidt-Erfurth et al., Ophtalmology, 2014;121:193-201).

Brolucizumab 6mg represents an important treatment option for patients with wet AMD, with an overall favorable benefit-risk profile. Following approval of brolucizumab 6 mg for the treatment of nAMD by the Food and Drug Administration (FDA) in October 2019, there were reports of retinal vasculitis and/or retinal artery occlusion (RAO) accompanied by IOI with intravitreal injections of brolucizumab (Safety of Beovu® (brolucizumab). https://www.brolucizumab.info/ Last accessed on 19 November 2020. 2020; Witkin et al., J Vitreoretin Dis 2020;4(4):269-79). Based on post-marketing reports, and post hoc review of Hawk & Harrier cases of interest, Novartis initiated internal review of these post-marketing safety case reports, including the establishment of an external Safety Review Committee (SRC) to provide an independent, unmasked post-hoc review of these cases (Beovu® (brolucizumab) Global Safety Information for Healthcare Professionals. https://www.brolucizumab.info/. Accessed October 2, 2020). Based on an unmasked post-hoc

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review, the SRC reported overall incidence of intraocular inflammation (IOI) of any form of 4.6% (50/1088), overall incidence of signs of retinal vasculitis of 3.3% (36/1088), overall incidence of concomitant signs of retinal vasculitis (RV) and retinal vascular occlusion (RO) of 2.1% (23/1088), and overall incidence of IOI of any form associated with losing 15 or more letters at the last visit / end of the study was 0.7% (8/1088) (Member Update: Novartis-Appointed Safety Review Committee Reports Initial Brolucizumab Findings. American Society of Retina Specialists. June 4, 2020; Mones et al., American Academy of Ophthalmology, 2020). Label updates have been approved by several health authorities, including the FDA and the EMA. RV and/or RO have been reported with the use of brolucizumab (Beovu® [US prescribing information]. East Hanover, NJ: Novartis Pharmaceuticals Corp, Oct 2019; Beovu® [summary of product characteristics], Basel, Switzerland, Novartis Pharma AG. Sept 2020). These events may result in severe vision loss and have typically occurred in the presence of IOI (Beovu® (brolucizumab) Global Safety Information for Healthcare Professionals. https://www.brolucizumab.info/. Accessed October 2, 2020).

Intraocular inflammation (IOI) is an adverse event that may lead to further complications if not identified, including severe vision loss. To increase patient's safety, there is a benefit to identifying susceptible patients before development of intraocular inflammation or at the very early onset of intraocular inflammation and adjust their treatment, for example by discontinuing the treatment with that particular drug that can cause intraocular inflammation or by altering / selecting the treatment (drug and treatment regimen) correspondingly. There is a continuing need in the art for predicting, determining, monitoring delayed drug-induced intraocular inflammation.

SUMMARY

Using advanced imaging methods (spectral-domain optical coherence tomography, SD-OCT) a post hoc analysis was conducted of the HAWK and HARRIER studies, which were 2-year, randomized, double-masked, multicenter, phase 3 trials comparing brolucizumab with aflibercept in treatment-naïve patients with nAMD. The post-hoc analysis identified a new biomarker that precedes or is associated with intraocular inflammation (IOI) adverse events in nAMD patients: a hyperreflective deposit (HRD). In particular, the post-hoc

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analysis identified that a formation of HRD, and in particular a formation of a HRD in vitreous compartment, e.g., preretinal, e.g., internal limiting membrane (ILM), precedes or is associated with intraocular inflammation (IOI) adverse events in nAMD patients. . The reported hyperreflective deposits were in a form of heterogeneity observed on the OCT scan including hyperreflective debris, hyperreflective round- or rod-shaped dots, e.g., stalagmite-like preretinal deposits. The stalagmite-like deposits are discrete HRD, e.g., PHRD, that resemble the elevated speleothems. Previously, the stalagmite-like preretinal deposits have been described and associated with vitreous inflammation in patients with vitrectomized eyes and flares of posterior uveitis (Yonekawa et al., Digital Journal of Ophthalmology, 2017, Vol. 23, pp. 18-22). In accordance specific aspects of the present invention, it has been surprisingly found that there is a correlation between presence of HRD, e.g., VHRD, e.g., PHRD e.g., stalagmite-like preretinal hyperreflective material, with posterior inflammation in nAMD patients treated with a VEGF inhibitor, e.g., brolucizumab. Based on this finding, it is proposed that HRD, e.g., VHRD, e.g., PHRD , in particular stalagmite-like preretinal hyperreflective material, may be used as a safety biomarker for the selection of the patients suitable for treatment with a VEGF inhibitor, e.g., brolucizumab, or for monitoring the patients during the course of treatment with a VEGF inhibitor, e.g., brolucizumab. Use of this new safety biomarker thus advances management of ophthalmic disorders such as nAMD in patients.

In one aspect, the present invention provides a method of treating ocular disease, e.g., neovascular age-related macular degeneration (nAMD), comprising selectively administering a VEGF antagonist, e.g., brolucizumab, to a subject in need thereof on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD.

In one aspect, the present invention provides a method of treating ocular disease, e.g., nAMD, comprising: (a) selecting a subject for treatment with a VEGF antagonist, e.g., brolucizumab, on the basis said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and (b) thereafter, selectively administering the VEGF antagonist, e.g., brolucizumab, to the subject.

In a another aspect, the present invention provides a method of selectively treating a subject having ocular disease, e.g., nAMD, the method comprising:

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(a) selectively administering a therapeutically effective amount of a VEGF antagonist, e.g., brolucizumab, to the subject on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; or

(b) selectively administering a therapeutically effective amount of a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, to the subject on the basis of said subject having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD

.

In a another aspect, the present invention provides a method for determining whether a subject with ocular disease, e.g., nAMD, should be treated with a VEGF antagonist, e.g., brolucizumab, the method comprising:

(a) performing analysis, e.g., OCT, SD-OCT analysis, on a subject's eye to assess the presence or absence of a HRD, e.g., VHRD, e.g., PHRD; and

(b) determining that the subject should be treated with a VEGF antagonist, e.g., brolucizumab, if no said HRD, e.g., VHRD, e.g., PHRD, is observed in the subject's eye;

(c) determining that the subject should not be treated with a VEGF antagonist, e.g., brolucizumab, if the HRD, e.g., VHRD, e.g., PHRD, is observed in the subject's eye.

In a another aspect, the present invention provides a method of predicting the likelihood that a subject having ocular disease, e.g., nAMD, will develop intraocular inflammation (IOI), e.g., intraocular inflammation with retinal vasculitis and / or retinal occlusion (IOI+RV/RO), in response to a treatment with a VEGF antagonist, e.g., brolucizumab, the method comprising determining if the subject has a HRD in a subject's eye, e.g., VHRD, e.g., PHRD. Suitably, the method comprises determining by OCT or SD-OCT if the subject has a HRD in a subject's eye, e.g., VHRD, e.g., PHRD, wherein:

(a) the presence of the HRD, e.g., VHRD, e.g., PHRD, is indicative of an increased likelihood that the patient will develop IOI, e.g., IOI+RV/RO, in response to the treatment with the VEGF antagonist, e.g., brolucizumab; and|

(b) the absence of the HRD, e.g., VHRD, e.g., PHRD, is indicative of a decreased likelihood that the patient will develop IOI, e.g., IOI+RV/RO, in response to the treatment with the VEGF antagonist, e.g., brolucizumab.

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In one aspect, the present invention provides a method of monitoring a subject during a course of treatment with a VEGF antagonist for presence of a HRD in a subject's eye, e.g., VHRD, e.g., PHRD, e.g., by performing OCT or SD-OCT analysis, and (i) altering or discontinuing the treatment with the VEGF antagonist if, during the course of treatment, the subject is determined to have the HRD, e.g., VHRD, e.g., PHRD, in the treatment eye; or (ii) maintaining the treatment with the VEGF antagonist if, during the course of treatment, the subject is determined not to have the HRD, e.g., VHRD, e.g., PHRD, in the treatment eye.

In one aspect, the present invention provides a VEGF antagonist, e.g., brolucizumab, or a pharmaceutical composition comprising said VEGF antagonist for the use in treatment of ocular disease, e.g., nAMD, in a subject, characterized in that a therapeutically effective amount of the VEGF antagonist, e.g., brolucizumab, is to be administered to the subject on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD.

In a another aspect, the present invention provides a VEGF antagonist, e.g., brolucizumab, or a pharmaceutical composition comprising said VEGF antagonist for the use in treatment of ocular disease, e.g., nAMD, in a subject, characterized in that:
(a) a subject is selected for treatment with the VEGF antagonist, e.g., brolucizumab, on the basis of the subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and
(b) thereafter, a therapeutically effective amount of the VEGF antagonist, e.g., brolucizumab, is administered to the subject.

In a another aspect, the present invention provides a VEGF antagonist, e.g., brolucizumab, or a pharmaceutical composition comprising said VEGF antagonist for the use in treatment of ocular disease, e.g., nAMD, in a subject, wherein the use comprises
(a) determining if the subject has a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and
(b) if the HRD, e.g., VHRD, e.g., PHRD, is not present, a therapeutically effective amount of the VEGF antagonist, e.g., brolucizumab, is to be administered to the subject; and
(c) if the HRD, e.g., VHRD, e.g., PHRD, is present, a therapeutically effective amount of a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, is to be administered to the subject.

In a another aspect, the present invention provides a VEGF antagonist, e.g., brolucizumab, or a pharmaceutical composition comprising said VEGF antagonist for the use

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in treatment of ocular disease, e.g., nAMD, in a subject, wherein the use comprises:
(a) performing analysis, e.g., OCT, SD-OCT analysis, on a subject's eye to assess the presence or absence of a HRD, e.g., VHRD, e.g., PHRD; and
(b) determining if the subject has the HRD, e.g., VHRD, e.g., PHRD; and
(c) if the HRD, e.g., VHRD, e.g., PHRD, is not present, a therapeutically effective amount of the VEGF antagonist, e.g., brolucizumab, is to be administered to the subject; and
(d) if the HRD, e.g., VHRD, e.g., PHRD, is present, a therapeutically effective amount of a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, is to be administered to the subject.

In one aspect, the present invention provides use of a VEGF antagonist, e.g., brolucizumab, or a pharmaceutical composition comprising said VEGF antagonist for the manufacture of a medicament for treating ocular disease, e.g., nAMD, in a subject, characterized in that a therapeutically effective amount of the VEGF antagonist, e.g., brolucizumab, is to be administered to the subject on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD.

In a another aspect, the present invention provides use of a VEGF antagonist, e.g., brolucizumab, or a pharmaceutical composition comprising said VEGF antagonist for the manufacture of a medicament for treating ocular disease, e.g., nAMD, in a subject, characterized in that:
(a) a subject is selected for treatment with the VEGF antagonist, e.g., brolucizumab, on the basis of the subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and
(b) thereafter, a therapeutically effective amount of the VEGF antagonist, e.g., brolucizumab, is administered to the subject.

In a another aspect, the present invention provides use of a VEGF antagonist, e.g., brolucizumab, or a pharmaceutical composition comprising said VEGF antagonist for the manufacture of a medicament for treating ocular disease, e.g., nAMD, in a subject, wherein the use comprises
(a) determining if the subject has a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and
(b) if the HRD, e.g., VHRD, e.g., PHRD, is not present, a therapeutically effective amount of the VEGF antagonist, e.g., brolucizumab, is to be administered to the subject; and

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(c) if the HRD, e.g., VHRD, e.g., PHRD, is present, a therapeutically effective amount of a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, is to be administered to the subject.

In a another aspect, the present invention provides use of a VEGF antagonist, e.g., brolucizumab, or a pharmaceutical composition comprising said VEGF antagonist for the manufacture of a medicament for treating ocular disease, e.g., nAMD, in a subject, wherein the use comprises:
(a) performing analysis, e.g., OCT, SD-OCT analysis, on a subject's eye to assess the presence or absence of a HRD, e.g., VHRD, e.g., PHRD; and
(b) determining if the subject has a HRD, e.g., VHRD, e.g., PHRD; and
(c) if the HRD, e.g., VHRD, e.g., PHRD, is not present, a therapeutically effective amount of the VEGF antagonist, e.g., brolucizumab, is to be administered to the subject; and
(d) if the HRD, e.g., VHRD, e.g., PHRD, is present, a therapeutically effective amount of a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, is to be administered to the subject.

In one aspect, the present invention provides use of a VEGF antagonist, e.g., brolucizumab, or a pharmaceutical composition comprising said VEGF antagonist for treating ocular disease, e.g., nAMD, in a subject, characterized in that a therapeutically effective amount of the VEGF antagonist, e.g., brolucizumab, is to be administered to the subject on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD.

In a another aspect, the present invention provides use of a VEGF antagonist, e.g., brolucizumab, or a pharmaceutical composition comprising said VEGF antagonist for treating ocular disease, e.g., nAMD, in a subject, characterized in that:
(a) a subject is selected for treatment with the VEGF antagonist, e.g., brolucizumab, on the basis of the subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and
(b) thereafter, a therapeutically effective amount of the VEGF antagonist, e.g., brolucizumab, is administered to the subject.

In a another aspect, the present invention provides use of a VEGF antagonist, e.g., brolucizumab, or a pharmaceutical composition comprising said VEGF antagonist for

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treating ocular disease, e.g., nAMD, in a subject, wherein the use comprises
(a) determining if the subject has a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and
(b) if the HRD, e.g., VHRD, e.g., PHRD, is not present, a therapeutically effective amount of the VEGF antagonist, e.g., brolucizumab, is to be administered to the subject; and
(c) if the HRD, e.g., VHRD, e.g., PHRD, is present, a therapeutically effective amount of a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, is to be administered to the subject.

In a another aspect, the present invention provides use of a VEGF antagonist, e.g., brolucizumab, or a pharmaceutical composition comprising said VEGF antagonist for treating ocular disease, e.g., nAMD, in a subject, wherein the use comprises:
(a) performing analysis, e.g., OCT, SD-OCT analysis, on a subject's eye to assess the presence or absence of a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and
(b) determining if the subject has a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and
(c) if the HRD, e.g., VHRD, e.g., PHRD, is not present, a therapeutically effective amount of the VEGF antagonist, e.g., brolucizumab, is to be administered to the subject; and
(d) if the HRD, e.g., VHRD, e.g., PHRD, is present, a therapeutically effective amount of a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, is to be administered to the subject.

In one aspect, the present invention provides, a method for producing a transmittable form of information for determining whether a subject with ocular disease, e.g., nAMD, should be treated with a VEGF antagonist, e.g., brolucizumab, the method comprising:
(a) determining that the subject should be treated with a VEGF antagonist, e.g., brolucizumab, on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; or
(b) determining that the subject should be treated with a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, to the subject on the basis of said subject having HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and
(c) recording the result of the determining step on a tangible or intangible media form for use in transmission.

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In one aspect, the present invention provides, a method for producing a transmittable form of information for predicting whether a subject with ocular disease, e.g., nAMD, may develop intraocular inflammation (IOI), e.g., intraocular inflammation with retinal vasculitis/retinal occlusion (IOI+RV/RO), in response to a treatment with a VEGF antagonist, e.g., brolucizumab, the method comprising:

(a) determining an increased likelihood that the subject will develop IOI, e.g., IOI+RV/RO, in response to the treatment with the VEGF antagonist, e.g., brolucizumab, on the basis of said subject having HRD in a subject's eye, e.g., VHRD, e.g., PHRD; or

(b) determining a decreased likelihood that the subject will develop IOI, e.g., IOI+RV/RO, in response to the treatment with the VEGF antagonist, e.g., brolucizumab, on the basis of said subject not having HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and

(c) recording the result of the determining step on a tangible or intangible media form for use in transmission.

Non-limiting embodiments of the present disclosure are described in the following embodiments:

Embodiment 1: A method of treating ocular disease comprising selectively administering a VEGF antagonist to a subject in need thereof on the basis of said subject not having a hyperreflective deposit (HRD), e.g., a vitreous hyperreflective deposit (VHRD), e.g., a preretinal hyperreflective deposit (PHRD).

Embodiment 2: A method of treating ocular disease comprising:

(a) selecting a subject for treatment with a VEGF antagonist on the basis said subject not having a HRD, e.g., VHRD, e.g., PHRD; and

(b) thereafter, selectively administering the VEGF antagonist to the subject.

Embodiment 3: A method of selectively treating a subject having ocular disease the method comprising:

(a) selectively administering a therapeutically effective amount of a VEGF antagonist, e.g., brolucizumab, to the subject on the basis of said subject not having a HRD, e.g., VHRD, e.g., PHRD; or

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(b) selectively administering a therapeutically effective amount of a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, to the subject on the basis of said subject having a HRD, e.g., VHRD, e.g., PHRD.

Embodiment 4: The method of embodiment 2 or 3 further comprising assessing the presence or absence of the HRD, e.g., VHRD, e.g., PHRD, e.g., prior to the steps (a)-(b) of embodiment 2 or embodiment 3.

Embodiment 5: The method of any one of embodiments 1 to 4, wherein the presence or absence of the HRD, e.g., VHRD, e.g., PHRD, is assessed by performing OCT, e.g., SD-OCT, analysis on a subject's eye.

Embodiment 6: A method for determining whether a subject with ocular disease should be treated with a VEGF antagonist, the method comprising:
(a) performing analysis, e.g., OCT, SD-OCT analysis, on a subject's eye to assess the presence or absence of a HRD, e.g., VHRD, e.g., PHRD; and
(b) determining that the subject should be treated with a VEGF antagonist if no HRD, e.g., VHRD, e.g., PHRD, is observed in the subject's eye;
(c) determining that the subject should not be treated with a VEGF antagonist if the HRD, e.g., VHRD, e.g., PHRD, is observed in the subject's eye.

Embodiment 7: The method of embodiment 6 further comprising treating the subject with the VEGF antagonist if no HRD, e.g., VHRD, e.g., PHRD, is observed in the subject's eye.

Embodiment 8: The method of any one of the preceding embodiments, wherein the subject is assessed for presence of the HRD, e.g., VHRD, e.g., PHRD, prior to initiation of the treatment with the VEGF antagonist and / or during the treatment course with the VEGF antagonist.

Embodiment 9: The method of embodiment 8, further comprising:
(a) altering or discontinuing the treatment with the VEGF antagonist if, during the course of treatment, the subject is determined to have a HRD, e.g., VHRD, e.g., PHRD, in the treatment eye, or

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(b) maintaining the treatment with the VEGF antagonist if, during the course of treatment, the subject is determined not to have a HRD, e.g., VHRD, e.g., PHRD, in the treatment eye.

Embodiment 10: The method of any one of the preceding embodiments, wherein the HRD, e.g., VHRD, e.g., PHRD, has high reflectivity, e.g., has clearly delineated boundaries from the surrounding components of the retina.

Embodiment 11: The method of any one of the preceding embodiments, wherein the HRD, e.g., VHRD, e.g., PHRD, is hyperreflective round- or rod-shaped dot, e.g., stalagmite-like hyperreflective material, e.g., stalagmite-like preretinal hyperreflective material.

Embodiment 12: The method of any one of the preceding embodiments, wherein the VEGF antagonist is selected from the group consisting of brolucizumab, aflibercept, ranibizumab, faricimab, conbercept and abicipar.

Embodiment 13: The method of any one of the preceding embodiments, wherein the VEGF antagonist is an anti-VEGF antibody or a fragment thereof.

Embodiment 14: The method of embodiment 13, wherein the VEGF antibody comprises a variable heavy chain having the sequence as set forth in SEQ ID NO: 1 and a variable light chain having the sequence as set forth in SEQ ID NO: 2.

Embodiment 15: The method of embodiment 13, wherein the VEGF antibody comprises the sequence of SEQ ID NO: 3 or SEQ ID NO: 4.

Embodiment 16: The method of embodiment 13, wherein the VEGF antagonist is brolucizumab.

Embodiment 17: The method of any one of the preceding embodiments, wherein the method comprises administering to the subject three individual doses of the VEGF antagonist at 4-week intervals, and thereafter administering to the subject an additional dose every 8 to 12 weeks.

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Embodiment 18: The method of embodiment 17, wherein the method comprises administering to the subject three individual doses of the VEGF antagonist at 4-week intervals, and thereafter administering to the subject an additional dose every 12 weeks.

Embodiment 19: The method of embodiment 18, wherein a 12 week treatment interval is switched to an 8 week treatment interval if a disease activity is detected in the subject.

Embodiment 20: The method of embodiment 15, wherein the method comprises administering to the subject three individual doses of the VEGF antagonist at 4-week intervals, and thereafter administering to the subject an additional dose every 8 weeks.

Embodiment 21: The method of embodiment 20, wherein an 8 week treatment interval is switched to a 12 week treatment interval if no disease activity is detected in the subject.

Embodiment 22: The method of any one of embodiments 14 to 21, wherein each dose of the VEGF antagonist is at least 3 mg, e.g., 3 mg or 6 mg, in particular 6 mg.

Embodiment 23: The method of embodiment 22, wherein each dose of the VEGF antagonist is administered as a 50 µL intravitreal injection.

Embodiment 24: The method of any one of the preceding embodiments, wherein the ocular disease is selected from the list consisting of abnormal angiogenesis, choroidal neovascularization (CNV), retinal vascular permeability, retinal edema, diabetic retinopathy (particularly proliferative diabetic retinopathy (PDR) and non-proliferative diabetic retinopathy (NPDR)), macular edema (ME), diabetic macular edema (DME), neovascular (exudative) age-related macular degeneration (nAMD), choroidal neovascularization (CNV) associated with nAMD, sequela associated with retinal ischemia, Retinal Vein Occlusion (RVO), Central Retinal Vein Occlusion (CRVO), Branch Retinal Vein Occlusion (BRVO), macular edema following retinal vein occlusion, and posterior segment neovascularization.

Embodiment 25: The method of embodiment 24, wherein the ocular disease is neovascular age-related macular degeneration (nAMD).

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Embodiment 26: A method of predicting the likelihood that a subject having ocular disease will develop intraocular inflammation (IOI), e.g., intraocular inflammation with retinal vasculitis/retinal occlusion (IOI+RV/RO), in response to a treatment with a VEGF antagonist the method comprising determining if the subject has a HRD in a subject's eye, e.g., VHRD, e.g., PHRD , in particular the method comprising determining by OCT or SD-OCT if the subject has a HRD in a subject's eye, e.g., VHRD, e.g., PHRD, wherein:

(a) the presence of a HRD, e.g., VHRD, e.g., PHRD, is indicative of an increased likelihood that the patient will develop IOI, e.g., IOI+RV/RO, in response to the treatment with the VEGF antagonist; and

(b) the absence of a HRD, e.g., VHRD, e.g., PHRD, is indicative of a decreased likelihood that the patient will develop IOI, e.g., IOI+RV/RO, in response to the treatment with the VEGF antagonist.

Embodiment 27: The method of embodiment 26 further comprising treating the subject with the VEGF antagonist if no HRD, e.g., VHRD, e.g., PHRD, is observed in the subject's eye.

Embodiment 28: The method of any one of embodiments 26 to 27, wherein the HRD, e.g., VHRD, e.g., PHRD, has high reflectivity, e.g., has clearly delineated boundaries from the surrounding components of the retina.

Embodiment 29: The method of any one of embodiments 26 to 27, wherein the HRD, e.g., VHRD, e.g., PHRD, is a hyperreflective round- or rod-shaped dot, e.g., stalagmite-like hyperreflective material, e.g., stalagmite-like preretinal hyperreflective material.

Embodiment 30: The method of any one of embodiments 26 to 29, wherein the VEGF antagonist is selected from the group consisting of brolucizumab, aflibercept, ranibizumab, faricimab, conbercept and abicipar.

Embodiment 31: The method of any one of embodiments 26 to 29, wherein the VEGF antagonist is an anti-VEGF antibody or a fragment thereof.

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Embodiment 32: The method of embodiment 31, wherein the VEGF antibody comprises a variable heavy chain having the sequence as set forth in SEQ ID NO: 1 and a variable light chain having the sequence as set forth in SEQ ID NO: 2.

Embodiment 33: The method of embodiment 31, wherein the VEGF antibody comprises the sequence of SEQ ID NO: 3 or SEQ ID NO: 4.

Embodiment 34: The method of embodiment 31, wherein the VEGF antagonist is brolucizumab.

Embodiment 35: The method of any one of embodiments 32 to 34, wherein the method comprises administering to the subject three individual doses of the VEGF antagonist at 4-week intervals, and thereafter administering to the subject an additional dose every 8 to 12 weeks.

Embodiment 36: The method of embodiment 35, wherein the method comprises administering to the subject three individual doses of the VEGF antagonist at 4-week intervals, and thereafter administering to the subject an additional dose every 12 weeks.

Embodiment 37: The method of embodiment 36, wherein a 12 week treatment interval is switched to an 8 week treatment interval if a disease activity is detected in the subject.

Embodiment 38: The method of embodiment 35, wherein the method comprises administering to the subject three individual doses of the VEGF antagonist at 4-week intervals, and thereafter administering to the subject an additional dose every 8 weeks.

Embodiment 39: The method of embodiment 38, wherein an 8 week treatment interval is switched to a 12 week treatment interval if no disease activity is detected in the subject.

Embodiment 40: The method of any one of embodiments 32 to 39, wherein each dose of the VEGF antagonist is at least 3 mg, e.g., 3 mg or 6 mg, in particular 6 mg.

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Embodiment 41: The method of embodiment 40, wherein each dose of the VEGF antagonist is administered as a 50 μL intravitreal injection.

Embodiment 42: The method of any one of embodiments 26 to 41, wherein the ocular disease is selected from the list consisting of abnormal angiogenesis, choroidal neovascularization (CNV), retinal vascular permeability, retinal edema, diabetic retinopathy (particularly proliferative diabetic retinopathy (PDR) and non-proliferative diabetic retinopathy (NPDR)), macular edema (ME), diabetic macular edema (DME), neovascular (exudative) age-related macular degeneration (nAMD), choroidal neovascularization (CNV) associated with nAMD, sequela associated with retinal ischemia, Retinal Vein Occlusion (RVO), Central Retinal Vein Occlusion (CRVO), Branch Retinal Vein Occlusion (BRVO), macular edema following retinal vein occlusion, and posterior segment neovascularization.

Embodiment 43: The method of embodiment 42, wherein the ocular disease is neovascular age-related macular degeneration (nAMD).

Embodiment 44: A VEGF antagonist for the use in treatment of ocular disease in a subject, characterized in that a therapeutically effective amount of the VEGF antagonist is to be administered to the subject on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD.

Embodiment 45: A VEGF antagonist for the use in treatment of ocular disease in a subject, characterized in that:

(a) a subject is selected for treatment with the VEGF antagonist on the basis of the subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and

(b) thereafter, a therapeutically effective amount of the VEGF antagonist is administered to the subject.

Embodiment 46: A VEGF antagonist for the use in treatment of ocular disease in a subject, wherein the use comprises

(a) determining if the subject has a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and

(b) if the HRD, e.g., VHRD, e.g., PHRD, is not present, a therapeutically effective amount of the VEGF antagonist, e.g., brolucizumab, is to be administered to the subject; and

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(c) if the HRD, e.g., VHRD, e.g., PHRD, is present, a therapeutically effective amount of a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, is to be administered to the subject.

Embodiment 47: The VEGF antagonist for use of embodiment 45 or 46, wherein the use further comprises assessing the presence or absence of the HRD, e.g., VHRD, e.g., PHRD, e.g., prior to the steps (a)-(b) of embodiment 45 or embodiment 46.

Embodiment 48: The VEGF antagonist for use of any one of embodiments 44 to 47, wherein the presence or absence of the HRD, e.g., VHRD, e.g., PHRD, is assessed by performing OCT, e.g., SD-OCT, analysis on a subject's eye.

Embodiment 49: A VEGF antagonist for the use in treatment of ocular disease in a subject, wherein the use comprises:
(a) performing analysis, e.g., OCT, SD-OCT analysis, on a subject's eye to assess the presence or absence of a HRD, e.g., VHRD, e.g., PHRD; and
(b) determining if the subject has a HRD, e.g., VHRD, e.g., PHRD; and
(c) if the HRD, e.g., VHRD, e.g., PHRD, is not present, a therapeutically effective amount of the VEGF antagonist, e.g., brolucizumab, is to be administered to the subject; and
(d) if the HRD, e.g., VHRD, e.g., PHRD, is present, a therapeutically effective amount of a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, is to be administered to the subject.

Embodiment 50: The VEGF antagonist for use of embodiment 49, wherein a therapeutically effective amount of the VEGF antagonist is to be administered to the subject on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD.

Embodiment 51: The VEGF antagonist for use of any one of embodiments 44 to 50, wherein the subject is assessed for presence of the HRD in a subject's eye, e.g., VHRD, e.g., PHRD prior to initiation of the treatment with the VEGF antagonist and / or during the treatment course with the VEGF antagonist.

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Embodiment 52: The VEGF antagonist for use of embodiment 51, wherein the use further comprises:

(a) altering or discontinuing the treatment with the VEGF antagonist if, during the course of treatment, the subject is determined to have a HRD, e.g., VHRD, e.g., PHRD, in the treatment eye, or

(b) maintaining the treatment with the VEGF antagonist if, during the course of treatment, the subject is determined not to have a HRD, e.g., VHRD, e.g., PHRD, in the treatment eye.

Embodiment 53: The VEGF antagonist for use of any one of embodiments 44 to 52, wherein the HRD, e.g., VHRD, e.g., PHRD, has high reflectivity, e.g., has clearly delineated boundaries from the surrounding components of the retina.

Embodiment 54: The VEGF antagonist for use of any one of embodiments 44 to 53, wherein the HRD, e.g., VHRD, e.g., PHRD, is a hyperreflective round- or rod-shaped dot, e.g., stalagmite-like hyperreflective material, e.g., stalagmite-like preretinal hyperreflective material.

Embodiment 55: The VEGF antagonist for use of any one of embodiments 44 to 54, wherein the VEGF antagonist is selected from the group consisting of brolucizumab, aflibercept, ranibizumab, faricimab, conbercept and abicipar.

Embodiment 56: The VEGF antagonist for use of any one of embodiments 44 to 55, wherein the VEGF antagonist is an anti-VEGF antibody or a fragment thereof.

Embodiment 57: The VEGF antagonist for use of embodiment 56, wherein the VEGF antibody comprises a variable heavy chain having the sequence as set forth in SEQ ID NO: 1 and a variable light chain having the sequence as set forth in SEQ ID NO: 2.

Embodiment 58: The VEGF antagonist for use of embodiment 56, wherein the VEGF antibody comprises the sequence of SEQ ID NO: 3 or SEQ ID NO: 4.

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Embodiment 59: The VEGF antagonist for use of embodiment 56, wherein the VEGF antagonist is brolucizumab.

Embodiment 60: The VEGF antagonist for use of any one of embodiments 44 to 59, wherein the VEGF antagonist is administered to the subject in three individual doses of at 4-week intervals, and thereafter is administered to the subject as an additional dose every 8 to 12 weeks.

Embodiment 61: The VEGF antagonist for use of embodiment 60, wherein the VEGF antagonist is administered to the subject in three individual doses of at 4-week intervals, and thereafter is administered to the subject as an additional dose every 12 weeks.

Embodiment 62: The VEGF antagonist for use of embodiment 61, wherein a 12 week treatment interval is switched to an 8 week treatment interval if a disease activity is detected in the subject.

Embodiment 63: The VEGF antagonist for use of embodiment 60, wherein the VEGF antagonist is administered to the subject in three individual doses of at 4-week intervals, and thereafter is administered to the subject as an additional dose every 8 weeks.

Embodiment 64: The VEGF antagonist for use of embodiment 63, wherein an 8 week treatment interval is switched to a 12 week treatment interval if no disease activity is detected in the subject.

Embodiment 65: The VEGF antagonist for use of any one of embodiments 51 to 58, wherein each dose of the VEGF antagonist is at least 3 mg, e.g., 3 mg or 6 mg, in particular 6 mg.

Embodiment 66: The VEGF antagonist for use of embodiment 65, wherein each dose of the VEGF antagonist is administered as a 50 µL intravitreal injection.

Embodiment 67: The VEGF antagonist for use of any one of embodiments 44 to 66, wherein the ocular disease is selected from the list consisting of abnormal angiogenesis, choroidal neovascularization (CNV), retinal vascular permeability, retinal edema, diabetic retinopathy

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(particularly proliferative diabetic retinopathy (PDR) and non-proliferative diabetic retinopathy (NPDR)), macular edema (ME), diabetic macular edema (DME), neovascular (exudative) age-related macular degeneration (nAMD), choroidal neovascularization (CNV) associated with nAMD, sequela associated with retinal ischemia, Retinal Vein Occlusion (RVO), Central Retinal Vein Occlusion (CRVO), Branch Retinal Vein Occlusion (BRVO), macular edema following retinal vein occlusion, and posterior segment neovascularization.

Embodiment 68: The VEGF antagonist for use of embodiment 67, wherein the ocular disease is neovascular age-related macular degeneration (nAMD).

Embodiment 69: A method for producing a transmittable form of information for determining whether a subject with ocular disease, e.g., neovascular age-related macular degeneration (nAMD), should be treated with a VEGF antagonist, the method comprising:
(a) determining that the subject should be treated with a VEGF antagonist, e.g., brolucizumab, on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; or
(b) determining that the subject should be treated with a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, to the subject on the basis of said subject having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and
(c) recording the result of the determining step on a tangible or intangible media form for use in transmission.

Embodiment 70: A method for producing a transmittable form of information for predicting whether a subject with ocular disease, e.g., neovascular age-related macular degeneration (nAMD), may develop intraocular inflammation (IOI), e.g., intraocular inflammation with retinal vasculitis/retinal occlusion (IOI+RV/RO), in response to a treatment with a VEGF antagonist, e.g., brolucizumab, the method comprising:
(a) determining an increased likelihood that the subject will develop IOI, e.g., IOI+RV/RO, in response to the treatment with the VEGF antagonist, e.g., brolucizumab, on the basis of said subject having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; or
(b) determining a decreased likelihood that the subject will develop IOI, e.g., IOI+RV/RO, in response to the treatment with the VEGF antagonist, e.g., brolucizumab, on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and

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(c) recording the result of the determining step on a tangible or intangible media form for use in transmission.

Specific preferred embodiments of the invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Preretinal hyperreflective material. (A) Baseline OCT, smooth inner retinal boundary. (B) Pre-IOI event OCT, new appearance of preretinal hyperreflective material (arrow).

Figure 2. Preretinal hyperreflective material. (A) Baseline OCT: smooth inner retinal boundary; no preretinal hyperreflective material or vitreous debris observed . (B) V3/Pre-IOI event OCT: early signs of preretinal hyperreflective material (foci) / vitreous debris. (C) V7/IOI event OCT: vitreous debris with preretinal hyperreflective material (foci). (D) V3/Pre-IOI event OCT: numerous preretinal hyperreflective material (foci).

Figure 3. Radiomics evaluation.

Figure 4. Box and Whisker Plot of the most discriminating baseline feature.

Figure 5. Machine learning quantitative characterization of the vitreous compartment identified differences in the number of hyperreflective foci between eyes in the IOI group and the control group, including prior to the IOI reported event.

Figure 6. Machine learning quantitative characterization of the vitreous compartment identified differences in the number of hyperreflective preretinal foci (preretinal deposits) between eyes in the IOI group and the control group, including prior to the IOI reported event.

Figure 7. Machine learning quantitative characterization of the vitreous compartment identified differences in the number of hyperreflective vitreous foci (vitreous debris) between eyes in the IOI group and the control group, including prior to the IOI reported event.

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DETAILED DESCRIPTION

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this present disclosure pertains. Any references cited herein, including, e.g., all patents, published patent applications, and non-patent publications, are incorporated by reference in their entirety. To facilitate understanding of the disclosure, several terms and abbreviations as used herein are defined below as follows:

As used herein, all percentages are percentages by weight, unless stated otherwise.

As used herein and unless otherwise indicated, the terms "a" and "an" are taken to mean "one", "at least one" or "one or more". Unless otherwise required by context, singular terms used herein shall include pluralities and plural terms shall include the singular.

As used herein, the term "about" includes and describes the value or parameter per se. For example, "about x" includes and describes "x" per se. As used herein, the term "about" when used in association with a measurement, or used to modify a value, a unit, a constant, or a range of values, refers to variations of ±1-10% in addition to including the value or parameter per se. In some embodiments, the term "about" when used in association with a measurement, or used to modify a value, a unit, a constant, or a range of values, refers to variations of ±1, ±2, ±3, ±4, ±5, ±6, ±7, ±8, ±9, or ±10%.

The term "VEGF" refers to the 165-amino acid vascular endothelial cell growth factor, and related 121-, 189-, and 206-amino acid vascular endothelial cell growth factors, as described by Leung et al., Science 246:1306 (1989), and Houck et al., Mol. Endocrin. 5:1806 (1991) together with the naturally occurring allelic and processed forms of those growth factors. The term "VEGF", in particular, refers to the human VEGF.

The term "VEGF receptor" or "VEGFr" refers to a cellular receptor for VEGF, ordinarily a cell-surface receptor found on vascular endothelial cells, as well as variants thereof retaining the ability to bind hVEGF. One example of a VEGF receptor is the fms-like tyrosine kinase (flt), a transmembrane receptor in the tyrosine kinase family. DeVries et al.,

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Science 255:989 (1992); Shibuya et al., Oncogene 5:519 (1990). The flt receptor comprises an extracellular domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The extracellular domain is involved in the binding of VEGF, whereas the intracellular domain is involved in signal transduction. Another example of a VEGF receptor is the flk-1 receptor (also referred to as KDR). Matthews et al., Proc. Nat. Acad. Sci. 88:9026 (1991); Terman et al., Oncogene 6:1677 (1991); Terman et al., Biochem. Biophys. Res. Commun. 187:1579 (1992). Binding of VEGF to the flt receptor results in the formation of at least two high molecular weight complexes, having an apparent molecular weight of 205,000 and 300,000 Daltons. The 300,000 Dalton complex is believed to be a dimer comprising two receptor molecules bound to a single molecule of VEGF.

As used herein, a "VEGF antagonist" refers to a compound that can diminish or inhibit VEGF activity *in vivo*. A VEGF antagonist can bind to a VEGF receptor(s) or block VEGF protein(s) from binding to VEGF receptor(s). A VEGF antagonist can be, for example, a small molecule, an anti-VEGF antibody or antigen-binding fragments thereof, fusion protein (such as aflibercept or other such soluble decoy receptor), an aptamer, an antisense nucleic acid molecule, an interfering RNA, receptor proteins, and the like that can bind specifically to one or more VEGF proteins or one or more VEGF receptors. Several VEGF antagonists are described in WO 2006/047325. In one embodiment, the VEGF antagonist is any licensed anti-VEGF drug such as brolucizumab, ranibizumab or aflibercept. In one embodiment, the VEGF antagonist is an anti-VEGF antibody (such as brolucizumab or ranibizumab or bevacizumab or a bi-specific antibody such as faricimab) or an anti-VEGF DARPin (such as abicipar) or a soluble VEGF receptor (e.g., a fusion protein composed of the VEGF receptor domains, such as a fusion protein composed of the combination between VEGF receptor domains with the Fc fragment of human immunoglobulin with the Fc fragment of human immunoglobulin, e.g., conbercept, aflibercept) or AAV containing a sequence encoding for an anti-VEGF antibody (such as RGX-314 from Regenxbio), or AAV containing a sequence encoding the VEGF receptor domains, e.g., conbercept (such as ADVM-022 from Adverum) or any licensed anti-VEGF drug (such as brolucizumab, ranibizumab or aflibercept).

The term "antibody" as used herein includes whole antibodies and any antigen binding fragment (*i.e.*, "antigen-binding portion", "antigen binding polypeptide", or

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"immunobinder") or single chain thereof. An "antibody" includes a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The structures and locations of antibody variable domains, e.g., CDRs, may be defined using well known numbering schemes, e.g., the Kabat numbering scheme, the Chothia numbering scheme, or a combination of Kabat and Chothia (see, e.g., Sequences of Proteins of Immunological Interest, U.S. Department of Health and Human Services (1991), eds. Kabat et al.; Al Lazikani et al., (1997) J. Mol. Bio. 273:927 948); Kabat et al., (1991) Sequences of Proteins of Immunological Interest, 5th edit., NIH Publication no. 91-3242 U.S. Department of Health and Human Services; Chothia et al., (1987) J. Mol. Biol. 196:901-917; Chothia et al., (1989) Nature 342:877-883; and Al-Lazikani et al., (1997) J. Mal. Biol. 273:927-948), and numbering scheme described in Honegger and Pluckthun, J. Mol. Biol. 309 (2001) 657-670 ("AHo" numbering). For example, for classic formats, under Kabat, the CDR amino acid residues in the heavy chain variable domain (VH) are numbered 31-35 (HCDR1, also referred to as CDRH1), 50-65 (HCDR2, also referred to as CDRH2), and 95-102 (HCDR3, also referred to as CDRH3); and the CDR amino acid residues in the light chain variable domain (VL) are numbered 24-34 (LCDRl, also referred to as CDRL1), 50-56 (LCDR2, also referred to as CDRL2), and 89-97 (LCDR3, also referred to as CDRL3). Under Chothia the CDR amino acids in the VH are numbered 26-32 (HCDR1, also referred to as CDRH1), 52-56 (HCDR2, also referred to as CDRH2), and 95-102 (HCDR3, also referred to as CDRH3); and the amino acid residues in VL are numbered 24-34 (LCDRl, also referred to as CDRL1), 50-56 (LCDR2, also referred to as CDRL2), and 89-97 (LCDR3, also referred to as CDRL3). By combining the CDR definitions of both Kabat and Chothia, the CDRs consist of amino acid residues 26-35 (HCDR1, also referred to as CDRH1), 50-65 (HCDR2, also referred to as CDRH2), and 95-

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102 (HCDR3, also referred to as CDRH3) in human VH and amino acid residues 24- 34 (LCDRl, also referred to as CDRL1), 50-56 (LCDR2, also referred to as CDRL2), and 89-97 (LCDR3, also referred to as CDRL3) in human VL. Under IMGT the CDR amino acid residues in the VH are numbered approximately 26-35 (HCDR1, also referred to as CDRH1), 51-57 (HCDR2, also referred to as CDRH2) and 93-102 (HCDR3, also referred to as CDRH3), and the CDR amino acid residues in the VL are numbered approximately 27-32 (LCDRl, also referred to as CDRL1), 50-52 (LCDR2, also referred to as CDRL2), and 89-97 (LCDR3, also referred to as CDRL3) (numbering according to "Kabat"). Under IMGT, the CDRs of an antibody can be determined using the program IMGT/DomainGap Align. In the context of the present invention, the numbering system suggested by Honegger and Pluckthun ("AHo) is used (Honegger and Pluckthun, J. Mol. Biol. 309 (2001) 657-670), unless specifically mentioned otherwise. Furthermore, the following residues are defined as CDRs according to AHo numbering scheme: LCDRl (also referred to as CDRL1): L24-L42; LCDR2 (also referred to as CDRL2): L58-L72; LCDR3 (also referred to as CDRL3): L107-L138; HCDR1 (also referred to as CDRH1): H27-H42; HCDR2 (also referred to as CDRH2): H57-H76; HCDR3 (also referred to as CDRH3): H108-H138. For the sake of clarity, the numbering system according to Honegger and Pluckthun takes the length diversity into account that is found in naturally occurring antibodies, both in the different VH and VL subfamilies and, in particular, in the CDRs, and provides for gaps in the sequences. Thus, in a given antibody variable domain usually not all positions 1 to 149 will be occupied by an amino acid residue. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system.

The term “single chain antibody”, “single chain Fv” or “scFv” is intended to refer to a molecule comprising an antibody heavy chain variable domain (or region; V_H) and an antibody light chain variable domain (or region; V_L) connected by a linker. Such scFv molecules can have the general structures: NH2-VL-linker-VH-COOH or NH2-VH-linker-VL-COOH.

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The term "antigen-binding portion" of an antibody (or simply "antibody portion" or "antibody fragment") refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., VEGF). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a single domain or dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR) or (vii) a combination of two or more isolated CDRs which may optionally be joined by a synthetic linker. Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 242:423-426; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies. Antigen-binding portions can be produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins. Antibodies can be of different isotype, for example, an IgG (e.g., an IgG1, IgG2, IgG3, or IgG4 subtype), IgA1, IgA2, IgD, IgE, or IgM antibody.

The term "homologous" or "identity" refers to the subunit sequence identity between two polymeric molecules, e.g., between two nucleic acid molecules, such as, two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit; e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous or identical at that position. The homology between two sequences is a direct function of the number of matching or homologous positions; e.g., if half (e.g., five positions in a polymer ten subunits in length) of the positions in two sequences are homologous, the two sequences

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are 50% homologous; if 90% of the positions (e.g., 9 of 10), are matched or homologous, the two sequences are 90% homologous. Percentage of "sequence identity" can be determined by comparing two optimally aligned sequences over a comparison window, where the fragment of the amino acid sequence in the comparison window may comprise additions or deletions (e.g., gaps or overhangs) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage can be calculated by determining the number of positions at which the identical amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity. The output is the percent identity of the subject sequence with respect to the query sequence.

As used herein, a "mammal" includes any animal classified as a mammal, including, but not limited to, humans, domestic animals, farm animals, and companion animals, etc.

As used herein, the term "subject" or "patient" refers to human and non-human mammals, including but, not limited to, primates, pigs, horses, dogs, cats, sheep, and cows. Preferably, a subject or patient is a human. In certain embodiments, a patient is at least 18 years of age. In certain embodiments, a patient is at least 50 years of age. In certain embodiments, a patient is at least 65 years of age.

The term "treat", "treating" or "treatment" includes therapeutic treatments, prophylactic treatments and applications in which one reduces the risk that a subject will develop a disorder or other risk factor. Treatment does not require the complete curing of a disorder and encompasses the reduction of the symptoms or underlying risk factors. As used herein, the terms "treat", "treatment" and "treating" refer to the reduction or amelioration of the progression or severity of an ocular disease or the amelioration of one or more symptoms, suitably of one or more discernible symptoms of an ocular disease. In specific embodiments, the terms "treat", "treatment" and "treating" refer to the amelioration of at least one measurable physical parameter of an ocular disease (such as achieve or at least partially achieve a desired effect (e.g. the partial or complete regression of retinal neovascularization, decrease of retinal fluid or achieving retinal fluid-free status, e.g., intraretinal fluid (IRF) and subretinal fluid (SRF), decrease of Central Subfield Thickness (CSFT), improvement in

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vision, e.g., a change of BCVA > 1, > 2, > 3, > 4 or > 5 letters, or a DRSS score < 61), wherein the physical parameter is not necessarily discernible by the patient.

A term "ocular disease" or "neovascular ocular disease", as used herein, refers to a condition, disease, or disorder associated with ocular neovascularization. An "ocular disease" or "neovascular ocular disease" that can be treated using a method of the disclosure includes, a condition, disease, or disorder associated with ocular neovascularization, including, but not limited to, abnormal angiogenesis, choroidal neovascularization (CNV), choroidal neovascularization (CNV) associated with nAMD, retinal vascular permeability, retinal edema, diabetic retinopathy (particularly proliferative diabetic retinopathy (PDR) and non-proliferative diabetic retinopathy (NPDR)), macular edema (ME), diabetic macular edema (DME), neovascular (exudative) age-related macular degeneration (nAMD), sequela associated with retinal ischemia, Retinal Vein Occlusion (RVO), Central Retinal Vein Occlusion (CRVO), Branch Retinal Vein Occlusion (BRVO), macular edema following retinal vein occlusion, and posterior segment neovascularization.

The term "loading phase" refers to the first 2, 3, 4, 5, or 6 doses of a VEGF antagonist administered at q4w or q6w intervals. Suitably, the term "loading phase" refers to the first 2 to 3 doses of a VEGF antagonist administered at q4w.

The term "maintenance phase" refers to additional doses at 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 week intervals, and can be adjusted as described herein based on Disease Activity Assessments as described herein. Suitably, the term "maintenance phase" refers to additional doses at 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 week intervals, and can be adjusted as described herein based on Disease Activity Assessments as described herein.

As used herein, an administration interval can be referred to as qXw, where the "X" is a number of weeks between administered doses. For example, q6w is an interval of 6 weeks.

As used herein, the term "week" means 7 days ± 1 day. As used herein, the term "month" means 25 to 31 days. Also, as used herein, the term "month" means 4 weeks.

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As used herein, the terms "effective amount" or "therapeutically effective amount" or "therapeutically effective dose" refer to an amount of a therapy (e.g., a VEGF antagonist, e.g., brolucizumab, or a pharmaceutical composition provided herein) which is sufficient to reduce and/or ameliorate the severity of a given condition, disorder, or disease and/or a symptom related thereto. The term "effective amount" or "therapeutically effective amount" or "therapeutically effective dose" is defined as an amount sufficient to achieve or at least partially achieve a desired effect (e.g. the partial or complete regression of retinal neovascularization, decrease of retinal fluid or achieving retinal fluid-free status, e.g., intraretinal fluid (IRF) and subretinal fluid (SRF), decrease of Central Subfield Thickness (CSFT), improvement in vision, e.g., a change of BCVA > 1, > 2, > 3, > 4 or > 5 letters, or a DRSS score < 61). A therapeutically effective dose is sufficient if it can produce even an incremental change in the symptoms or conditions associated with the disease. The therapeutically effective dose does not have to completely cure the disease or completely eliminate symptoms. Preferably, a therapeutically effective dose can at least partially arrest the disease and/or its complications in a patient already suffering from the disease. In specific embodiments, a therapeutic effective dose may involve repeated administration over a period of time. Amounts effective for this use will depend upon the severity of the disorder being treated and the general state of the patient's own immune system.

As used herein, the term "adverse event" refers to any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product, whether or not related to the medicinal (investigational) product. In particular, adverse events in ocular disease, e.g., nAMD, patients may include blurred visiona, cataract, conjunctival hemmorage, eye pain, vitreous floaters, intraocular inflammation, e.g., e.g., intraocular inflammation with retinal vasculitis/retinal occlusion (IOI+RV/RO). Specifically, the term refers to the effect that leads to intraocular inflammation, e.g., intraocular inflammation with retinal vasculitis and/or retinal occlusion.

As used herein, the term "intraocular inflammation" encompasses anterior chamber cell, anterior chamber flare, anterior chamber inflammation, chorioretinitis, eye inflammation, iridocyclitis, iritis, uveitis, vitreous haze, vitritis. As used herein, the term "hyperreflective deposit" or "HRD" or"hyperreflective material" or "hyperreflective foci" or "hyperreflective foci formation" refers to a region of increased reflectivity on the

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tomographic scan, e.g., on spectral domain optical coherence tomography (SD-OCT). Hyperreflective material (hyperreflective deposit) may be classified according to its morphological appearance and location; hyperreflective material (hyperreflective deposit) with high reflectivity whose boundaries are clearly delineated from the surrounding neural components of the retina is classified as well-defined; hyperreflective material (hyperreflective deposit) with low reflectivity and whose borders are less well defined and therefore not easily distinguishable from surrounding neural components is classified as undefined (Casalino et al., Expert Review of Ophthalmology, 2020, Volume 15, No. 2, pp. 83-91). In one embodiment, a "hyperreflective deposit" or "HRD" or "hyperreflective material" or "hyperreflective foci formation" is a well-defined (with high reflectivity) hyperreflective material. Morphological appearance of hyperreflective material (hyperreflective deposit) includes a heterogeneity observed on the OCT scan including hyperreflective debris, hyperreflective round- or rod-shaped dots, e.g., stalagmite-like deposit or material. As used herein, the term "stalagmite-like hyperreflective deposit" or "stalagmite-like hyperreflective material" or "stalagmite-like hyperreflective foci formation" refers to hyperreflective material that resemble the elevated speleothems, in particular as determined on spectral domain optical coherence tomography (SD-OCT). According to the present disclosure, the HRD, including debris, round- or rod-shaped dots and stalagmite-like HRD, is observed in vitreous compartment - a vitreous hyperreflective deposit (VHRD). In particular, according to the present disclosure, the HRD, including stalagmite-like hyperreflective material, is observed in preretinal compartment - a preretinal hyperreflective deposit (PHRD), e.g., internal limiting membrane (ILM) hyperreflective deposit (ILMHRD).

As used herein, "likelihood" and "likely" is a measurement of how probable an event is to occur. It may be used interchangeably with "probability". Likelihood refers to a probability that is more than speculation, but less than certainty. Thus, an event is likely if a reasonable person using common sense, training or experience concludes that, given the circumstances, an event is probable. In some embodiments, once likelihood has been ascertained, the patient may be treated (or treatment continued) with the compound or the treatment may need to be altered or discontinued. In one embodiment, the "likelihood" and "likely" denote a chance in percent of how probable an event is to occur.

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The phrase "increased likelihood" refers to an increase in the probability that an event will occur. For example, some methods herein allow prediction of whether a patient will display an increased likelihood of developing intraocular inflammation, e.g., intraocular inflammation with retinal vasculitis and/or retinal occlusion, in response to the administration of a VEGF antagonist, e.g., brolucizumab . In one embodiment the increased likelihood means that there is more than 50% chance, more than 60 % chance, more than 70 % or more than 80 % chance that an event will occur. Equally, a "decreased likelihood" means, that the chance is lower than 50%, lower than 60 %, lower than 70 % or lower than 80 %, respectively, that an event will occur.

As used herein, "predicting" indicates that the methods described herein provide information to enable a health care provider to determine the likelihood that an individual subjected to a treatment will develop with a higher likelihood intraocular inflammation, e.g., intraocular inflammation with retinal vasculitis and/or retinal occlusion. It does not refer to the ability to predict response with 100% accuracy. Instead, the skilled artisan will understand that it refers to an increased probability.

Methods of treatment

In one aspect, the disclosure provides a method of treating ocular disease, e.g., neovascular age-related macular degeneration (nAMD), comprising selectively administering a VEGF antagonist, e.g., brolucizumab, to a subject in need thereof on the basis of said subject not having or having a low level of a hyperreflective deposit (HRD) in a subject's eye, e.g., a vitreous hyperreflective deposit (VHRD), e.g., a preretinal hyperreflective deposit (PHRD), e.g., internal limiting membrane (ILM) hyperreflective deposit (ILMHRD). In a specific embodiment, the disclosure provides a method of treating ocular disease, e.g., neovascular age-related macular degeneration (nAMD), comprising selectively administering a VEGF antagonist, e.g., brolucizumab, to a subject in need thereof on the basis of said subject not having or having a low level of a preretinal hyperreflective deposit (PHRD), e.g., internal limiting membrane (ILM) hyperreflective deposit (ILMHRD).

In one aspect, the disclosure provides a method of treating ocular disease, e.g., nAMD, comprising: (a) selecting a subject for treatment with a VEGF antagonist, e.g., brolucizumab, on the basis said subject not having a HRD in a subject's eye, e.g., VHRD,

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e.g., PHRD, or having a low level of a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and (b) thereafter, selectively administering the VEGF antagonist, e.g., brolucizumab, to the subject. In a specific embodiment, the disclosure provides a method of treating ocular disease, e.g., nAMD, comprising: (a) selecting a subject for treatment with a VEGF antagonist, e.g., brolucizumab, on the basis said subject not having a PHRD or having a low level of a PHRD; and (b) thereafter, selectively administering the VEGF antagonist, e.g., brolucizumab, to the subject.

In a another aspect, the disclosure n provides a method of selectively treating a subject having ocular disease, e.g., nAMD, the method comprising: (a) selectively administering a therapeutically effective amount of a VEGF antagonist, e.g., brolucizumab, to the subject on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD, or having a low level of a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; or (b) selectively administering a therapeutically effective amount of a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, to the subject on the basis of said subject having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD. In a specific embodiment, the disclosure n provides a method of selectively treating a subject having ocular disease, e.g., nAMD, the method comprising: (a) selectively administering a therapeutically effective amount of a VEGF antagonist, e.g., brolucizumab, to the subject on the basis of said subject not having a PHRD or having a low level of a PHRD; or (b) selectively administering a therapeutically effective amount of a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, to the subject on the basis of said subject having a PHRD.

In one embodiment, the method further comprises assessing the presence or absence of HRD in a subject's eye, e.g., VHRD, e.g., PHRD. Suitably, the assessment for the presence or absence of the HRD in a subject's eye, e.g., VHRD, e.g., PHRD, is performed prior to the steps (a)-(b). Suitably, the HRD in a subject's eye, e.g., VHRD, e.g., PHRD, e.g., presence or absence or level, is assessed by performing optical coherence tomography, e.g., spectral-domain optical coherence tomography, SD-OCT, analysis on a subject's eye.

In a another aspect, the disclosure provides a method for determining whether a subject with ocular disease, e.g., nAMD, should be treated with a VEGF antagonist, e.g.,

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brolucizumab, the method comprising:
(a) performing analysis, e.g., OCT, SD-OCT analysis, on a subject's eye to assess the presence or absence or level of a HRD in a subject's eye, e.g., VHRD, preferably PHRD; and
(b) determining that the subject should be treated with a VEGF antagonist, e.g., brolucizumab, if no HRD in a subject's eye, e.g., VHRD, preferably PHRD, or a low level of a HRD in a subject's eye, e.g., VHRD, preferably PHRD, is observed in the subject's eye;
(c) determining that the subject should not be treated with a VEGF antagonist, e.g., brolucizumab, if a HRD in a subject's eye, e.g., VHRD, preferably PHRD, or a high level of a HRD in a subject's eye, e.g., VHRD, preferably PHRD, is observed in the subject's eye.

In a another aspect, the present invention provides a method of predicting the likelihood that a subject having ocular disease, e.g., nAMD, will develop intraocular inflammation (IOI), e.g., intraocular inflammation with retinal vasculitis and / or retinal occlusion (IOI+RV/RO), in response to a treatment with a VEGF antagonist, e.g., brolucizumab, the method comprising determining if the subject has a HRD in a subject's eye, e.g., VHRD, preferably PHRD, wherein: (a) the presence of a HRD in a subject's eye, e.g., VHRD, preferably PHRD, or the presence of a high level of a HRD in a subject's eye, e.g., VHRD, preferably PHRD, is indicative of an increased likelihood that the patient will develop IOI, e.g., IOI+RV/RO, in response to the treatment with the VEGF antagonist, e.g., brolucizumab; and| (b) the absence of a HRD in a subject's eye, e.g., VHRD, preferably PHRD, or the presence of a low level of a HRD in a subject's eye, e.g., VHRD, preferably PHRD, is indicative of a decreased likelihood that the patient will develop IOI, e.g., IOI+RV/RO, in response to the treatment with the VEGF antagonist, e.g., brolucizumab. In one embodiment, the method further comprises treating the subject with the VEGF antagonist if no HRD in a subject's eye, e.g., VHRD, preferably PHRD, or a low level of a HRD in a subject's eye, e.g., VHRD, preferably PHRD, is observed in the subject's eye. In a specific embodiment, the method comprises determining by OCT or SD-OCT if the subject has a HRD in a subject's eye, e.g., VHRD, preferably PHRD.

Suitably, according to the present disclosure, the subject may be assessed for presence of the HRD in a subject's eye, e.g., VHRD, e.g., PHRD, prior to initiation of the treatment with the VEGF antagonist and / or during the treatment course with the VEGF antagonist. In one embodiment, the subject is assessed for presence of the HRD in a subject's eye, e.g.,

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VHRD, e.g., PHRD, during the treatment course with the VEGF antagonist. In one embodiment, the subject is assessed for presence of the HRD in a subject's eye, e.g., VHRD, e.g., PHRD, during the treatment course with the VEGF antagonist and prior to an administration of the VEGF antagonist, e.g., on the same day and prior to the administration or 1, 2, 3, 4, 5, 6, or 7 days prior to the administration of the VEGF antagonist or 1, 2, 3, 4, 5, 6, or 7 weeks prior the administration of the VEGF antagonist. In one embodiment, the subject is assessed for presence of the HRD in a subject's eye, e.g., VHRD, e.g., PHRD, during the treatment course with the VEGF antagonist and after an administration of the VEGF antagonist, e.g., on the same day and after the administration or on the 1st, 2nd, 3rd, 4th, 5th, 6th, or 7th day after the administration of the VEGF antagonist or 1, 2, 3, 4, 5, 6, or 7 weeks after the administration of the VEGF antagonist. In one embodiment, the subject is assessed for presence of the HRD in a subject's eye, e.g., VHRD, e.g., PHRD, during the treatment course with the VEGF antagonist and one month after the initial dose of the VEGF antagonist was administered to the subject.

According to the present disclosure, a HRD in a subject's eye, e.g., VHRD, e.g., PHRD, may be used as a biomarker for the selection of the patients suitable for treatment with a VEGF inhibitor, e.g., brolucizumab, or for monitoring a patient during the course of treatment with a VEGF inhibitor, e.g., brolucizumab, and adjusting, if necessary, the treatment (e.g., treatment regimen (frequency and /or amount), choice of medicament). Thus, in one embodiment, the method of the present disclosure further comprises: (a) altering or discontinuing the treatment with the VEGF antagonist, e.g., brolucizumab, if, during the course of treatment, the subject is determined to have a HRD, e.g., VHRD, e.g., PHRD, or a high level of a HRD, e.g., VHRD, e.g., PHRD, in the treatment eye, or (b) maintaining the treatment with the VEGF antagonist, e.g., brolucizumab, if, during the course of treatment, the subject is determined not to have a HRD, e.g., VHRD, e.g., PHRD, or a low level of a HRD, e.g., VHRD, e.g., PHRD, in the treatment eye.

In one embodiment, the HRD, e.g., VHRD, e.g., PHRD, suitable for the methods of present invention has enough reflectivity to be determined as heterogeneity in comparison to the baseline level or in comparison to the surrounding components of the retina. In one embodiment, the HRD, e.g., VHRD, e.g., PHRD, suitable for the methods of present invention has high reflectivity, e.g., has clearly delineated boundaries from the surrounding

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components of the retina. In one embodiment, the HRD, e.g., VHRD, e.g., PHRD, suitable for the methods of present invention is as a hyperreflective debris or a hyperreflective round- or rod-shaped dot. In a specific embodiment, the HRD, e.g., VHRD, e.g., PHRD, suitable for the methods of present invention is stalagmite-like hyperreflective material, e.g., stalagmite-like preretinal hyperreflective material. In a specific embodiment, the HRD is PHRD having high reflectivity and is round- or rod-shaped dot, e.g., stalagmite-like. In a more specific embodiment, the PHRD has high reflectivity and is stalagmite-like.

Suitably, the methods of the present disclosure are suitable for treating ocular disease or for predicting the likelihood that a subject having ocular disease will develop intraocular inflammation (IOI), wherein the ocular disease is selected from the list consisting of abnormal angiogenesis, choroidal neovascularization (CNV), retinal vascular permeability, retinal edema, diabetic retinopathy (particularly proliferative diabetic retinopathy (PDR) and non-proliferative diabetic retinopathy (NPDR)), macular edema (ME), diabetic macular edema (DME), neovascular (exudative) age-related macular degeneration (nAMD), choroidal neovascularization (CNV) associated with nAMD, sequela associated with retinal ischemia, Retinal Vein Occlusion (RVO), Central Retinal Vein Occlusion (CRVO), Branch Retinal Vein Occlusion (BRVO), macular edema following retinal vein occlusion, and posterior segment neovascularization.

In a specific embodiment, ocular disease is nAMD. Age-related macular degeneration (AMD) is the leading cause of severe vision loss in people affecting 10%-13% of individuals over the age of 65 in North America, Europe, and Australia (Kawasaki 2010, Rein et al., Arch Ophthalmol. 2009;127:533-40, Smith 2001). Genetic, environmental and health factors play an important role in the pathogenesis of the disease. AMD is classified into 2 clinical subtypes: the non-neovascular (atrophic) or dry form and the neovascular (exudative) or wet form (Ferris et al., Arch Ophthalmol. 1984;102:1640-2, Lim et al., Lancet. 2012;379:1728-38, Miller et al., Am J Ophthalmol. 2013;155:1-35). VEGF has been shown to be elevated in patients with nAMD and is thought to play a key role in the neovascularization process (Spilsbury et al., Am J Pathol. 2000;157:135-44). The use of intravitreal (IVT) pharmacotherapy targeting VEGF has significantly improved visual outcomes in patients with nAMD (Bloch et al., Am J Ophthalmol. 2012;153:209-13, Campbell et al., Arch Ophthalmol. 2012;130:794-5). Anti-VEGF treatments, such as ranibizumab (LUCENTIS®),

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aflibercept (EYLEA®), and brolucizumab (Beovu®), inhibit VEGF signaling pathways and have been shown to halt the growth of neovascular lesions and resolve retinal edema.

Diabetes mellitus (DM) is the most common endocrine disease in developed countries, with prevalence estimates ranging between 2 to 5% of the world population. Diabetic retinopathy (DR) and diabetic macular edema (DME) are common microvascular complications in patients with diabetes and may have a debilitating impact on visual acuity (VA), eventually leading to blindness.

In one embodiment, ocular disease is DME. DME is a frequent manifestation of DR (Riordan-Eva, 2004, Eye (Lond). 2004, 18:1161-8) and is the major cause of visual loss in patients with DR. For anti-VEGF agents like ranibizumab or aflibercept a favorable benefit risk ratio was demonstrated with superior efficacy versus the previous standard of care (laser photocoagulation) in large Phase 3 programs that consequently led to their approval for the treatment of DME. Anti-VEGF treatment led to clinically relevant improvements of BCVA, reduction of fluid accumulation and decreased severity of diabetic retinopathy.

In one embodiment, ocular disease is DR. DR is the most common cause of vision loss among people with diabetes and the leading cause of vision impairment and blindness in working aged adults. DR occurs when high blood glucose levels cause damage to blood vessels in the retina. These blood vessels can swell and leak or they can close, stopping blood from passing through. Sometimes abnormal new blood vessels can also grow on the retina. Diabetic retinopathy includes both non-proliferative diabetic retinopathy (nPDR) and proliferative diabetic retinopathy (PDR), the more advanced form of the disease. DME is a frequent manifestation of DR (Riordan-Eva, 2004, Eye (Lond). 2004, 18:1161-8) and is the major cause of visual loss in patients with DR. While diabetic macular edema (DME) may occur at any stage of DR, it is more likely to manifest following severe nPDR and PDR. Currently, care providers actively monitor mild to moderate nPDR and reserve treatment for severe nPDR and PDR. Both LUCENTIS® and EYLEA® have recently gained approval for DR in the United States. For treating DR, the recommended dose of EYLEA® is 2 mg (0.05 mL) administered every 4 weeks for 5 injections followed by an injection once every 8 weeks. For treating DR, the recommended dose of LUCENTIS® is 0.3 mg (0.05 mL) administered once a month.

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In one embodiment, ocular disease is Retinal Vein Occlusion (RVO), e.g., Central Retinal Vein Occlusion (CRVO), Branch Retinal Vein Occlusion (BRVO). Retinal vein occlusion is a blockage of the small veins that carry blood away from the retina. The retina is the layer of tissue at the back of the inner eye that converts light images to nerve signals and sends them to the brain. Retinal vein occlusion is most often caused by hardening of the arteries (atherosclerosis) and the formation of a blood clot. Both CRVO and BRVO are both related to occlusion of the retinal vein, however the cause of the occlusion differs based on location. Central Retinal Vein Occlusion (CRVO) occurs with a thrombus occludes the central retinal vein near the lamina cribosa (Green et al., Retina. 1:27-55, 1981). Branch Retinal Vein Occlusion (BRVO) occurs when a thrombus occurs at the arteriovenous crossing point secondary to atherosclerosis of the retinal artery causing compression and occlusion of the retinal vein. (Frangieh et al., Archives of Ophthalmology. 100:1132-40, 1982). Blockage of retinal veins may cause other eye problems, including: (i) glaucoma (high pressure in the eye), caused by new, abnormal blood vessels growing in the front part of the eye; (ii) macular edema, caused by the leakage of fluid in the retina. VEGF has a leading role in RVO pathogenesis and symptoms. Use of anti-VEGF agents by intravitreal injections has become common with the aim to improve the clinical outcomes in these patients. Currently 2 anti-VEGF agents (ranimizumab and aflibercept) have been FDA (Food and Drug Administration) and EMA (European Medicine Agency) approved for the treatment of RVO, while another VEGF inhibitor (bevacizumab) is often used "off-label" in clinical practice.

In a another aspect, the present invention provides a method for producing a transmittable form of information for determining whether a subject with ocular disease, e.g., nAMD, should be treated with a VEGF antagonist, e.g., brolucizumab, the method comprising:

(a) determining that the subject should be treated with a VEGF antagonist, e.g., brolucizumab, on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD, or having a low level of a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; or

(b) determining that the subject should be treated with a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, to the subject on the basis of said subject having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD, or having a high level of a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and

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(c) recording the result of the determining step on a tangible or intangible media form for use in transmission.

In a another aspect, the present invention provides a method for producing a transmittable form of information for predicting whether a subject with ocular disease, e.g., nAMD, may develop intraocular inflammation (IOI), e.g., intraocular inflammation with retinal vasculitis and/ or retinal occlusion (IOI+RV/RO), in response to a treatment with a VEGF antagonist, e.g., brolucizumab, the method comprising:
(a) determining an increased likelihood that the subject will develop IOI, e.g., IOI+RV/RO, in response to the treatment with the VEGF antagonist, e.g., brolucizumab, on the basis of said subject having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD, or having a high level of a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; or
(b) determining a decreased likelihood that the subject will develop IOI, e.g., IOI+RV/RO, in response to the treatment with the VEGF antagonist, e.g., brolucizumab, on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD, or having a low level of a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and
(c) recording the result of the determining step on a tangible or intangible media form for use in transmission.

Anti-VEGF Antagonists

The present disclosure provides methods and uses for treating ocular disease, e.g., nAMD, the method or the use comprising selectively administering a VEGF antagonist, e.g., brolucizumab, to a subject in need thereof on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD.

In one embodiment, the VEGF antagonist of the disclosure is any licensed anti-VEGF drug such as brolucizumab, ranibizumab or aflibercept. In one embodiment, the VEGF antagonist of the disclosure is an anti-VEGF antibody (such as brolucizumab or ranibizumab or bevacizumab or a bi-specific antibody such as faricimab) or an anti-VEGF DARPin (such as abicipar) or a soluble VEGF receptor (e.g., a fusion protein composed of the VEGF receptor domains, such as a fusion protein composed of the combination between VEGF receptor domains with the Fc fragment of human immunoglobulin with the Fc fragment of human immunoglobulin, e.g., conbercept, aflibercept) or AAV containing a sequence

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encoding for an anti-VEGF antibody (such as RGX-314 from Regenxbio), or AAV containing a sequence encoding the VEGF receptor domains, e.g., conbercept (such as ADVM-022 from Adverum) or any licensed anti-VEGF drug (such as brolucizumab, ranibizumab or aflibercept). In a preferred embodiment, the VEGF antagonist of the disclosure is brolucizumab or ranibizumab or aflibercept. In a more preferred embodiment, the VEGF antagonist of the disclosure is brolucizumab.

In certain embodiments, the VEGF antagonist of the disclosure is an anti-VEGF antibody, e.g., a single chain antibody (scFv) or Fab fragment.

In certain embodiments, the VEGF antagonist of the disclosure is an anti-VEGF antibody, particularly anti-VEGF antibodies described in WO 2009/155724, the entire contents of which are hereby incorporated by reference.

In one embodiment, the VEGF antagonist of the disclosure is an anti-VEGF antibody comprising a variable heavy chain having the sequence as set forth in SEQ ID NO: 1 and a variable light chain having the sequence as set forth in SEQ ID NO: 2.

VH: SEQ ID NO. 1

EVQLVESGGGLVQPGGSLRLSCTASGFSLTDYYYMTWVRQAPGKGLEWVGFIDPDDDPYYATWAKGRFT
ISRDNSKNTLYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTVSS

VL: SEQ ID NO. 2

EIVMTQSPSTLSASVGDRVIITCQASEIIHSWLAWYQQKPGKAPKLLIYLASTLASGVPSRFSGSGSGA
EFTLTISSLQPDDFATYYCQNVYLASTNGANFGQGTKLTVLG

In another embodiment, the VEGF antagonist of the disclosure is an anti-VEGF antibody comprising the sequence as set forth in SEQ ID NO: 3.

EIVMTQSPSTLSASVGDRVIITCQASEIIHSWLAWYQQKPGKAPKLLIYLASTLASGVPSRFSGSGSGA
EFTLTISSLQPDDFATYYCQNVYLASTNGANFGQGTKLTVLGGGGGSGGGGSGGGGSGGGGSEVQLVES
GGGLVQPGGSLRLSCTASGFSLTDYYYMTWVRQAPGKGLEWVGFIDPDDDPYYATWAKGRFTISRDNSK
NTLYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTVSS

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In a preferred embodiment, the VEGF antagonist of the disclosure is brolucizumab (which comprises the sequence of SEQ ID NO: 3). The sequence of brolucizumab is set forth in SEQ ID NO: 4. A methionine derived from the start codon in an expression vector is present in the final protein in cases where it has not been cleaved posttranslationally as follows.

MEIVMTQSPS TLSASVGDRV IITCQASEII HSWLAWYQQK PGKAPKLLIY LASTLASGVP
SRFSGSGSGA EFTLTISSLQ PDDFATYYCQ NVYLASTNGA NFGQGTKLTV LGGGGGSGGG
GSGGGGSGGG GSEVQLVESG GGLVQPGGSL RLSCTASGFS LTDYYYMTWV RQAPGKGLEW
VGFIDPDDDP YYATWAKGRF TISRDNSKNT LYLQMNSLRA EDTAVYYCAG GDHNSGWGLD
IWGQGTLVTV SS (SEQ ID NO: 4)

In another embodiment, the VEGF antagonist of the disclosure is an anti-VEGF antibody comprising three light chain CDRs (CDRL1, CDRL2, and CDRL3) and three heavy chain CDRs (CDRH1, a CDRH2, a CDRH3) as follows:

CDRL1	QASEIIHSWLA	SEQ ID NO: 5
CDRL2	LASTLAS	SEQ ID NO: 6
CDRL3	QNVYLASTNGAN	SEQ ID NO: 7
CDRH1	GFSLTDYYYMT	SEQ ID NO: 8
CDRH2	FIDPDDDPYYATWAKG	SEQ ID NO: 9
CDRH3	GDHNSGWGLDI	SEQ ID NO: 10

In another embodiment, the VEGF antagonist of the disclosure is an anti-VEGF antibody comprising a heavy chain complementarity determining region 1 (CDRH1) comprising or consisting of the amino acid sequence of SEQ ID NO: 8; a heavy chain complementarity determining region 2 (CDRH2) comprising or consisting of the amino acid sequence of SEQ ID NO: 9; a heavy chain complementarity determining region 3 (CDRH3) comprising or consisting of the amino acid sequence of SEQ ID NO: 10; a light chain complementarity determining region 1 (CDRL1) comprising or consisting of the amino acid sequence of SEQ ID NO: 5; a light chain complementarity determining region 2 (CDRL2) comprising or consisting of the amino acid sequence of SEQ ID NO: 6; and a light chain complementarity determining region 3 (CDRL3) comprising or consisting of the amino acid sequence of SEQ ID NO: 7. In one embodiment, the CDRH1, CDRH2, CDRH3, CDRL1, CDRL2 and CDRL3 are defined according to the AHo numbering scheme.

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In another embodiment, the VEGF antagonist of the disclosure is an anti-VEGF antibody comprising:

(i) a variable heavy chain comprising or having the sequence having at least 80% identity, e.g., 85%, 90%, 95%, to SEQ ID NO: 1, and wherein the variable heavy chain comprises CDRH1 comprising or consisting of the amino acid sequence of SEQ ID NO: 8, CDRH2 comprising or consisting of the amino acid sequence of SEQ ID NO: 9, CDRH3 comprising or consisting of the amino acid sequence of SEQ ID NO: 10,

and

(ii) a variable light chain comprising or having the sequence having at least 80% identity, e.g., 85%, 90%, 95%, to SEQ ID NO: 2, and wherein the variable light chain comprises CDRL1 comprising or consisting of the amino acid sequence of SEQ ID NO: 5, CDRL2 comprising or consisting of the amino acid sequence of SEQ ID NO: 6, and CDRL3 comprising or consisting of the amino acid sequence of SEQ ID NO: 7. In one embodiment, the CDRH1, CDRH2, CDRH3, CDRL1, CDRL2 and CDRL3 are defined according to the AHo numbering scheme.

In another embodiment, the VEGF antagonist of the disclosure is an anti-VEGF antibody comprising or having a sequence having at least 80% identity, e.g., 85%, 90%, 95%, to SEQ ID NO: 3 or SEQ ID NO: 3, and wherein the anti-VEGF antibody comprises CDRH1 comprising or consisting of the amino acid sequence of SEQ ID NO: 8; CDRH2 comprising or consisting of the amino acid sequence of SEQ ID NO: 9; CDRH3 comprising or consisting of the amino acid sequence of SEQ ID NO: 10; CDRL1 comprising or consisting of the amino acid sequence of SEQ ID NO: 5; CDRL2 comprising or consisting of the amino acid sequence of SEQ ID NO: 6; and CDRL3 comprising or consisting of the amino acid sequence of SEQ ID NO: 7. In one embodiment, the CDRH1, CDRH2, CDRH3, CDRL1, CDRL2 and CDRL3 are defined according to the AHo numbering scheme.

Brolucizumab, is a humanized single-chain Fv (scFv) antibody fragment inhibitor of VEGF with a molecular weight of ~26 kDa. It is an inhibitor of VEGF-A and works by binding to the receptor binding site of the VEGF-A molecule, thereby preventing the interaction of VEGF-A with its receptors VEGFR1 and VEGFR2 on the surface of endothelial cells. Increased levels of signaling through the VEGF pathway are associated with pathologic ocular angiogenesis and retinal edema. Inhibition of the VEGF pathway has

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been shown to inhibit the growth of neovascular lesions and resolve retinal edema in patients with nAMD.

In one embodiment, the disclosure provides a method of selectively treating a subject having ocular disease, e.g., nAMD, the method comprising: (a) selectively administering a therapeutically effective amount of a VEGF antagonist, e.g., brolucizumab, to the subject on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; or (b) selectively administering a therapeutically effective amount of a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, to the subject on the basis of said subject having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD.

In one embodiment, said different ocular disease agent is another VEGF antagonist, such as any licensed anti-VEGF drug, e.g., ranibizumab or aflibercept. In one embodiment, said different ocular disease agent is an anti-VEGF antibody (such as ranibizumab or bevacizumab or a bi-specific antibody such as faricimab) or an anti-VEGF DARPin (such as abicipar) or a soluble VEGF receptor (e.g., a fusion protein composed of the VEGF receptor domains, such as a fusion protein composed of the combination between VEGF receptor domains with the Fc fragment of human immunoglobulin with the Fc fragment of human immunoglobulin, e.g., conbercept, aflibercept) or AAV containing a sequence encoding for an anti-VEGF antibody (such as RGX-314 from Regenxbio), or AAV containing a sequence encoding the VEGF receptor domains, e.g., conbercept (such as ADVM-022 from Adverum) or any licensed anti-VEGF drug (such as ranibizumab or aflibercept). In a preferred embodiment, said different ocular disease agent is ranibizumab or aflibercept or faricimab. In a further embodiment, said different ocular disease agent is not a VEGF antagonist.

In certain embodiments, the VEGF antagonist of the disclosure is brolucizumab and is administered by an injection, e.g., an intravitreal injection.

In some embodiments, the VEGF antagonist of the disclosure is brolucizumab and is administered at a dose of about 1, about 2, about 3, about 4, about 5, or about 6 mg (e.g., about 6 mg/0.05 mL) as an intravitreal injection. In certain embodiments, the VEGF antagonist of the disclosure is brolucizumab and is administered at a dose of 1, 2, 3, 4, 5, or 6 mg (e.g., 6 mg/0.05 mL) as an intravitreal injection.

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In some embodiments, the VEGF antagonist of the disclosure is aflibercept and is administered at a dose of about 0.5, about 1 or about 2 mg (e.g., about 2 mg/0.05 mL) as an intravitreal injection. In certain embodiments, the VEGF antagonist of the disclosure is aflibercept and is administered at a dose of 0.5, 1 or 2 mg (e.g., 2 mg/0.05 mL) as an intravitreal injection. In some embodiments, the VEGF antagonist of the disclosure is aflibercept and is administered at a dose of about 0.2, about 0.3, about 0.4 or about 0.5 mg (e.g., about 0.5 mg/0.05 mL) as an intravitreal injection. In certain embodiments, the VEGF antagonist of the disclosure is aflibercept and is administered at a dose of 0.2, 0.3, 0.4 or 0.5 mg (e.g., 0.5 mg/0.05 mL) as an intravitreal injection.

Pharmaceutical Preparations

In one aspect, the methods or uses of the disclosure comprise the use of pharmaceutical formulations or pharmaceutical compositions comprising a VEGF antagonist, e.g., an anti-VEGF antibody. The term "pharmaceutical formulation" or "pharmaceutical composition" refers to preparations which are in such form as to permit the biological activity of the antagonist, e.g., antibody or antibody derivative, to be unequivocally effective, and which contain no additional components which are toxic to the subjects to which the formulation or composition would be administered. "Pharmaceutically acceptable" excipients (vehicles, additives) are those which can reasonably be administered to a subject mammal to provide an effective dose of the active ingredient employed.

A "stable" formulation is one in which a therapeutic agent, e.g. a VEGF antagonist, e.g., an anti-VEGF antibody or antibody derivative thereof, essentially retains its physical stability and/or chemical stability and/or biological activity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in Peptide and Protein Drug Delivery, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, N.Y., Pubs. (1991) and Jones, A. Adv. Drug Delivery Rev. 10: 29-90 (1993), for example. Stability can be measured at a selected temperature for a selected time period. Preferably, the formulation is stable at room temperature (about 30° C) or at 40° C for at least 1 week and/or stable at about 2-8° C for at least 3 months to 2 years. Furthermore, the formulation is preferably stable following freezing (to, *e.g.*, -70° C) and thawing of the formulation.

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An antagonist, e.g., an antibody or antibody derivative, "retains its physical stability" in a pharmaceutical formulation if it meets the defined release specifications for aggregation, degradation, precipitation and/or denaturation upon visual examination of color and/or clarity, or as measured by UV light scattering or by size exclusion chromatography, or other suitable art recognized methods.

An antagonist, e.g., an antibody or antibody derivative, "retains its chemical stability" in a pharmaceutical formulation, if the chemical stability at a given time is such that the compound, e.g., protein, is considered to still retain its biological activity as defined below. Chemical stability can be assessed by detecting and quantifying chemically altered forms of the protein. Chemical alteration may involve size modification (*e.g.* clipping) which can be evaluated using size exclusion chromatography, SDS-PAGE and/or matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI/TOF MS), for example. Other types of chemical alteration include charge alteration (*e.g.* occurring as a result of deamidation) which can be evaluated by ion-exchange chromatography, for example.

An antagonist, e.g., an antibody or antibody derivative, "retains its biological activity" in a pharmaceutical formulation, if the biological activity of the antibody at a given time is within about 10% (within the errors of the assay) of the biological activity exhibited at the time the pharmaceutical formulation was prepared as determined in an antigen binding assay, for example. Other "biological activity" assays for antibodies are elaborated herein below.

By "isotonic" is meant that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350 mOsm. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example.

A "polyol" is a substance with multiple hydroxyl groups, and includes sugars (reducing and non-reducing sugars), sugar alcohols and sugar acids. Preferred polyols herein have a molecular weight which is less than about 600 kD (*e.g.* in the range from about 120 to about 400 kD). A "reducing sugar" is one which contains a hemiacetal group that can reduce metal ions or react covalently with lysine and other amino groups in proteins and a "non-reducing sugar" is one which does not have these properties of a reducing sugar. Examples of reducing sugars are fructose, mannose, maltose, lactose, arabinose, xylose, ribose, rhamnose,

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galactose and glucose. Non-reducing sugars include sucrose, trehalose, sorbose, melezitose and raffinose. Mannitol, xylitol, erythritol, threitol, sorbitol and glycerol are examples of sugar alcohols. As to sugar acids, these include L-gluconate and metallic salts thereof. Where it is desired that the formulation is freeze-thaw stable, the polyol is preferably one which does not crystallize at freezing temperatures (*e.g.* –20° C) such that it destabilizes the antibody in the formulation. Non-reducing sugars such as sucrose and trehalose are the preferred polyols herein, with trehalose being preferred over sucrose, because of the superior solution stability of trehalose.

As used herein, "buffer" refers to a buffered solution that resists changes in pH by the action of its acid-base conjugate components. The buffer of this disclosure has a pH in the range from about 4.5 to about 8.0; preferably from about 5.5 to about 7. Examples of buffers that will control the pH in this range include acetate (*e.g.* sodium acetate), succinate (such as sodium succinate), gluconate, histidine, citrate and other organic acid buffers. Where a freeze-thaw stable formulation is desired, the buffer is preferably not phosphate.

In a pharmacological sense, in the context of the present disclosure, a "therapeutically effective amount" of a therapeutic agent, e.g., a VEGF antagonist, e.g., an anti-VEGF antibody or antibody derivative, refers to an amount effective in the prevention or treatment of a disorder for the treatment of which the antagonist, e.g., antibody or antibody derivative, is effective. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

A "preservative" is a compound which can be included in the formulation to essentially reduce bacterial action therein, thus facilitating the production of a multi-use formulation, for example. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol. The most preferred preservative herein is benzyl alcohol.

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The pharmaceutical compositions used in present disclosure comprise a VEGF antagonist A, preferably an anti-VEGF antibody (e.g., an anti-VEGF antibody comprising the variable light chain sequence of SEQ ID NO: 1 and the variable heavy chain sequence of SEQ ID NO: 2, such as brolucizumab), together with at least one physiologically acceptable carrier or excipient. Pharmaceutical compositions may comprise, for example, one or more of water, buffers (*e.g.,* neutral buffered saline or phosphate buffered saline), ethanol, mineral oil, vegetable oil, dimethylsulfoxide, carbohydrates (*e.g.,* glucose, mannose, sucrose or dextrans), mannitol, proteins, adjuvants, polypeptides or amino acids such as glycine, antioxidants, chelating agents such as EDTA or glutathione and/or preservatives. As noted above, other active ingredients may (but need not) be included in the pharmaceutical compositions provided herein.

A carrier is a substance that may be associated with an antibody or antibody derivative prior to administration to a patient, often for the purpose of controlling stability or bioavailability of the compound. Carriers for use within such formulations are generally biocompatible, and may also be biodegradable. Carriers include, for example, monovalent or multivalent molecules such as serum albumin (*e.g.,* human or bovine), egg albumin, peptides, polylysine and polysaccharides such as aminodextran and polyamidoamines. Carriers also include solid support materials such as beads and microparticles comprising, for example, polylactate polyglycolate, poly(lactide-co-glycolide), polyacrylate, latex, starch, cellulose or dextran. A carrier may bear the compounds in a variety of ways, including covalent bonding (either directly or via a linker group), noncovalent interaction or admixture.

Pharmaceutical compositions may be formulated for any appropriate manner of administration, including, for example, topical, intraocular, oral, nasal, rectal or parenteral administration. In certain embodiments, compositions in a form suitable for intraocular injection, such as intravitreal injection, are preferred. Other forms include, for example, pills, tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs. Within yet other embodiments, compositions provided herein may be formulated as a lyophilizate. The term parenteral as used herein includes subcutaneous, intradermal, intravascular (*e.g.,* intravenous), intramuscular, spinal, intracranial, intrathecal and intraperitoneal injection, as well as any similar injection or infusion technique.

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The pharmaceutical composition may be prepared as a sterile injectible aqueous or oleaginous suspension in which the active agent (i.e. VEGF antagonist), depending on the vehicle and concentration used, is either suspended or dissolved in the vehicle. Such a composition may be formulated according to the known art using suitable dispersing, wetting agents and/or suspending agents such as those mentioned above. Among the acceptable vehicles and solvents that may be employed are water, 1,3-butanediol, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils may be employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed, including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectible compositions, and adjuvants such as local anesthetics, preservatives and/or buffering agents can be dissolved in the vehicle.

An aqueous formulation of a VEGF antagonist, e.g., an anti-VEGF antibody (e.g., brolucizumab), used in the methods or uses of the disclosure is prepared in a pH-buffered solution. Preferably, the buffer of such aqueous formulation has a pH in the range from about 4.5 to about 8.0, preferably from about 5.5 to about 7.0, most preferably about 6.75. In one embodiment, the pH of an aqueous pharmaceutical composition of the disclosure is about 7.0-7.5, or about 7.0-7.4, about 7.0-7.3, about 7.0-7.2, about 7.1-7.6, about 7.2-7.6, about 7.3-7.6 or about 7.4-7.6. In one embodiment, an aqueous pharmaceutical composition of the disclosure has a pH of about 7.0, about 7.1, about 7.2, about 7.3, about 7.4, about 7.5 or about 7.6. In a preferred embodiment, the aqueous pharmaceutical composition has a pH of $\geq$7.0 In a preferred embodiment, the aqueous pharmaceutical composition has a pH of about 7.2. In another preferred embodiment, the aqueous pharmaceutical composition has a pH of about 7.4. In another preferred embodiment, the aqueous pharmaceutical composition has a pH of about 7.6. Examples of buffers that will control the pH within this range include acetate (*e.g.* sodium acetate), succinate (such as sodium succinate), gluconate, histidine, citrate and other organic acid buffers. The buffer concentration can be from about 1 mM to about 50 mM, preferably from about 5 mM to about 30 mM, depending, for example, on the buffer and the desired isotonicity of the formulation.

A polyol, which acts as a tonicifier, may be used to stabilize an antibody in an aqueous formulation. In preferred embodiments, the polyol is a non-reducing sugar, such as sucrose or trehalose. If desired, the polyol is added to the formulation in an amount that may

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vary with respect to the desired isotonicity of the formulation. Preferably the aqueous formulation is isotonic, in which case suitable concentrations of the polyol in the formulation are in the range from about 1% to about 15% w/v, preferably in the range from about 2% to about 10% w/v, for example. However, hypertonic or hypotonic formulations may also be suitable. The amount of polyol added may also alter with respect to the molecular weight of the polyol. For example, a lower amount of a monosaccharide (*e.g.* mannitol) may be added, compared to a disaccharide (such as trehalose).

A surfactant is also added to an aqueous antibody formulation. Exemplary surfactants include nonionic surfactants such as polysorbates (*e.g.* polysorbates 20, 80 etc) or poloxamers (*e.g.* poloxamer 188). The amount of surfactant added is such that it reduces aggregation of the formulated antibody/antibody derivative and/or minimizes the formation of particulates in the formulation and/or reduces adsorption. For example, the surfactant may be present in the formulation in an amount from about 0.001% to about 0.5%, preferably from about 0.005% to about 0.2% and most preferably from about 0.01% to about 0.1%.

In one embodiment, an aqueous antibody formulation used in the methods or uses of the disclosure is essentially free of one or more preservatives, such as benzyl alcohol, phenol, m-cresol, chlorobutanol and benzethonium Cl. In another embodiment, a preservative may be included in the formulation, particularly where the formulation is a multidose formulation. The concentration of preservative may be in the range from about 0.1% to about 2%, most preferably from about 0.5% to about 1%. One or more other pharmaceutically acceptable carriers, excipients or stabilizers such as those described in Remington's Pharmaceutical Sciences 21st edition, Osol, A. Ed. (2006) may be included in the formulation provided that they do not adversely affect the desired characteristics of the formulation. Acceptable carriers, excipients or stabilizers are non-toxic to recipients at the dosages and concentrations employed and include: additional buffering agents, co-solvents, antioxidants including ascorbic acid and methionine, chelating agents such as EDTA, metal complexes (*e.g.* Zn-protein complexes), biodegradable polymers such as polyesters, and/or salt-forming counterions such as sodium.

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Formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to, or following, preparation of the formulation.

In one embodiment, the VEGF antagonist of the disclosure is administered to an eye of a subject in need of treatment in accordance with known methods for ocular delivery. Preferably, the subject is a human, the VEGF antagonist is an anti-VEGF antibody (preferably brolucizumab), and the antibody is administered directly to an eye. Administration to a patient can be accomplished, for example, by intravitreal injection.

The VEGF antagonist in the methods and uses of the disclosure can be administered as the sole treatment or in conjunction with other drugs or therapies useful in treating the condition in question.

A preferred formulation for brolucizumab for intravitreal injection comprises about 4.5% to 11% (w/v) sucrose, 5-20 mM sodium citrate, and 0.001% to 0.05% (w/v) polysorbate 80, wherein the pH of the formulation is about 7.0 to about 7.4. One such formulation comprises 5.9% (w/v) sucrose, 10 mM sodium citrate, 0.02% (w/v) polysorbate 80, pH of 7.2, and 6 mg of brolucizumab. Another such formulation comprises 6.4% (w/v) or 5.8% sucrose, 12 mM or 10 mM sodium citrate, 0.02% (w/v) polysorbate 80, pH of 7.2, and 3 mg of brolucizumab. Preferred concentrations of brolucizumab are about 120 mg/ml and about 60 mg/ml. Doses can be delivered, for example as 6 mg/50 µL and 3 mg/50 µL concentrations.

Dosage and dosing regimen

A dose used in the methods or uses of the disclosure is based on the specific disease or condition being treated, and is therapeutically effective dose. Amounts effective for this use will depend upon the severity of the disorder being treated and the general state of the patient's own immune system. The dose amount can be readily determined using known dosage adjustment techniques by a physician having ordinary skill in treatment of the disease or condition. The therapeutically effective amount of a VEGF antagonist used in the methods or uses of the disclosure is determined by taking into account the desired dose volumes and

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mode(s) of administration, for example. Typically, therapeutically effective compositions are administered in a dosage ranging from 0.001 mg/ml to about 200 mg/ml per dose.

In one embodiment of the present disclosure, the VEGF antagonist used in the methods or uses of the disclosure is brolucizumab, and a dosage thereof used in the methods or uses of the disclosure is about 60 mg/ml to about 120 mg/ml (for example, a dosage is 60, 70, 80, 90, 100, 110, or 120 mg/ml). In a preferred embodiment, the dosage of the VEGF antagonist used in the methods or uses of the disclosure is 60 mg/ml or 120 mg/ml.

In certain embodiments, a dose of the VEGF antagonist is administered directly to an eye of a patient. In one embodiment, a dose of the VEGF antagonist per eye is at least about 0.5 mg up to about 6 mg. Preferred doses per eye include about 0.5 mg, 0.6 mg, 0.7 mg, 0.8 mg, 0.9 mg, 1.0 mg, 1.2 mg, 1.4 mg, 1.6 mg, 1.8 mg, 2.0 mg, 2.5 mg, 3.0 mg, 3.5 mg, 4.0 mg, 4.5 mg, 5.0 mg, 5.5 mg, and 6.0 mg. In one embodiment, a dose per eye is at least about 3 mg up to about 6 mg, e.g., about 3 mg or about 6 mg. Doses can be administered in various volumes suitable for ophthalmic administration, such as 50 µl or 100 µl, for example, including 3 mg/50 µl or 6 mg/50 µl. Smaller volumes can also be used, including 20 µl or less, for example about 20 µl, about 10 µl, or about 8.0 µl. In certain embodiments, a dose of 2.4 mg/20 µl, 1.2 mg/10 µl or 1 mg/8.0 µl (e.g., 1 mg/8.3 µl) is delivered to an eye of a patient for treating or ameliorating one or more of the diseases and disorders described above. Delivery can be, for example, by an injections, e.g., an intravitreal injection.

In specific embodiments, the VEGF antagonist of the disclosure is brolucizumab and is administered at a dose of about 1, about 2, about 3, about 4, about 5, or about 6 mg (e.g., about 6 mg/0.05 mL), e.g., 1, 2, 3, 4, 5, or 6 mg (e.g., 6 mg/0.05 mL), as an injections, e.g., an intravitreal injection.

In specific embodiments, the VEGF antagonist of the disclosure is brolucizumab and is administered to the subject in three individual doses at 4-week intervals, and thereafter administered to the subject as an additional dose every 8 to 12 weeks, e.g., every 8 weeks or every 12 weeks. In some cases, a patient might be on a 12-week interval regimen for some time, and then switch to an 8-week interval, and then switch back to the 12-week interval. Thus, patients may not stay on one interval regimen, and may go back and forth depending on assessments according to the criteria set forth herein. For example, a 12 week treatment

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interval may be switched to an 8 week treatment interval if a disease activity is detected in the subject, or an 8 week treatment interval may be switched to a 12 week treatment interval if no disease activity is detected in the subject.

Suitably, the disease activity may be assessed based on visual function, retinal structure and leakage. An assessment as described herein preferably includes one or more of the following tests to assess activity of a VEGF antagonist (e.g., brolucizumab) on visual function, retinal structure and leakage: (i) best corrected visual acuity (BCVA), e.g., best corrected visual acuity with ETDRS-like chart at 4 meters, (ii) visual acuity (VA), (iii) central subfield thickness (CSFT), (iv) presence of intraretinal cysts/fluid, (v) ETDRS DRSS score based on 7-field stereo Color Fundus Photography (CFP), (vi) anatomical retinal evaluation by Optical Coherence Tomography (OCT), standard or wide-field Fluorescein Angiography (FA), OCT angiography, and/or wide-field CFP/FA, (vii) peripheral visual field assessed by perimetry, (viii) contrast sensitivity, (viii) severity of diabetic retinopathy.

Visual acuity can be assessed using best correction determined from protocol refraction (BCVA). BCVA measurements can be taken, for example, in a sitting position using ETDRS–like visual acuity testing charts.

Optical Coherence Tomography (OCT), color fundus photography and fluorescein angiography can be assessed according to methods known to those of skill in the art.

The CST is the average thickness of circular 1 mm area centered around the fovea measured from retinal pigment epithelium (RPE) to the internal limiting membrane (ILM), inclusively. CST can be measured, for example, using spectral domain Optical Coherence Tomography (SD-OCT).

Means of performing the above tests are well understood and commonly used by those skilled in the art.

Suitably, the disease activity may be assessed based on one or more of the following: (i) best corrected visual acuity (BCVA), (ii) visual acuity (VA), (iii) central subfield thickness (CSFT), and (iv) presence of intraretinal cysts/fluid. The presence of ocular disease activity, e.g., nAMD disease activity, includes one or more of the following: (i) decrease in

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Best Corrected Visual Acuity (BCVA), (ii) decrease in Visual Acuity (VA), (iii) increase or lack of reduction in Central Subfield Thickness (CSFT), (iv) new or persistent or recurrent Intraretinal Cysts (IRC) and/or Intraretinal Fluid (IRF) and/or Subretinal Fluid (SRF). Fluid measured in the eye can be intraretinal and/or subretinal fluid.

In specific embodiments, the presence of ocular disease activity, e.g., nAMD disease activity, includes one or more of the following:

(i) decrease in BCVA of ≥2 letters, e.g., decrease in BCVA of ≥3 letters, decrease in BCVA of ≥4 letters, e.g., decrease in BCVA of ≥5 letters, more particularly wherein:
- the decrease in BCVA is observed at Week 8 or Week 12 after the last administration of the VEGF antagonist (e.g., brolucizumab) compared to a baseline BCVA, wherein the baseline BCVA was assessed prior to the last administration of the VEGF antagonist, or
- the decrease in BCVA is observed after 4 months or longer, e.g., after 6 months or longer, of the administration of the VEGF antagonist compared to a baseline BCVA, wherein the baseline BCVA was assessed 4 months or longer, e.g., 6 months or longer, prior to the last administration of the VEGF antagonist;

(ii) decrease in VA of ≥1 letters, e.g., decrease in VA of ≥2 letters, e.g., decrease in VA of ≥3 letters, more particularly wherein:
- the decrease in VA is observedat Week 8 or Week 12 after the last administration of the VEGF antagonist (e.g., brolucizumab) compared to a baseline VA, wherein the baseline VA was assessed prior to the last administration of the VEGF antagonist, or
- the decrease in VA is observed after 4 months or longer, e.g., after 6 months or longer, of the administration of the VEGF antagonist compared to a baseline VA, wherein the baseline VA was assessed 4 months or longer, e.g., 6 months or longer, prior to the last administration of the VEGF antagonist;

(iii) CSFT increase ≥25μm, e.g., CSFT increase ≥50μm, e.g., CSFT increase ≥75μm, more particularly wherein:
- the CSFT increase is observed at Week 8 or Week 12 after the last administration of the VEGF antagonist (e.g., brolucizumab) compared to a baseline CSFT,

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wherein the baseline CSFT was assessed prior to the last administration of the VEGF antagonist, or

- the CSFT increase is observed after 4 months or longer, e.g., after 6 months or longer, of the administration of the VEGF antagonist compared to a baseline CSFT, wherein the baseline CSFT was assessed 4 months or longer, e.g., 6 months or longer, prior to the last administration of the VEGF antagonist;

(iv) new or persistent or recurrent intraretinal cysts (IRC) and/or intraretinal fluid (IRF) and/or subretinal fluid (SRF), e.g., wherein:

- new or persistent or recurrent intraretinal cysts (IRC) and/or intraretinal fluid (IRF) and/or subretinal fluid (SRF) are observed at at Week 8 or Week 12 after the last administration of the VEGF antagonist (e.g., brolucizumab) compared to a baseline RC and/or IRF and/or SRF, wherein the baseline RC and/or IRF and/or SRF was assessed prior to the last administration of the VEGF antagonist, or
- new or persistent or recurrent intraretinal cysts (IRC) and/or intraretinal fluid (IRF) and/or subretinal fluid (SRF) are observed after 4 months or longer, e.g., after 6 months or longer, of the administration of the VEGF antagonist compared to a baseline IRC and/or IRF and/or SRF, wherein the baseline IRC and/or IRF and/or SRF was assessed 4 months or longer, e.g., 6 months or longer, prior to the last administration of the VEGF antagonist.

Where disease activity is present (for example, loss of letters measured by BCVA, increase in CST, increased fluid accumulation, and or increased severity of ocular disease compared with baseline reading for the patient or compared with any previous assessment), a more frequent dosing interval is prescribed going forward. Where improvement of disease activity is observed, a less frequent dosing interval is prescribed.

In some embodiments, the VEGF antagonist of the disclosure is aflibercept and is administered at a dose of about 0.5, about 1 or about 2 mg (e.g., about 2 mg/0.05 mL), e.g., 0.5, 1 or 2 mg (e.g., 2 mg/0.05 mL), as an injections, e.g., an intravitreal injection.

In some embodiments, the VEGF antagonist used in the methods or uses of the disclosure is ranibizumab and is administered at a dose of about 0.2, about 0.3, about 0.4 or

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about 0.5 mg (e.g., about 0.5 mg/0.05 mL), e.g., 0.2, 0.3, 0.4 or 0.5 mg (e.g., 0.5 mg/0.05 mL), as an injections, e.g., an intravitreal injection.

The various features, aspects and embodiments of the present invention, referred to in individual sections above apply, as appropriate, to other sections, mutatis mutandis. Consequently features specified in one section may be combined with features specified in other sections, as appropriate.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific aspects and embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

All references cited herein, including patents, patent applications, papers, publications, text books, and the like, and the references cited therein, to the extent that they are not already, are hereby incorporated herein by reference in their entirety

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 discovered by the inventor 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, in light of the present disclosure, appreciate 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.

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EXAMPLES

Example 1: Safety-specific Next Generation HAWK OCT Analysis

Purpose: To perform a comparative discovery assessment to evaluate the presence of OCT features that may precede or develop in association with intraocular inflammation (IOI), which might serve as OCT biomarkers for IOI.

Methods: HAWK is a randomized, double-masked, 96-week phase 3 studies comparing brolucizumab to aflibercept in nAMD. The Safety Review Committee (SRC) performed an independent unmasked post hoc review of all cases of investigator-reported IOI, retinal vascular occlusions and endophthalmitis in the phase 3 HAWK and HARRIER studies. Following this analysis, a higher order comparative discovery analysis was initiated evaluating 34 eyes from the HAWK study identified through the SRC analysis and compared to 34 propensity-matched controls. This analysis includes comparative higher-order OCT, radiomics assessment, and machine learning classification. This report provides an initial assessment of the qualitative comparative discovery analysis of the Safety next Generation imaging study (SAGE). Images were reviewed frame-by-frame for specific features prior to or during the IOI event.

Results:

Qualitative review of the cases of interest identified a characteristic preretinal hyperreflective deposit, "stalagmite" in 20 of 34 eyes (59%) with IOI compared to 1 of 34 eyes (3%) in the controls ($p < 0.001$) (Figure 1). This one control subject was noted to have an adverse event secondary to floaters at the time the "stalagmites" were noted on OCT, suggesting a possible underlying IOI event. The presence of the "stalagmites" were identified at the time IOI was initially noted in 11 subjects and was visualized at visits prior to the reported IOI event in 9 subjects. When "stalagmites" were identified at visits prior to the reported IOI event, 77% of these eyes ultimately developed retinal vasculitis and/or vascular occlusive event.

Further qualitative review of the cases of interest identified a characteristic preretinal hyperreflective deposit (PHRD) and / or vitreous debris in eyes with IOI compared to in the controls (Figure 2). Qualitative review of the cases of interest identified a characteristic

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preretinal hyperreflective deposit or vitreous debris in 20 of 34 eyes (59%) with IOI compared to 1 of 34 eyes (3%) in the controls ($p < 0.001$). Out of 20 eyes with preretinal hyperreflective deposit or vitreous debris, in 11/20 eyes preretinal hyperreflective deposit and/or vitreous debris were observed at time of the IOI event with 8/11 eyes (72%) defined as having IOI + RV/RO, and in 9/20 eyes preretinal hyperreflective deposit and/or vitreous debris were observed prior to the IOI event with 8/14 eyes (57%) defined as having IOI + RV/RO.

Example 2: Predictive Assessment of Radiomic features to identify eyes with Intraocular Inflammation (IOI) on HAWK-SAGE data

In addition, radiomics assessment has been performed (Figure 3) to evaluate potential pre-inflammatory event signals within the vitreous compartment. Specifically, the assessment was conducted to identify and characterize vitreous compartment features that might provide early insights into pre-inflammatory event signals.

To evaluate whether radiomics features extracted from vitreous compartment can distinguish eyes (N=68) with intraocular inflammation (IOI). Last timepoint represented the "safety" event or when inflammation develops. The last timepoint vitreous compartment between the Safety group and the Control group was compared. In addition, an analysis of the timepoint immediately before the final timepoint was done to assess if we can tell a difference between the groups prior to actual events. Finally, assessment at the month one timepoint was conducted to assess whether after a single injection it is possible to identify a difference between those two groups.

The Control group was N=34; the Safety group (N=34) was divided into two subgroups: S1 (N=20) - with visible OCT findings, and S2 (N=14) - without visible OCT findings.

Supervised Classification for distinguishing Control and Safety groups

A 3-fold cross validation was implemented for supervised classification. Different machine learning (ML) classifiers (linear discriminant analysis (LDA), quadratic discriminant analysis (QDA), support vector machine (SVM), random forest (RF)) were trained using the topmost 7

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features selected by different feature selection methods (t-test, Wilcoxon, mRmR) on the baseline images. LDA+ttest yielded best AUC of 0.75±0.06 on baseline images. Using the topmost 7 features the same model was evaluated on the other timepoint images, AUC (Area Under the Curve) values shown in Table 1.

Table 1: AUC values yielded by LDA in classifying Control and Safety group.

Timepoint	**AUC**
Baseline	0.75±0.06
After 1st injection (Timepoint 1)	0.76±0.07
Prior to Event	0.79±0.04
Last Timepoint (Event)	0.81±0.08

- 7 most discriminating features:
    1. skewness-Gabor XY-θ=1.178, XZ-θ=0.000, λ=3.261, BW=1 ($p<0.001$)
    2. skewness-Laws E3E3L3($p<0.001$)
    3. skewness-Gabor XY-θ=2.356, XZ-θ=0.000, λ=3.261, BW=1($p<0.001$)
    4. skewness-Gabor XY-θ=0.393, XZ-θ=2.356, λ=3.261, BW=1($p<0.001$)
    5. skewness-Gabor XY-θ=0.785, XZ-θ=0.000, λ=3.261, BW=1($p<0.001$)
    6. kurtosis-Gabor XY-θ=0.785, XZ-θ=0.000, λ=3.261, BW=1($p<0.001$)
    7. skewness-Laws S3E3S3($p<0.001$)

13/20 (65%) eyes of S1 were always classified correctly at each of these timepoints. The box and whisker plot of the most discriminating baseline feature significantly distinguishes the two groups of patients (Figure 4).

Feature map for the most discriminating feature at prior to event timepoint (Figure 3) shows that for both the Control and Safety patients the feature expression of the low frequency Gabor feature 'Gabor XY-θ=1.178, XZ-θ=0.000, λ=3.261, BW=1' ($p=2.2186e-11$) within the vitreous compartment is low. However, higher feature expression is observed (Figure 3e) within the inflammation regions and the surface of ILM and vitreous compartment (Figure 3b, 3c, 3d) for Safety patients.

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Thus, even if the actual event is late, i.e, when the clinicians actually observe the inflammation in the OCT scans, prior to that the presence of inflammatory safety signal is observed within the texture of the vitreous compartment and surface of ILM and vitreous compartment in the form of heterogeneity.

Unsupervised Classification for distinguishing Control and Safety groups

Using Unsupervised Hierechical Clustering on texture-based radiomics features, a heatmap analysis was generated, showing that the patients in S2 and S1 aggregated together in two clusters, Cluster 1 and 2, respectively.

K-means Clustering was also conducted. Three clusters were formed: 60% of S1s grouped within Cluster 1; 79% of S2s grouped within Cluster 2; 62% Controls grouped within Cluster 3.

Supervised Classification betweeen Control and S1 group

The AUC values yielded by LDA are presented in Table 2 on different timepoint images.

Table 2: AUC values yielded by LDA in classifying Control and S1 group.

Timepoint	**AUC**
Baseline	0.67±0.04
After 1st injection (Timepoint 1)	0.74±0.07
Prior to Event	0.78±0.04
Last Timepoint (Event)	0.87±0.08

When the subset S2 was excluded from the Safety group, initially at baseline, after 1st injection and prior-to-event timepoint, the AUC values were comparatively less than that of when all Safety group of patients were considered (Table 2). However, the AUC value drastically increased for the last (event) timepoint.

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Machine learning quantitative characterization of the vitreous compartment identified differences in the number of hyperreflective vitreous and preretinal foci between eyes in the IOI group and the control group, including prior to the IOI reported event (Figures 5 to 7).

Conclusion: In this preliminary discovery evaluation for OCT biomarkers for IOI-related events in the HAWK dataset, hyperreflective preretinal foci (i.e., preretinal stalagmites) appear to be a potential important objective OCT finding of IOI and may provide an important signal for clinicians assessing for underlying posterior inflammation.

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CLAIMS

1. A method of treating ocular disease comprising selectively administering a VEGF antagonist, e.g., brolucizumab, to a subject in need thereof on the basis of said subject not having a hyperreflective deposit (HRD) in a subject's eye, e.g., a vitreous hyperreflective deposit (VHRD), e.g., a preretinal hyperreflective deposit (PHRD).

2. A method of treating ocular disease comprising:
(a) selecting a subject for treatment with a VEGF antagonist, e.g., brolucizumab, on the basis said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and
(b) thereafter, selectively administering the VEGF antagonist, e.g., brolucizumab, to the subject.

3. A method of selectively treating a subject having ocular disease the method comprising:
(a) selectively administering a therapeutically effective amount of a VEGF antagonist, e.g., brolucizumab, to the subject on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; or
(b) selectively administering a therapeutically effective amount of a different ocular disease agent, e.g., a different VEGF antagonist, e.g., aflibercept, ranibizumab, faricimab, to the subject on the basis of said subject having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD.

4. The method of claim 2 or 3 further comprising assessing the presence or absence of the HRD in a subject's eye, e.g., VHRD, e.g., PHRD, e.g., prior to the steps (a)-(b) of claim 2 or claim 3.

5. The method of any one of claims 1 to 4, wherein the presence or absence of the HRD, e.g., VHRD, e.g., PHRD, is assessed by performing OCT, e.g., SD-OCT, analysis on a subject's eye.

6. A method for determining whether a subject with ocular disease should be treated with a VEGF antagonist, e.g., brolucizumab, the method comprising:

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(a) performing analysis, e.g., OCT, SD-OCT analysis, on a subject's eye to assess the presence or absence of a HRD, e.g., VHRD, e.g., PHRD;

(b) determining that the subject should be treated with a VEGF antagonist, e.g., brolucizumab, if no HRD, e.g., VHRD, e.g., PHRD, is observed in the subject's eye;

(c) determining that the subject should not be treated with a VEGF antagonist, e.g., brolucizumab, if the HRD, e.g., VHRD, e.g., PHRD, is observed in the subject's eye.

7. The method of claim 6 further comprising treating the subject with the VEGF antagonist, e.g., brolucizumab, if no HRD, e.g., VHRD, e.g., PHRD, is observed in the subject's eye.

8. The method of any one of the preceding claims, wherein the subject is assessed for presence of the HRD in a subject's eye, e.g., VHRD, e.g., PHRD, prior to initiation of the treatment with the VEGF antagonist and / or during the treatment course with the VEGF antagonist.

9. The method of claim 8, further comprising:
(a) altering or discontinuing the treatment with the VEGF antagonist if, during the course of treatment, the subject is determined to have a HRD, e.g., VHRD, e.g., PHRD, in the treatment eye, or
(b) maintaining the treatment with the VEGF antagonist if, during the course of treatment, the subject is determined not to have a HRD, e.g., VHRD, e.g., PHRD, in the treatment eye.

10. A method of predicting the likelihood that a subject having ocular disease will develop intraocular inflammation (IOI), e.g., intraocular inflammation with retinal vasculitis and / or retinal occlusion (IOI+RV/RO), in response to a treatment with a VEGF antagonist, e.g., brolucizumab, the method comprising determining if the subject has a HRD in a subject's eye, e.g., VHRD, e.g., PHRD, wherein:
(a) the presence of a HRD, e.g., VHRD, e.g., PHRD, is indicative of an increased likelihood that the patient will develop IOI, e.g., IOI+RV/RO, in response to the treatment with the VEGF antagonist, e.g., brolucizumab; and
(b) the absence of a HRD, e.g., VHRD, e.g., PHRD, is indicative of a decreased likelihood that the patient will develop IOI, e.g., IOI+RV/RO, in response to the

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treatment with the VEGF antagonist, e.g., brolucizumab.

11. The method of claim 10 further comprising treating the subject with the VEGF antagonist, e.g., brolucizumab, if no HRD, e.g., VHRD, e.g., PHRD, is observed in the subject's eye.

12. The method of any one of the preceding claims, wherein the HRD, e.g., VHRD, e.g., PHRD, has high reflectivity, e.g., has clearly delineated boundaries from the surrounding components of the retina.

13. The method of any one of the preceding claims, wherein the HRD, e.g., VHRD, e.g., PHRD, is a hyperreflective round- or rod-shaped dot, e.g., stalagmite-like hyperreflective material.

14. The method of any one of the preceding claims, wherein the VEGF antagonist is an anti-VEGF antibody.

15. The method of claim 14, wherein the VEGF antibody comprises a variable heavy chain having the sequence as set forth in SEQ ID NO: 1 and a variable light chain having the sequence as set forth in SEQ ID NO: 2.

16. The method of claim 14, wherein the VEGF antibody comprises the sequence of SEQ ID NO: 3 or SEQ ID NO: 4.

17. The method of claim 14, wherein the VEGF antagonist is brolucizumab.

18. The method of any one of claims 15 to 17, wherein the method comprises administering to the subject three individual doses of the VEGF antagonist at 4-week intervals, and thereafter administering to the subject an additional dose every 8 to 12 weeks.

19. The method of claim 18, wherein the method comprises administering to the subject three individual doses of the VEGF antagonist at 4-week intervals, and thereafter administering

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to the subject an additional dose every 12 weeks.

20. The method of claim 19, wherein a 12 week treatment interval is switched to an 8 week treatment interval if a disease activity is detected in the subject.

21. The method of claim 18, wherein the method comprises administering to the subject three individual doses of the VEGF antagonist at 4-week intervals, and thereafter administering to the subject an additional dose every 8 weeks.

22. The method of claim 21, wherein an 8 week treatment interval is switched to a 12 week treatment interval if no disease activity is detected in the subject.

23. The method of any one of claims 15 to 22, wherein each dose of the VEGF antagonist is at least 3 mg, e.g., 3 mg or 6 mg, e.g., 6 mg.

24. The method of claim 23, wherein each dose of the VEGF antagonist is administered as a 50 μL intravitreal injection.

25. The method of any one of preceding claims, wherein the ocular disease is selected from the list consisting of abnormal angiogenesis, choroidal neovascularization (CNV), retinal vascular permeability, retinal edema, diabetic retinopathy (particularly proliferative diabetic retinopathy (PDR) and non-proliferative diabetic retinopathy (NPDR)), macular edema (ME), diabetic macular edema (DME), neovascular (exudative) age-related macular degeneration (nAMD), choroidal neovascularization (CNV) associated with nAMD, sequela associated with retinal ischemia, Retinal Vein Occlusion (RVO), Central Retinal Vein Occlusion (CRVO), Branch Retinal Vein Occlusion (BRVO), macular edema following retinal vein occlusion, and posterior segment neovascularization.

26. The method of claim 25, wherein the ocular disease is neovascular age-related macular degeneration (nAMD).

27. A method for producing a transmittable form of information for determining whether a subject with ocular disease, e.g., nAMD, should be treated with a VEGF antagonist, e.g.,

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brolucizumab, the method comprising:

(a) determining that the subject should be treated with a VEGF antagonist on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; or

(b) determining that the subject should be treated with a different ocular disease agent, e.g., nAMD agent, on the basis of said subject having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and

(c) recording the result of the determining step on a tangible or intangible media form for use in transmission.

28. A method for producing a transmittable form of information for predicting whether a subject with ocular disease, e.g., nAMD, may develop intraocular inflammation (IOI), e.g., intraocular inflammation with retinal vasculitis/retinal occlusion (IOI+RV/RO), in response to a treatment with a VEGF antagonist, e.g., brolucizumab, the method comprising:

(a) determining an increased likelihood that the subject will develop IOI, e.g., IOI+RV/RO, in response to the treatment with the VEGF antagonist, e.g., brolucizumab, on the basis of said subject having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; or

(b) determining a decreased likelihood that the subject will develop IOI, e.g., IOI+RV/RO, in response to the treatment with the VEGF antagonist, e.g., brolucizumab, on the basis of said subject not having a HRD in a subject's eye, e.g., VHRD, e.g., PHRD; and

(c) recording the result of the determining step on a tangible or intangible media form for use in transmission.

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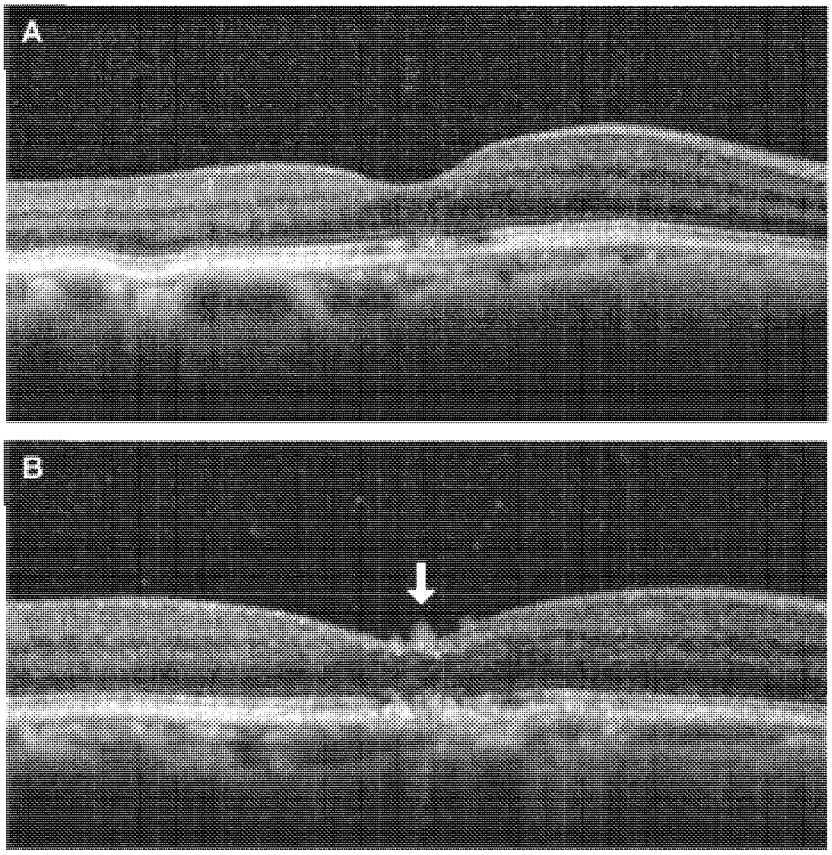


Figure 1

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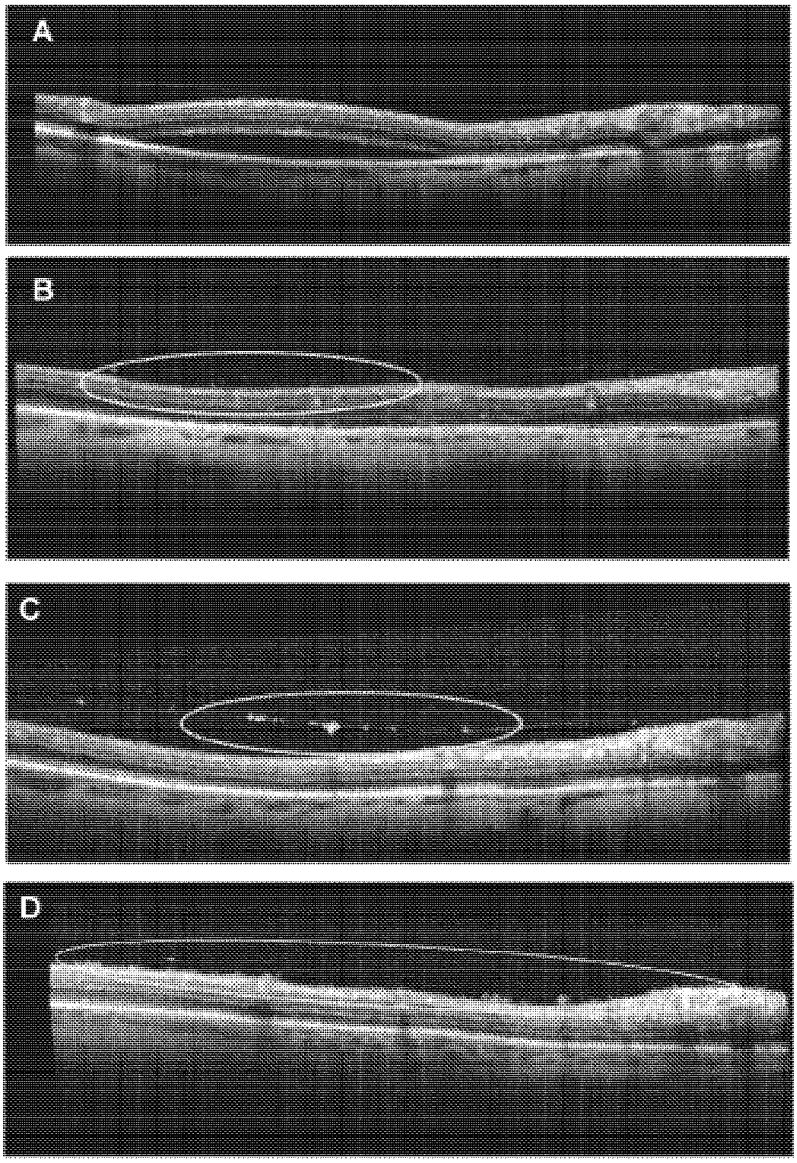


Figure 2

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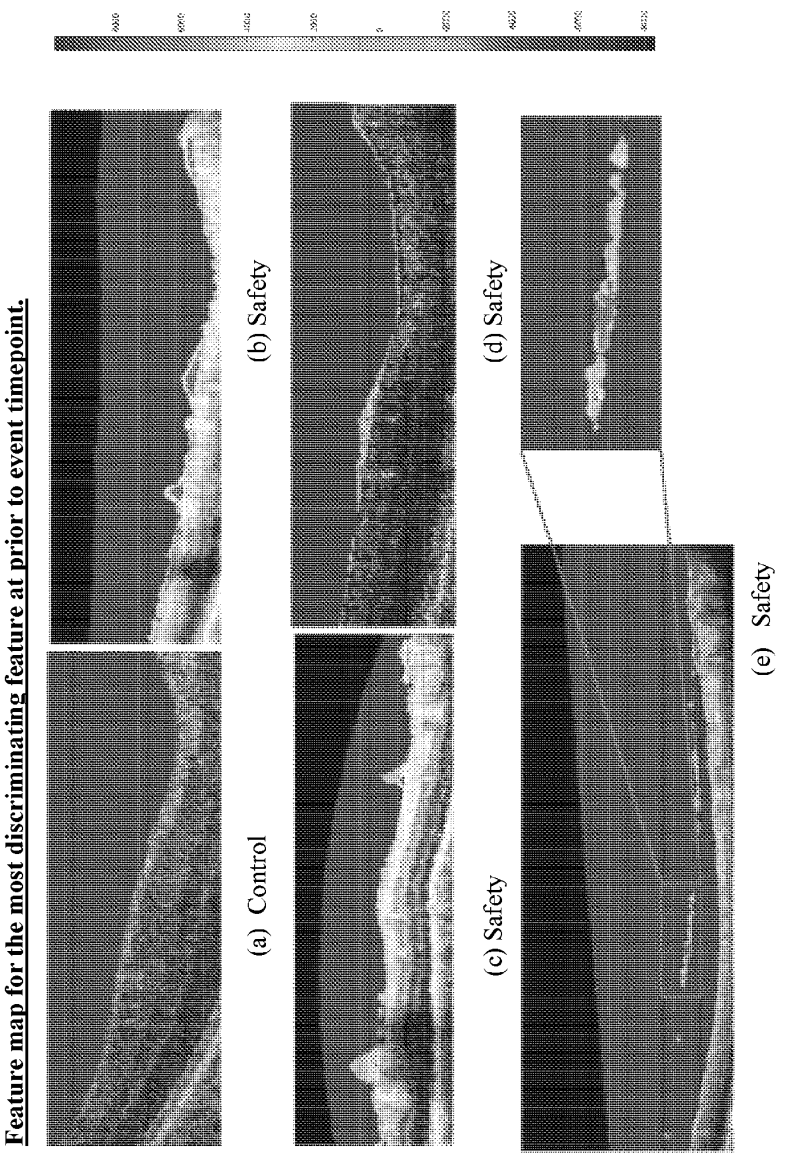


Figure 3

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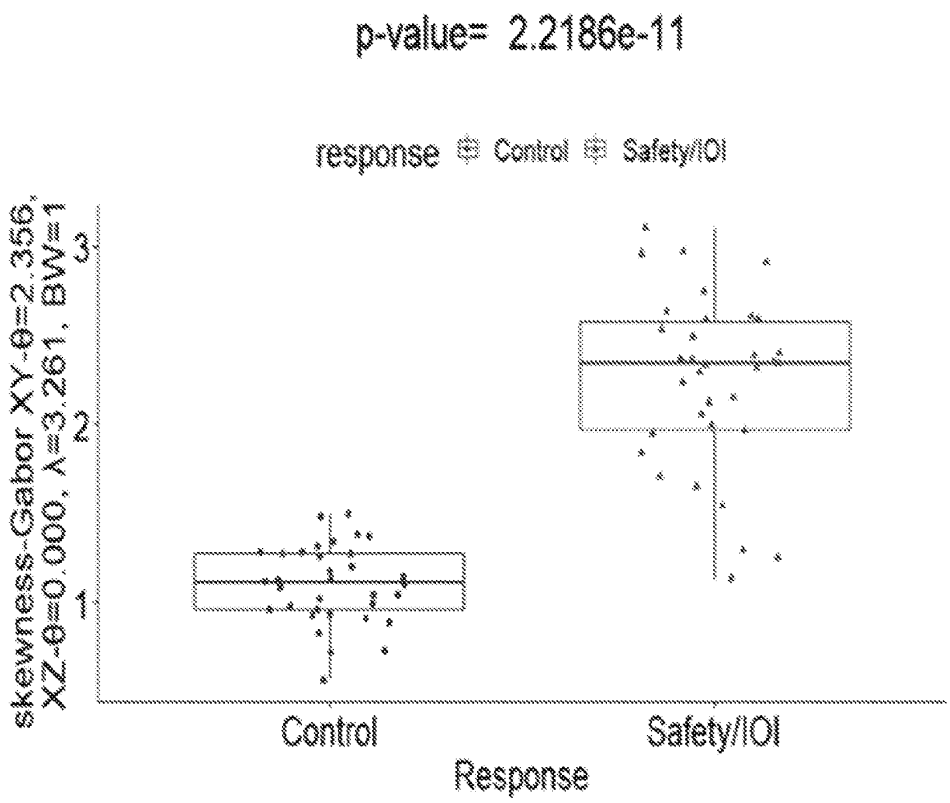


Figure 4

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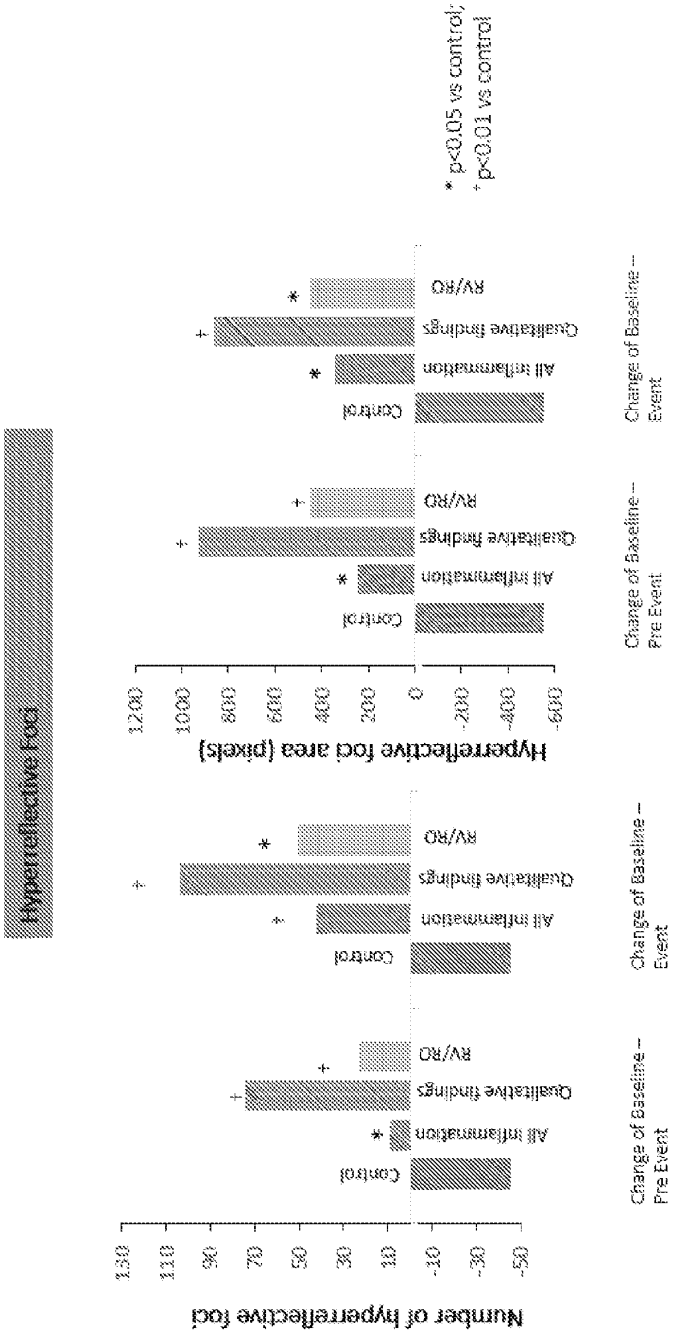


Figure 5

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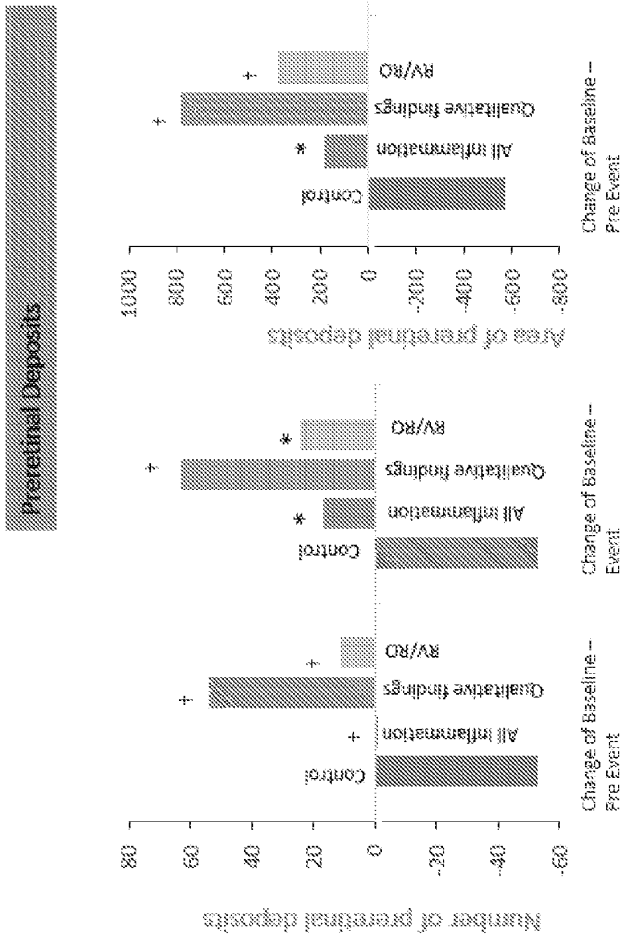


Figure 6

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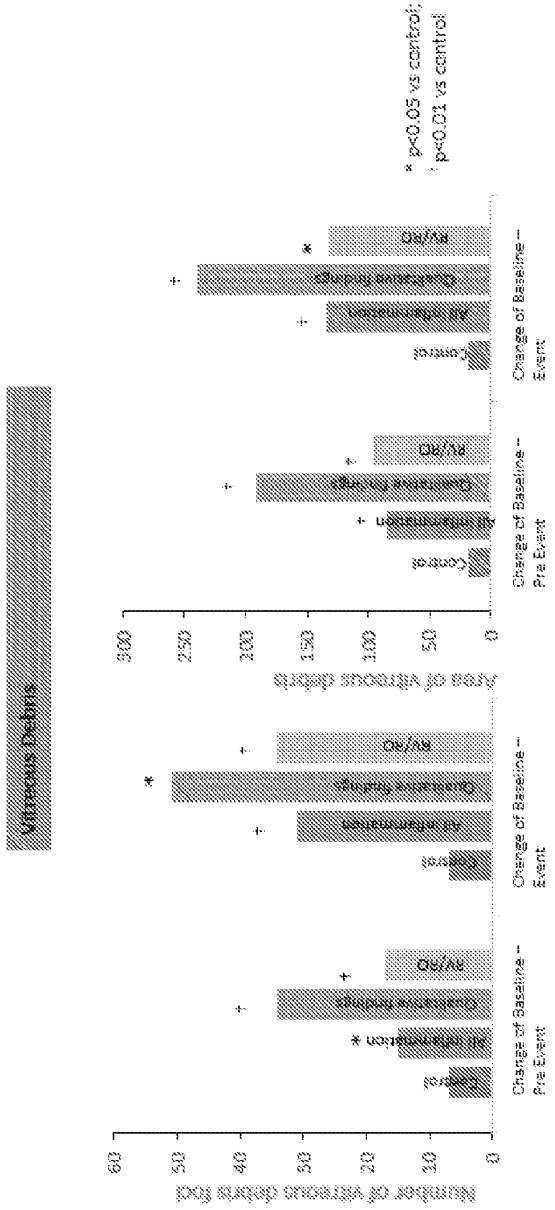


Figure 7

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(54) Title: COMPOSITIONS AND METHODS FOR OCULAR TRANSGENE EXPRESSION

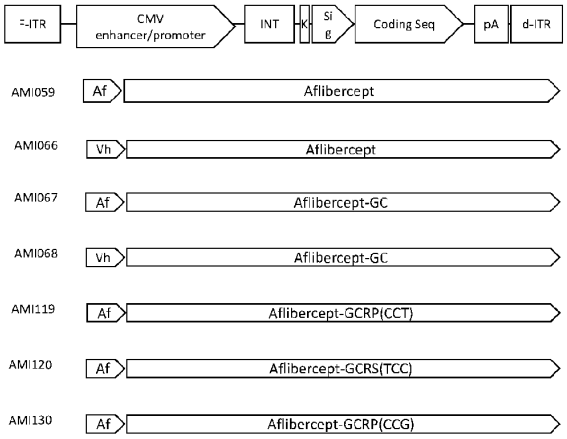


Fig. 1

(57) Abstract: Provided herein are compositions and methods comprising non-naturally occurring nucleic acid sequences that encode biologics. Also provided are methods of utilizing the provided compositions and methods as ocular therapeutics for prevention and treatment of various diseases and conditions.

[Continued on next page]

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TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

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Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

COMPOSITIONS AND METHODS FOR OCULAR TRANSGENE EXPRESSION

CROSS-REFERENCE

[0001] This application claims the benefit of US Provisional Application Serial Number 63/173,280 filed on April 9, 2021, the entirety of which is hereby incorporated by reference herein.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on April 8, 2022, is named 59561-703_601_SL.txt and is 159,002 bytes in size.

BACKGROUND

[0003] Aflibercept (VEGF-Trap) is a recombinant fusion protein that acts as a decoy receptor for vascular endothelial growth factor subtypes A (VEGF-A), VEGF-B, and placental growth factor (PIGF). By binding to these ligands, aflibercept is able to prevent these ligands from binding to vascular endothelial growth factor receptors (VEGFR), VEGFR-1 and VEGFR-2, to suppress neovascularization and decrease vascular permeability. Aflibercept consists of domain 2 of VEGFR-1 and domain 3 of VEGFR-2 fused with the Fc fragment of IgG1. Aflibercept is commercially marketed under the trade name EYLEA® (aflibercept), which is an ophthalmic intravitreal aflibercept fusion protein injection.

SUMMARY

[0004] Current treatments employing aflibercept suffer from short durability and repeated monthly injections in order to suppress the neovascularization. Several gene therapy studies using AAV vectors carrying the coding sequence for VEGF-Trap has been investigated for long term treatment of the neovascularization. However, these studies suffer from low expression levels of the agent and therefore reduced treatment efficacy. Accordingly, it has become increasingly clear that the full potential of this technology will only be realized after modifications are made for improved delivery and expression of VEGF-Trap and other anti-angiogenic agents.

[0005] Described herein, in some aspects, is an isolated, non-naturally occurring nucleic acid comprising a sequence encoding a biologic comprising an anti-angiogenic agent, said sequence comprising a modification in a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region, said modification comprising a replacement of at least four non-AGG arginine codons with AGG. In some embodiments, the sequence that encodes the anti-angiogenic agent further comprises a second modification. In

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some embodiments, the second modification is in at least one codon of the coding region of the sequence, and wherein the second modification is selected from the group consisting of: (a) replacement of at least one non-CCC proline codon with CCC; (b) replacement of at least one non-TCC serine codon with TCC; (c) replacement of at least one non-CCG proline codon with CCG; and (d) any combination of (a)-(c). In some embodiments, the second modification comprises (a). In some embodiments, the at least one non-CCC proline codon is CCT. In some embodiments, the second modification comprises (b). In some embodiments, the at least one non-TCC serine codon is AGC. In some embodiments, the second modification comprises (c). In some embodiments, the at least one non-CCG proline codon is CCC. In some embodiments, the second modification comprises (d). In some embodiments, the at least one non-CCC proline codon of (a) is CCT; the at least one non-TCC serine codon of (b) is AGC; and the at least one non-CCG proline codon of (c) is CCC. In some embodiments, the anti-angiogenic agent is selected from the group consisting of: a VEGF inhibitor, a multi-tyrosine kinase inhibitor, a receptor tyrosine kinase inhibitor, an inhibitor of Akt phosphorylation, a PDGF-1 inhibitor, a PDGF-2 inhibitor, a NP-1 inhibitor, a NP-2 inhibitor, a Del 1 inhibitor, and an integrin inhibitor. In some embodiments, the anti-angiogenic agent comprises the VEGF inhibitor, and wherein the VEGF inhibitor is a non-antibody inhibitor. In some embodiments, the non-antibody inhibitor is a fusion protein that comprises human VEGF receptors 1 and 2. In some embodiments, the fusion protein comprises VEGF-Trap or a modified version thereof. In some embodiments, the isolated, non-naturally occurring nucleic acid further comprises a signal peptide. In some embodiments, the signal peptide is selected from the group consisting of: human antibody heavy chain (Vh), human antibody light chain (Vl), and VEGF-Trap. In some embodiments, the signal peptide is from the human antibody heavy chain. In some embodiments, the signal peptide is derived from VEGF-Trap. In some embodiments, the isolated, non-naturally occurring nucleic acid further comprises an intronic sequence. In some embodiments, the intronic sequence is selected from the group consisting of: hCMV intron A, adenovirus tripartite leader sequence intron, SV40 intron, hamster EF-1 alpha gene intron 1, intervening sequence intron, human growth hormone intron, and human beta globin intron. In some embodiments, the intronic sequence is the SV40 intron. In some embodiments, the isolated, non-naturally occurring nucleic acid further comprises a promoter. In some embodiments, the promoter is selected from the group consisting of: a cytomegalovirus (CMV) promoter, an elongation factor 1 alpha (EF1α) promoter, a simian vacuolating virus (SV40) promoter, a phosphoglycerate kinase (PGK1) promoter, a ubiquitin C (Ubc) promoter, a human beta actin promoter, a CAG promoter, a Tetracycline response element (TRE) promoter, a UAS promoter, an Actin 5c (Ac5) promoter, a polyhedron promoter, a

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Ca2+/calmodulin-dependent protein kinase II (CaMKIIa) promoter, a GAL1 promoter, a GAL 10 promoter, a TEF1 promoter, a glyceraldehyde 3-phosphage dehydrogenase (GDS) promoter, an ADH1 promoter, a CaMV35S promoter, a Ubi promoter, a human polymerase III RNA (H1) promoter, a U6 promoter, a polyadenylated construct thereof, and any combination thereof. In some embodiments, the promoter is the CMV promoter. In some embodiments, the sequence is modified to replace non-AGG arginine codon with AGG in at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, or at least 16 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace every non-AGG arginine codon with AGG of the coding region of the sequence. In some embodiments, the sequence is modified to replace non-AGG arginine codon with AGG in at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, or at least 16 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace every non-AGG arginine codon with AGG as compared to **SEQ ID NO: 70**. In some embodiments, the non-AGG arginine codon comprises CGT, CGC, CGA, CGG, or AGA. In some embodiments, the non-AGG arginine codon is AGA. In some embodiments, the sequence is modified to replace AGA with AGG in at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, or at least 16 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace every AGA with AGG of the coding region of the sequence. In some embodiments, the sequence is modified to replace AGA with AGG in at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, or at least 16 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace every AGA with AGG as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace non-CCC proline codons with CCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or at least 30 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace every non-CCC proline codons with CCC of the coding region of the sequence. In some embodiments, the sequence is modified to replace non-CCC proline codon with CCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or up to 30 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace every non-CCC proline codon with CCC as compared to **SEQ ID NO: 70**. In some embodiments, the non-CCC proline codon comprises CCT or CCA. In some embodiments, the non-CCC proline codon is CCT. In some embodiments, the sequence is modified to replace CCT with CCC in at least 3, at least 6, at least 9, at least 12,

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at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or at least 30 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace every CCT with CCC of the coding region of the sequence. In some embodiments, the sequence is modified to replace CCT with CCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or at least 30 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace every CCT with CCC as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace non-TCC serine codon with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30, at least 33, or at least 36 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace every non-TCC serine codon with TCC of the coding region of the sequence. In some embodiments, the sequence is modified to replace non-TCC serine codon with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30, at least 33, or at least 36 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace every non-TCC serine codon with TCC as compared to **SEQ ID NO: 70**. In some embodiments, the non-TCC serine codon comprises TCT, TCA, TCG, AGT, or AGC. In some embodiments, the non-TCC serine codon is AGC. In some embodiments, the sequence is modified to replace AGC with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30, at least 33, or at least 36 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace every AGC with TCC of the coding region of the sequence. In some embodiments, the sequence is modified to replace AGC with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30, at least 33, or at least 36 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace every AGC with TCC as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace non-CCG proline codon with CCG in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, or at least 30 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace every non-CCG proline codon with CCG of the coding region of the sequence. In some embodiments, the sequence is modified to replace non-CCG proline codon with CCG in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace every non-CCG proline codon with CCG as compared to **SEQ ID NO: 70**. In some embodiments, the non-CCG proline codon comprises CCC or CCA. In some embodiments, the

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non-CCG proline codon is CCC. In some embodiments, the sequence is modified to replace CCC with CCG in in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, or at least 30 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace every CCC with CCG of the coding region of the sequence. In some embodiments, the sequence is modified to replace CCC with CCG in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, or at least 30 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace every CCC with CCG as compared to **SEQ ID NO: 70**. In some embodiments, the nucleic acid comprises a viral vector sequence. In some embodiments, the viral vector sequence is a scAAV vector sequence. In some embodiments, the AAV vector sequence is of serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, or any combination thereof. In some embodiments, the AAV vector sequence is of the AAV2 serotype. In some embodiments, the viral vector sequence comprises sequences of at least two AAV serotypes. In some embodiments, the at least two serotypes are selected from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV8, AAV9, AAV11, and AAV12. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises at least about 60% sequence identity or similarity with any one of **SEQ ID NOS: 13-19, 21-27, 31, 62, 64, 66,** or **68**. In some embodiments, the sequence identity is from about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100%. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100% sequence identity to **SEQ ID NO: 31**. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises the nucleic acid sequence of **SEQ ID NO: 31**. In some embodiments, the isolated, non-naturally occurring nucleic acid consists of the nucleic acid sequence of **SEQ ID NO: 31**. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100% sequence identity to **SEQ ID NO: 66**. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises the nucleic acid sequence of **SEQ ID NO: 66**. In some embodiments, the isolated, non-naturally occurring nucleic acid consists of the nucleic acid sequence of **SEQ ID NO: 66**. In some embodiments, the viral vector sequence is single-stranded. In some embodiments, the viral vector sequence is double-stranded. In some embodiments, the isolated, non-naturally occurring nucleic acid is single-stranded. In some embodiments, the isolated, non-naturally occurring nucleic acid is double-stranded. In some embodiments, the isolated, non-naturally occurring nucleic acid, upon

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contacting with a plurality of cells, increases expression of the biologic post transfection or post transduction in the plurality of cells as compared to an otherwise comparable isolated, non-naturally occurring nucleic acid that lacks the otherwise comparable sequence lacking the modification in a comparable plurality of cells. In some embodiments, the increased expression comprises at least a 5-fold, at least a 10-fold, at least a 20-fold, at least a 50-fold, at least a 100-fold, at least a 200-fold, or at least a 500-fold increase as determined by enzyme-linked immunoassay (ELISA) assay.

[0006] Described herein, in some aspects, is an isolated, non-naturally occurring nucleic acid that comprises at least about 60% sequence identity or similarity with any one of the nucleic acid sequences of **SEQ ID NOS: 13-19, 21-27, 31, 62, 64, 66,** or **68**. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises the nucleic acid sequence of **SEQ ID NO: 31**. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises the nucleic acid sequence of **SEQ ID NO: 66**.

[0007] Described herein, in some aspects, is a biologic encoded by an isolated non-naturally occurring nucleic acid described herein. In some embodiments, the biologic comprises at least about 60% sequence identity with **SEQ ID NO: 12**. In some embodiments, the sequence identity is from about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100%.

[0008] Described herein, in some aspects, is an engineered cell comprising an isolated, non-naturally occurring nucleic acid described herein.

[0009] Described herein, in some aspects, is a plurality of adeno-associated viral (AAV) particles comprising an isolated, non-naturally occurring nucleic acid described herein.

[0010] Described herein, in some aspects, is a composition comprising a plurality of AAV particles described herein in unit dosage form. In some embodiments, the composition is cryopreserved.

[0011] Described herein, in some aspects, is a container comprising: an isolated, non-naturally occurring nucleic acid described herein; a biologic described herein; an engineered cell described herein, or a plurality of AAV particles described herein.

[0012] Described herein, in some aspects, is a method of modifying cells, the method comprising contacting: (a) a plurality of cells with the isolated non-naturally occurring nucleic acid of any one of claims 1-88; (b) a plurality of cells with the plurality of adeno-associated viral (AAV) particles of claim 93; or (c) both (a) and (b).

[0013] Described herein, in some aspects, is a pharmaceutical composition comprising: an isolated, non-naturally occurring nucleic acid described herein; a biologic described herein; or a

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plurality of AAV particles described herein. In some embodiments, the pharmaceutical composition is for treating an ocular disease or condition. In some embodiments, the ocular disease or condition is selected from the group consisting of: Achromatopsia, Age-related macular degeneration (AMD), Diabetic retinopathy (DR), Glaucoma, Bardet-Biedl Syndrome, Best Disease, Choroideremia, Leber Congenital Amaurosis, Macular degeneration, Polypoidal choroidal vasculopathy (PCV), Retinitis pigmentosa, Refsum disease, Stargardt disease, Usher syndrome, X-linked retinoschisis (XLRS), Rod-cone dystrophy, Cone-rod dystrophy, Oguchi disease, Malattia Leventinese (Familial Dominant Drusen), and Blue-cone monochromacy. In some embodiments, the ocular disease or condition is AMD.

[0014] Described herein, in some aspects, is a method of treating a disease or condition in a subject in need thereof, the method comprising administering an effective amount of a pharmaceutical composition described herein to the subject, thereby treating the disease.

[0015] Described herein, in some aspects, is a method for treating a disease or condition in a subject in need thereof, the method comprising administering a therapeutically effective amount of a pharmaceutical composition that comprises an isolated, non-naturally occurring nucleic acid that comprises a sequence that encodes a biologic that comprises an anti-angiogenic agent, wherein the sequence is modified to replace non-AGG arginine codons with AGG in at least four codons of a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region, thereby treating the disease or condition in the subject in need thereof.

[0016] Described herein, in some aspects, is a method for treating a disease or condition in a subject in need thereof, the method comprising: administering a therapeutically effective amount of a pharmaceutical composition that comprises an isolated, non-naturally occurring nucleic acid that comprises a sequence that encodes a biologic that comprises an anti-angiogenic agent, wherein the sequence is modified to replace non-AGG arginine codons with AGG in at least four codons of a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region, and wherein the modification is effective in increasing a level of the biologic in the subject in need thereof as compared to an otherwise comparable subject administered an otherwise comparable isolated, non-naturally occurring nucleic acid lacking the modification. In some embodiments, the increased level of the biologic in the subject is at least a 5-fold, at least a 10-fold, at least a 20-fold, at least a 50-fold, at least a 100-fold, at least a 200-fold, or at least a 500-fold increased, as determined by a diagnostic assay. In some embodiments, the sequence that encodes the anti-angiogenic agent further comprises a second modification. In some embodiments, the second modification is in at least one codon of

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the coding region of the sequence, and wherein the second modification is selected from the group consisting of: (a) replacement of at least one non-CCC proline codon with CCC; (b) replacement of at least one non-TCC serine codon with TCC; (c) replacement of at least one non-CCG proline codon with CCG; and (d) any combination of (a)-(c). In some embodiments, the second modification comprises (a). In some embodiments, the second modification comprises (b). In some embodiments, the second modification comprises (c). In some embodiments, the second modification comprises (d).

[0017] Described herein, in some aspects, is a method for treating a disease or condition in a subject in need thereof, the method comprising administering a therapeutically effective amount of a pharmaceutical composition that comprises an isolated, non-naturally occurring nucleic acid that comprises a sequence that encodes a biologic that comprises an anti-angiogenic agent, wherein the sequence is modified to replace AGA with AGG in at least four codons of a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region, thereby treating the disease or condition in the subject in need thereof.

[0018] Described herein, in some aspects, is a method for treating a disease or condition in a subject in need thereof, the method comprising: administering a therapeutically effective amount of a pharmaceutical composition that comprises an isolated, non-naturally occurring nucleic acid that comprises a sequence that encodes a biologic that comprises an anti-angiogenic agent, wherein the sequence is modified to replace AGA with AGG in at least four codons of a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region, and wherein the modification is effective in increasing a level of the biologic in the subject in need thereof as compared to an otherwise comparable subject administered an otherwise comparable isolated, non-naturally occurring nucleic acid lacking the modification. In some embodiments, the increased level of the biologic in the subject is at least a 5-fold, at least a 10-fold, at least a 20-fold, at least a 50-fold, at least a 100-fold, at least a 200-fold, or at least a 500-fold increased, as determined by a diagnostic assay. In some embodiments, the sequence that encodes the anti-angiogenic agent further comprises a second modification. In some embodiments, the second modification is in at least one codon of the coding region of the sequence, and wherein the second modification is selected from the group consisting of: (a) CCT to CCC; (b) AGC to TCC; (c) CCC to CCG; and (d) any combination of (a)-(c). In some embodiments, the second modification comprises (a). In some embodiments, the second modification comprises (b). In some embodiments, the second modification comprises (c). In some embodiments, the second modification comprises (d). In some embodiments, the anti-

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angiogenic agent comprises: a VEGF inhibitor, a multi-tyrosine kinase inhibitor, a receptor tyrosine kinase inhibitor, or an inhibitor of Akt phosphorylation. In some embodiments, the anti-angiogenic agent comprises the VEGF inhibitor. In some embodiments, the VEGF inhibitor is a non-antibody inhibitor. In some embodiments, the non-antibody inhibitor comprises a fusion protein that comprises human VEGF receptors 1 and 2, and wherein the fusion protein comprises VEGF-Trap or a modified version thereof. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises at least about 60% sequence identity or similarity with any one **SEQ ID NOS: 13-19, 21-27, 31, 62, 64, 66,** or **68**. In some embodiments, the sequence identity is from about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100%. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100% sequence identity to **SEQ ID NO: 31**. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises the nucleic acid sequence of **SEQ ID NO: 31**. In some embodiments, the isolated, non-naturally occurring nucleic acid consists of the nucleic acid sequence of **SEQ ID NO: 31**. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100% sequence identity to **SEQ ID NO: 66**. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises the nucleic acid sequence of **SEQ ID NO: 66**. In some embodiments, the isolated, non-naturally occurring nucleic acid consists of the nucleic acid sequence of **SEQ ID NO: 66**. In some embodiments, the nucleic acid comprises a viral vector sequence. In some embodiments, the viral vector sequence is a scAAV vector sequence. In some embodiments, the AAV vector sequence is of serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, or any combination thereof. In some embodiments, the AAV vector sequence is of the AAV2 serotype. In some embodiments, the administering is via intravitreal injection, subretinal injection, microinjection, or supraocular injection. In some embodiments, the administering is via intravitreal injection. In some embodiments, the ocular disease or condition is selected from the group consisting of: Achromatopsia, Age-related macular degeneration (AMD), Diabetic retinopathy (DR), Glaucoma, Bardet-Biedl Syndrome, Best Disease, Choroideremia, Leber Congenital Amaurosis, Macular degeneration, Polypoidal choroidal vasculopathy (PCV), Retinitis pigmentosa, Refsum disease, Stargardt disease, Usher syndrome, X-linked retinoschisis (XLRS), Rod-cone dystrophy, Cone-rod dystrophy, Oguchi disease, Malattia Leventinese (Familial Dominant Drusen), and Blue-cone monochromacy. In some embodiments, the ocular disease or condition is AMD. In

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some embodiments, the AMD is wet AMD. In some embodiments, the AMD is dry AMD. In some embodiments, the administering is sufficient to reduce at least a symptom of the disease or condition, treat the disease or condition, and/or eliminate the disease or condition. In some embodiments, the administering comprises delivering a dosage of the isolated, non-naturally occurring nucleic acid of about 1.0×10^9 vg, about 1.0×10^{10}, about 1.0×10^{11} vg, about 3.0×10^{11} vg, about 6×10^{11} vg, about 8.0×10^{11} vg, about 1.0×10^{12} vg, about 1.0×10^{13} vg, about 1.0×10^{14} vg, or about 1.0×10^{15} vg. In some embodiments, the administering is repeated. In some embodiments, the administering is performed: twice daily, every other day, twice a week, bimonthly, trimonthly, once a month, every other month, semiannually, annually, or biannually. In some embodiments, prior to the administering, the subject undergoes genetic testing. In some embodiments, the genetic testing detects a mutation in a gene sequence as compared to an otherwise comparable wild type sequence. In some embodiments, the method further comprises administering a secondary therapy. In some embodiments, the secondary therapy comprises at least one of: photodynamic therapy (PDT), an anti-inflammatory agent, an anti-microbial agent, and laser photocoagulation therapy (LPT).

[0019] Disclosed herein are isolated non-naturally occurring nucleic acids that can comprise a sequence that encodes a biologic that can comprise an anti-angiogenic agent. In some embodiments, a sequence can be modified to replace AGA with AGG in at least one codon of a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region. In some embodiments, a sequence that encodes an anti-angiogenic agent can further comprise a second modification. In some embodiments, a second modification can be in at least one codon of the coding region of the sequence. In some embodiments, a second modification can be selected from the group comprising: a) CCT to CCC; b) AGC to TCC; c) CCC to CCG; or d) a combination of any of these. In some embodiments, a second modification can comprise CCT to CCC. In some embodiments, a second modification can comprise AGC to TCC. In some embodiments, a second modification can comprise CCC to CCG. In some embodiments, a second modification can comprise a combination of: CCT to CCC; AGC to TCC; or CCC to CCG. In some embodiments, an anti-angiogenic agent can comprise: a VEGF inhibitor, a multi-tyrosine kinase inhibitor, a receptor tyrosine kinase inhibitor, an inhibitor of Akt phosphorylation, a PDGF-1 inhibitor, a PDGF-2 inhibitor, a NP-1 inhibitor, a NP-2 inhibitor, a Del 1 inhibitor, or an integrin inhibitor. In some embodiments, an anti-angiogenic agent can comprise a VEGF inhibitor, and the VEGF inhibitor can be a non-antibody inhibitor. In some embodiments, a non-antibody inhibitor can be a fusion protein that can comprise human VEGF receptors 1 and 2. In some embodiments, a fusion protein can

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comprise VEGF-Trap or a modified version thereof. In some embodiments, an isolated non-naturally occurring nucleic acid can further comprise a signal peptide. In some embodiments, a signal peptide can be selected from the group consisting of: human antibody heavy chain (Vh), human antibody light chain (Vl), and VEGF-Trap. In some embodiments, a signal peptide can be from a human antibody heavy chain. In some embodiments, a signal peptide can be from an VEGF-Trap. In some embodiments, an isolated non-naturally occurring nucleic acid can further comprise an intronic sequence. In some embodiments, an intronic sequence can be selected from the group consisting of: CMV intron A, adenovirus tripartite leader sequence intron, SV40 intron, hamster EF-1 alpha gene intron 1, intervening sequence intron, human growth hormone intron, and human beta globin intron. In some embodiments, an intronic sequence can be a SV40 intron. In some embodiments, an isolated non-naturally occurring nucleic acid can further comprise a promoter. In some embodiments, a promoter can be selected from the group consisting of: a cytomegalovirus (CMV) promoter, an elongation factor 1 alpha (EF1α) promoter, a simian vacuolating virus (SV40) promoter, a phosphoglycerate kinase (PGK1) promoter, a ubiquitin C (Ubc) promoter, a human beta actin promoter, a CAG promoter, a Tetracycline response element (TRE) promoter, a UAS promoter, an Actin 5c (Ac5) promoter, a polyhedron promoter, a Ca2+/calmodulin-dependent protein kinase II (CaMKIIa) promoter, a GAL1 promoter, a GAL 10 promoter, a TEF1 promoter, a glyceraldehyde 3-phosphage dehydrogenase (GDS) promoter, an ADH1 promoter, a CaMV35S promoter, a Ubi promoter, a human polymerase III RNA (H1) promoter, a U6 promoter, a polyadenylated construct thereof, and any combination thereof. In some embodiments, a promoter can be a CMV promoter. In some embodiments, a sequence can be modified to replace AGA with AGG in at least 2, at least 4, at least 6, at least 8, at least 10, at least 12, at least 14, at least 16, at least 18, or up to 20 codons of the coding region of the sequence. In some embodiments, a sequence can be modified to replace AGA with AGG in 16 codons of the coding region of the sequence.

[0020] In some embodiments, a sequence can be modified to replace AGA with AGG at positions: X1-X16 as compared to **SEQ ID NO: 28**. In some embodiments, a sequence can be modified to replace CCT with CCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or up to 30 codons of the coding region of the sequence. In some embodiments, a sequence can be modified to replace CCT with CCC in 30 codons of the coding region of the sequence. In some embodiments, a sequence can be modified to replace CCT with CCC at positions: X1-X30 as compared to **SEQ ID NO: 28**. In some embodiments, a sequence can be modified to replace AGC with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30, at least

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33, or up to 36 codons of the coding region of the sequence. In some embodiments, a sequence can be modified to replace AGC with TCC in 36 codons of the coding region of the sequence. In some embodiments, a sequence can be modified to replace AGC with TCC at positions: X1-X36 as compared to **SEQ ID NO: 28**. In some embodiments, a sequence can be modified to replace CCC with CCG in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or up to 30 codons of the coding region of the sequence. In some embodiments, a sequence can be modified to replace CCC with CCG in 29 codons of the coding region of the sequence. In some embodiments, a sequence can be modified to replace CCC with CCG at positions: X1-X29 as compared to **SEQ ID NO: 28**. In some embodiments, a nucleic acid can comprise a viral vector sequence. In some embodiments, a viral vector sequence can be a self-complementary AAV (scAAV) vector sequence. In some embodiments, a AAV vector sequence can be of serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, or any combination thereof. In some embodiments, an AAV vector sequence can be of the AAV2 serotype. In some embodiments, a viral vector sequence can comprise sequences of at least 2 AAV serotypes. In some embodiments, at least two serotypes can be selected from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV8, AAV9, AAV11, and AAV12. In some embodiments, an isolated non-naturally occurring nucleic acid can comprise a sequence having at least 60% sequence identity or similarity with any one of **SEQ ID NO: 13 - SEQ ID NO: 19**. In some embodiments, a sequence identity can be from about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100%. In some embodiments, an isolated non-naturally occurring nucleic acid, upon contacting with a plurality of cells, can have increased expression of a biologic post transfection or post transduction in a plurality of cells as compared to an otherwise comparable isolated non-naturally occurring nucleic acid that may lack the otherwise comparable sequence lacking the modification in a comparable plurality of cells. In some embodiments, increased expression can comprise at least a 5-fold, at least a 10-fold, at least a 20-fold, at least a 50-fold, at least a 100-fold, at least a 200-fold, or at least a 500-fold increase as determined by enzyme-linked immunoassay (ELISA) assay.

[0021] Also described herein are isolated non-naturally occurring nucleic acids that can comprise at least 60% sequence identity or similarity with any one of the nucleic acid sequences of **SEQ ID NO: 13 - SEQ ID NO: 19** or **SEQ ID NO: 21 - SEQ ID NO: 27**. In some embodiments, a biologic can be encoded by an isolated non-naturally occurring nucleic acid. In some embodiments, a biologic can comprise at least 60% sequence identity with **SEQ ID NO: 12**. In

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some embodiments, the sequence identity can be from about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100%.

[0022] Also described herein is an engineered cell comprising an isolated non-naturally occurring nucleic acid described herein.

[0023] Also described herein are a plurality of adeno-associated viral (AAV) particles that can be isolated from an engineered cell.

[0024] Also described herein are compositions that can comprise AAV particles in unit dosage form. In some embodiments, a composition can be cryopreserved.

[0025] Also described herein are containers that can comprise a) an isolated non-naturally occurring nucleic acid; b) a biologic; c) an engineered cell, or d) a plurality of AAV particles.

[0026] Also described herein are methods of modifying cells that can comprise contacting: a) a plurality of cells with an isolated non-naturally occurring nucleic acid; and/or b) a plurality of cells with a plurality of adeno-associated viral (AAV) particles.

[0027] Also described herein are pharmaceutical compositions that can comprise a) an isolated non-naturally occurring nucleic acid b) a biologic; c) or a plurality of AAV particles. In some embodiments, a pharmaceutical can be for treating an ocular disease or condition. In some embodiments, an ocular disease or condition can be selected from the group consisting of: Achromatopsia, Age-related macular degeneration (AMD), Diabetic retinopathy (DR), Glaucoma, Bardet-Biedl Syndrome, Best Disease, Choroideremia, Leber Congenital Amaurosis, Macular degeneration, Polypoidal choroidal vasculopathy (PCV), Retinitis pigmentosa, Refsum disease, Stargardt disease, Usher syndrome, X-linked retinoschisis (XLRS), Rod-cone dystrophy, Cone-rod dystrophy, Oguchi disease, Malattia Leventinese (Familial Dominant Drusen), and Blue-cone monochromacy. In some embodiments, an ocular disease or condition can be AMD.

[0028] Also described herein are methods of treating a disease or condition in a subject in need thereof that can comprise administering an effective amount of a pharmaceutical composition to the subject, thereby treating the disease.

[0029] Also described herein are methods for treating a disease or condition in a subject in need thereof. In some embodiments, a method can comprise administering a therapeutically effective amount of a pharmaceutical composition that can comprise an isolated non-naturally occurring nucleic acid that can comprise a sequence that encodes a biologic that can comprise an anti-angiogenic agent. In some embodiments, a sequence can be modified to replace AGA with AGG in at least one codon of a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region. In some embodiments, a method can treat a disease or condition in a subject in need thereof.

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[0030] Also described herein are methods for treating a disease or condition in a subject in need thereof. In some embodiments, a method can comprise administering a therapeutically effective amount of a pharmaceutical composition that can comprise an isolated non-naturally occurring nucleic acid that can comprise a sequence that encodes a biologic that can comprise an anti-angiogenic agent. In some embodiments, a sequence can be modified to replace AGA with AGG in at least one codon of a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region. In some embodiments, a modification can be effective in increasing a level of the biologic in a subject in need thereof as compared to an otherwise comparable subject administered an otherwise comparable isolated non-naturally occurring nucleic acid lacking the modification. In some embodiments, an increased level of the biologic in the subject can be at least a 5-fold, at least a 10-fold, at least a 20-fold, at least a 50-fold, at least a 100-fold, at least a 200-fold, or at least a 500-fold increased, as determined by a diagnostic assay. In some embodiments, a sequence that can encode an antiangiogenic agent can further comprise a second modification. In some embodiments, a second modification can be in at least one codon of the coding region of the sequence. In some embodiments, a second modification can be selected from the group comprising: a) CCT to CCC; b) AGC to TCC; c) CCC to CCG; or d) a combination of any of these. In some embodiments, a second modification can comprise CCT to CCC. In some embodiments, a second modification can comprise AGC to TCC. In some embodiments, a second modification can comprise CCC to CCG. In some embodiments, a second modification can comprise any combination of: CCT to CCC, AGC to TCC, or CCC to CCG . In some embodiments, an anti-angiogenic agent can comprise: a VEGF inhibitor, a multi-tyrosine kinase inhibitor, a receptor tyrosine kinase inhibitor, or an inhibitor of Akt phosphorylation. In some embodiments, an anti-angiogenic agent can comprise a VEGF inhibitor. In some embodiments, an anti-angiogenic can comprise a VEGF inhibitor. In some embodiments, an VEGF inhibitor can be a non-antibody inhibitor. In some embodiments, a non-antibody inhibitor can comprise a fusion protein that can comprise human VEGF receptors 1 and 2. In some embodiments, a fusion protein can comprise VEGF-Trap or a modified version thereof. In some embodiments, an isolated non-naturally occurring nucleic acid can comprise at least 60% sequence identity or similarity with any one of **SEQ ID NO: 13 - SEQ ID NO: 19** or **SEQ ID NO: 21 - SEQ ID NO: 27**. In some embodiments, the sequence identity can be from about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100%. In some embodiments, a nucleic acid can comprise a viral vector sequence. In some embodiments, a viral vector sequence can be a scAAV vector sequence. In some embodiments, an AAV vector sequence can be of serotype AAV1, AAV2,

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AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, or any combination thereof. In some embodiments, an AAV vector sequence can be of the AAV2 serotype. In some embodiments, administering can be via intravitreal injection, subretinal injection, microinjection, or supraocular injection. In some embodiments, administering can be via intravitreal injection. In some embodiments, an ocular disease or condition can be selected from the group consisting of: Achromatopsia, Age-related macular degeneration (AMD), Diabetic retinopathy (DR), Glaucoma, Bardet-Biedl Syndrome, Best Disease, Choroideremia, Leber Congenital Amaurosis, Macular degeneration, Polypoidal choroidal vasculopathy (PCV), Retinitis pigmentosa, Refsum disease, Stargardt disease, Usher syndrome, X-linked retinoschisis (XLRS), Rod-cone dystrophy, Cone-rod dystrophy, Oguchi disease, Malattia Leventinese (Familial Dominant Drusen), and Blue-cone monochromacy. In some embodiments, an ocular disease or condition can be AMD.

[0031] In some embodiments, AMD can be wet AMD. In some embodiments, AMD can be dry AMD. In some embodiments, administering can be sufficient to reduce at least a symptom of a disease or condition, treat a disease or condition, and/or eliminate a disease or condition. In some embodiments, administering can comprise delivering a dosage of an isolated non-naturally occurring nucleic acid of about 1.0×10^9 vg, about 1.0×10^{10}, about 1.0×10^{11} vg, about 3.0×10^{11} vg, about 6×10^{11} vg, about 8.0×10^{11} vg, about 1.0×10^{12} vg, about 1.0×10^{13} vg, about 1.0×10^{14} vg, or about 1.0×10^{15} vg. In some embodiments, administering can be repeated. In some embodiments, administering can be performed: twice daily, every other day, twice a week, bimonthly, trimonthly, once a month, every other month, semiannually, annually, or biannually. In some embodiments, prior to administering a subject can undergo genetic testing. In some embodiments, genetic testing can detect a mutation in a gene sequence as compared to an otherwise comparable wild type sequence. In some embodiments, a method can further comprise administering a secondary therapy. In some embodiments, a secondary therapy can comprise at least one of: photodynamic therapy (PDT), an anti-inflammatory agent, an anti-microbial agent, or laser photocoagulation therapy (LPT).

INCORPORATION BY REFERENCE

[0032] 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

[0033] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained

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by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0034] Fig. 1 shows scAAV constructs carrying the CMV enhancer/promoter, SV40 intron (INT), Kozak sequence (K), original (Af) or human IgG1 heavy chain (Vh) signal peptide (Sig), coding sequence, and synthetic polyadenylation signal (pA) flanked by a full AAV2 ITR (F-ITR) and a truncated AAV2 ITR (d-ITR). AMI059, AMI066, AMI067, AMI068, AMI119, AMI120, and AMI130 show different VEGF-Trap coding sequences with different signal peptides. AMI059 has an Af signal peptide and a low GC content coding sequence. AMI066 has a Vh signal peptide and a low GC content coding sequence. AMI067 has an Af signal peptide and a high GC content coding sequence. AMI068 has a Vh signal peptide and a high GC content coding sequence. AMI119 has an Af signal peptide and a high GC content coding sequence containing 16 Arg codon changes from AGA to AGG and 29 Pro codon changes from CCC to CCT. AMI120 has an Af signal peptide and a high GC content coding sequence containing 16 Arg codon changes from AGA to AGG and 36 Ser codon changes from AGC to TCC. AMI130 has an Af signal peptide and a high GC content coding sequence containing 16 Arg codon changes from AGA to AGG and 29 Pro codon changes from CCC to CCG.

[0035] Fig. 2 shows a Western blot analysis of VEGF-Trap expression in HEK293 cells transfected with plasmid DNA. Lanes 1-5 are shown on a non-reducing gel; lanes 6-10 are shown on a reducing gel; lanes 1 and 6 were loaded with supernatants from plasmid AMI059 transfected cells; lanes 2 and 7 were loaded with supernatants from plasmid AMI066 transfected cells; lanes 3 and 8 were loaded with supernatants from plasmid AMI067 transfected cells; lanes 4, 5, 9, and 10 were loaded with supernatants from plasmid AMI068 transfected cells.

[0036] Figs. 3A and **3B** show an SDS-PAGE and Simply Blue Staining analysis of low-GC and high-GC AAV vector production in Sf9 cells. In both figures, M indicates the lane loaded with a size marker and sizes are indicated for kilodaltons. In **Fig. 3A**, lane 1 shows AAV5 vector loaded as control; lanes 2, 3, 4 and 5 show AMI059, AMI066, AMI067, and AMI068, respectively. In **Fig. 3B**, Lane 1 shows AAV2 vector loaded as control; Lanes 2, 3, and 4 show AMI119, AMI120, and AMI130, respectively.

[0037] Figs. 4A and **4B** show a Western blot analysis of VEGF-Trap expression in HEK293 cells transduced with scAAV2 variant (AAV2.N53) vectors (AMI059, AMI066, AMI067, AMI068, AMI120, AMI119, AMI067, and AMI130). Twenty microliters of supernatants from AAV2 variant transduced HEK293 cells were loaded in each lane. In **Fig. 4A,** lanes 1, 2, 3 and 4 show AMI059, AMI066, AMI067, and AMI068, respectively. In **Fig. 4B**, lanes 1, 2, 3 and 4 show, AMI120, AMI119, AMI067, and AMI130, respectively.

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[0038] Fig. 5A shows an SDS-PAGE and Simply Blue Staining analysis of VEGF-Trap purified from the supernatant of scAAV2-AMI067 transduced HEK293 cells. Commercially available aflibercept (A) was used as control. M indicates the lane loaded with a size marker and sizes are indicated for kilodaltons. 1.5 µg/lane of purified protein was loaded in the lane labeled V.

[0039] Fig. 5B and **Fig. 5C** illustrate binding affinity of AAV2.N54.120 (AVMX-110) derived VEGF-Trap to VEGF-A_{165}.

[0040] Fig. 6 shows amino acid sequence (**SEQ ID NO: 61**) and nucleic sequence (**SEQ ID NO: 62**) of AMI068-pFB-scCMV-SV40-intron-kozak-Vh-VEGF-Trap-GC. The bold letters indicate the human antibody heavy chain signal peptide.

[0041] Fig. 7 shows amino acid sequence (**SEQ ID NO: 63**) and nucleic acid sequence (**SEQ ID NO: 64**) of AMI119-pFB-scCMV-SV40-intron-kozak-Af-VEGF-Trap-GCRP (CCT). The bold letters indicate the VEGF-Trap signal peptide, an underlines indicate the 16 AGA to AGG changes and italics indicates the 30 CCC to CCT changes.

[0042] Fig. 8 shows amino acid sequence (**SEQ ID NO: 65**) and nucleic acid sequence (**SEQ ID NO: 66**) of AMI120-pFB-scCMV-SV40-intron-kozak-Af-VEGF-Trap-GCRS (TCC). The bold letters indicate the VEGF-Trap signal peptide, the underlines indicate the 16 AGA to AGG changes and italics indicate the 36 AGC to TCC changes.

[0043] Fig. 9 shows amino acid sequence (**SEQ ID NO: 67**) and nucleic acid sequence (**SEQ ID NO: 68**) of AMI130-pFB-scCMV-SV40-intron-kozak-Af-VEGF-Trap-GCRP (CCG). The bold letters indicate the VEGF-Trap signal peptide, the underlines indicate the 16 AGA to AGG changes and italics indicates the 29 CCC to CCG changes.

[0044] Fig. 10A illustrates determination of AVMX-110 DNA and protein concentration.

[0045] Fig. 10B shows a time course experiment of AAV2.VEGF-Trap expression level in HEK93 cell culture.

[0046] Fig. 11 shows durability of AAV2.N53-VEGF-Trap and AAV2.N54-VEGF-Trap expression in HEK293 cells (top panel) and human retina cells (ARPE-19, bottom panel).

[0047] Fig. 12 shows AAV2.054-AMI120 derived VEGF-Trap was glycosylated similarly to aflibercept on reducing or non-reducing gel (reducing Gel: with and without PNGase F treatment. non-reducing gel: with and without PNGase F treatment). Lane 1 and 3 show AAV2-VEGF-Trap, while lane 2 depicts commercial aflibercept. Multiple bands in the PNGase F treatment lane indicate that the deglycosylation was not complete.

[0048] Fig. 13 shows binding affinity of AAV2.054-AMI120 derived VEGF-Trap to human VEGF-A_{165} detected by Biacore assay.

[0049] Fig. 14 shows binding affinity of vector expressed VEGF-Trap to rhVEGF-A_{165}.

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[0050] Fig. 15 shows comparison of vector derived VEGF-Trap to VEGF-Trap in inhibition of HUVEC cell proliferation.

[0051] Fig. 16 illustrates day 7 GFP fundus images prior to angiography in Example 11.

[0052] Fig. 17 illustrates day 7 fluorescein angiography in Example 11. Representative images from each group are shown.

[0053] Fig. 18 illustrates quantification of fluorescein leakage on day 7 post-laser treatment plotted as averages (top) and individual values (bottom). * signifies p=0.0113 and *** signifies p=0.0002.

[0054] Fig. 19 illustrates representative lesion images from Flatmount on day 7 post-laser treatment.

[0055] Fig. 20 illustrates quantification of Flatmount lesion area on day 7 post-laser treatment plotted as averages (top) and individual values (bottom).

[0056] Fig. 21 illustrates fundus images of in Example 12.

[0057] Fig. 22 illustrates IHC images of mouse eyes administered with selected constructs in Example 12.

[0058] Fig. 23 illustrates fundus imaging of mouse eyes administered with selected AAV constructs performed on day 24 for the study.

[0059] Fig. 24 illustrates IHC images of pig eyes administered with selected constructs in Example 12.

[0060] Fig. 25 illustrates fundus imaging of pig eyes administered with selected constructs performed on day 24 after injection.

[0061] Fig. 26 illustrates immunohistochemistry images on day 28 of the pig study.

[0062] Fig. 27 illustrates confocal microscopic images comparing AMI054 and V226 capsid penetrability.

[0063] Fig. 28A illustrates AAV construct details for AVMX-110/116.

[0064] Fig. 28B illustrates an exemplary chromatogram showing the manufacturing of a vector described herein (AVMX-110) as indicated by a single and sharp fraction (arrow).

[0065] Fig. 28C illustrates an exemplary SDS-PAGE showing the expression of AAV VP1, VP2, and VP3.

[0066] Fig. 28D illustrates retention difference of empty and full AAV in separation column process.

[0067] Fig. 28E illustrates separation of empty capsid for the purified AVMX-110.

[0068] Fig. 28F illustrates exemplary SDS-PAGE detected with mouse anti-AAV2 antibody. Samples in lanes 1 and 2 were CsCl purified AVMX-110l; lanes 3 and 4 were samples from 2L

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shake; 5-8 were samples from 2L bioreactors; and lanes 9 and 10 were the final purified drug substance at 10 & 20 µL per lane. The Western blot with AAV2 specific antibody reaction demonstrates AVMX-110 as an AAV2 specific serotype.

[0069] Fig. 28G. illustrates exemplary silver stain image of AVMX-110 separated by 10% SDS-PAGE. Lanes 1 and 5: samples of capture eluate. Lanes 2 and 6: empty. Lanes 3 and 7: non-reducing samples from peak 2 of the column chromatography. The purity of AVMX-110 process intermediates was analyzed by SDS-PAGE stained with silver stain gel. AAV VP1, VP2 and VP3 were clearly visualized, and no single impurity high than 4% was found.

[0070] Fig. 29 illustrates representative images of the fluorescence angiograph (FA) data from different study groups and bar graph showing the efficacy. Statistical analysis: one-way ANOVA followed by Tukey's multiple comparison (* = 0.0332; *** = 0.0002).

[0071] Fig. 30 illustrates *in vitro* cell based assay for aflibercept expression in different serotypes.

[0072] Fig. 31 illustrates FA data for different AAV serotypes. ΔAflibercept was sham vector that did not generate protein. Statistics was performed by one-way ANOVA followed by Dunnett's multiple comparison (* = 0.0332; ** = 0.0021; *** = 0.0002).

[0073] Fig. 32 illustrates ELISA to quantify the aflibercept expression in ocular sample and serum samples. Statistics was performed using one-way ANOVA followed by Dunnett's multiple comparison (*** = 0.0002; **** = <0.0001).

[0074] Fig. 33 illustrates FA data for mouse CNV study with different AAV6.N54-Aflibercept vectors.

[0075] Fig. 34 illustrates comparison of AAV1, AAV2 and AAV6-GFP expression using IHC images.

[0076] Fig. 35 illustrates comparison of the expression of GFP in different serotypes injected in pig eyes.

[0077] Fig. 36 illustrates *in vitro* comparison of wild type (wt) AAV2 and N54-AAV2.

[0078] Fig. 37A illustrates comparison of GFP expression in HEK293 cells transduced with different lots of N54-GFP.

[0079] Fig. 37B illustrates GFP expression in HEK293 transduced with different lots of N54-GFP.

[0080] Fig. 38A illustrates FA measurement comparison and statistical analysis.

[0081] Fig. 38B illustrates FA representative images in Example 13. Arrows indicate the leaser lesions (bubbles).

[0082] Fig. 39A illustrates comparison of VEGF-Trap level in serum sample.

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[0083] Fig. 39B illustrates comparison VEGF-Trap level in ocular sample.

[0084] Fig. 40A illustrates correlation between VEGF-Trap expression and lesion area.

[0085] Fig. 40B illustrates comparison of the AAV2 capsid protein level in ocular sample.

[0086] Fig. 41A illustrates that the aflibercept expression in the animals treated with the sham vector control and untreated animals was similar. In Group 3 animals that were injected with AAV6.N54-Aflibercept, about 2.3 pg aflibercept/mg of ocular homogenate was measured (**Fig. 41A**). AAV2 and the mid-range level for AAV6.N54-Aflibercept had about 8.8 and about 25 pg aflibercept/mg of ocular homogenate respectively, which was a threefold-elevated expression for the AAV6-treated animals. High dose-AAV6 treated animals showed the highest level of aflibercept, with about 400 pg aflibercept/mg of ocular homogenate. Serum samples followed a similar trend as the ocular tissue samples. Groups 1 (Sham), 2 (AAV2-Aflibercept) and 3 (AAV6-low dose) had no detectable aflibercept. Both the medium and high dose AAV6-Aflibercept treated animals showed 2-3 ng/mL aflibercept in the serum (**Fig. 41B**). The data were analyzed in GraphPad Prism software using a one-way ANOVA followed by Dunnett's multiple comparison (** = 0.005 and **** = <0.0001).

[0087] Fig. 42 illustrates representative images from day 0 fundus imaging (Groups 6-8).

[0088] Fig. 43 illustrates representative images from day 7 fluorescein angiography.

[0089] Fig. 44 illustrates quantification of average fluorescein Leakage on day 7.

[0090] Fig. 45 illustrates representative images of Isolectin lesion area.

[0091] Fig. 46 illustrates Isolectin area measurements

[0092] Fig. 47 illustrates representative Images of Day 0 IHC (Groups 6-8 Only).

[0093] Fig. 48A illustrates Flatmount analysis of the images obtained from the dose response study (Example 14).

[0094] Fig. 48B illustrates FA analysis of the images obtained from the dose response study (Example 14).

[0095] Fig. 49A illustrates laser-caused lesion area and AVMX-110 dose responsive curve.

[0096] Fig. 49B illustrates FA analysis of the images from the AVMX-110 dosing study.

[0097] Fig. 50 illustrates fluorescent angiography images of AVMX-110 in mouse LCNV model.

[0098] Fig. 51A illustrates VEGF-Trap levels in sera of animals (n=6 per group).

[0099] Fig. 51B illustrates that VEGF-Trap levels in retina tissue was also measured in the retina homogenates using the ELISA. In the retina tissue suspension, the expression of VEGF-Trap was in a dose dependent manner to the AVMX-110 injected intravitreally. An average of 13 ng/mL of VEGF-Trap was detected for the high dose group of 1.6e10 vg/eye. The highest expression level

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reached over 40 ng/mL.

DETAILED DESCRIPTION

[0100] Provided herein are compositions and methods for use in ocular therapies. In an aspect, provided herein is an isolated, non-naturally occurring nucleic acid. The nucleic acid can be utilized in a composition for use in ocular therapy of various ocular diseases and conditions. In some cases, nucleic acids can comprise one or more modifications that confer certain advantages over unmodified otherwise comparable nucleic acids.

[0101] In an aspect, an isolated, non-naturally occurring nucleic acid is provided herein that encodes a biologic. Biologics include a wide range of products such as vaccines, blood and blood components, allergenics, cells, gene therapies, tissues, and recombinant therapeutic proteins. Biologics can be composed of sugars, proteins, or nucleic acids or complex combinations of these substances, or may be living entities such as cells and tissues. Biologics are isolated from a variety of natural sources (e.g., human, animal, microorganism) and may be produced using various methods. Gene-based and cellular biologics, for example, often are at the forefront of biomedical research, and may be used to treat a variety of medical conditions for which no other treatments are available.

[0102] Biologics as disclosed herein can comprise an anti-angiogenic agent. Angiogenesis refers to the formation of new blood vessels and/or maintenance of existing vasculature. The process involves the migration, growth, and/or differentiation of endothelial cells, which line the inside wall of blood vessels. The process of angiogenesis is modulated at least in part by chemical signals in the body. Some of these signals, such as vascular endothelial growth factor (VEGF), bind to receptors on the surface of normal endothelial cells. When VEGF and other endothelial growth factors bind to their receptors on endothelial cells, signals within these cells are initiated that promote the growth and survival of new blood vessels. Other chemical signals, for example anti-angiogenics can interfere with blood vessel formation. Normally, the angiogenesis-stimulating and -inhibiting effects of these signals are balanced so that blood vessels form only when and where they are needed, such as during growth and healing. In cases of disease or various conditions, these signals can become unbalanced, causing increased or aberrant blood vessel growth that can lead to abnormal conditions or disease. For example, aberrant angiogenesis is a cause of wet age-related macular degeneration.

[0103] Anti-angiogenic agents can reduce or eliminate vascularization. Anti-angiogenic agents can reduce or eliminate neovascularization. Anti-angiogenic agents can also reduce or eliminate existing vasculature. Angiogenesis inhibitors interfere in several ways with various steps in blood

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vessel growth. Some are biologics such as monoclonal antibodies that specifically recognize and bind to VEGF and/or other anti-angiogenic agents. For example, when VEGF is bound to these agents, it is unable to activate the VEGF receptor. Other anti-angiogenic agents bind to VEGF and/or its receptor as well as to other receptors on the surface of endothelial cells or to other proteins in the downstream signaling pathways, blocking their activities. Some anti-angiogenic agents are immunomodulatory drugs (e.g., agents that stimulate or suppress the immune system) that also have anti-angiogenic properties.

[0104] In an embodiment, an anti-angiogenic agent binds to a component of the VEGF signaling pathway. Components of the VEGF signaling pathway include but are not limited to: PLC, VRAP, Sck, Src, PI3K, PIP3, Akt, Bad, Caspase, eNOS, FAK, paxillin, Cdc42, p38 MAPK, Hsp27, GRB2, SOS, SHC, Ras, Raf, MEK1/2, ERK1/2, PKC, cPLA2, PGI2, IP3, and combinations thereof.

[0105] In an embodiment, a biologic is selected from a macromolecule such as a protein, peptide, aptamer, and/or non-translated RNAs, such as an antisense RNA, a ribozyme, an RNAi and/or an siRNA.

[0106] Biologics provided herein can comprise an anti-angiogenic agent. In some cases, a biologic is a protein or polypeptide. In some cases, a biologic comprises a polypeptide. A polypeptide can enhance and/or reduce one or more functions of an ocular cell, *e.g.*, a rod or cone photoreceptor cell, a retinal ganglion cell, a Müller cell, a bipolar cell, an amacrine cell, a horizontal cell, and/or a retinal pigmented epithelial cell.

[0107] Exemplary classes of polypeptides include but are not limited to: neuroprotective polypeptides (e.g., GDNF, CNTF, NT4, NGF, and NTN); anti-angiogenic polypeptides (e.g., a soluble vascular endothelial growth factor (VEGF) receptor; a VEGF-binding antibody; a VEGF-binding antibody fragment (e.g., a single chain anti-VEGF antibody); endostatin; tumstatin; angiostatin; a soluble Flt polypeptide (Lai et al. (2005) *Mol. Ther.* 12:659); an Fc fusion protein comprising a soluble Flt polypeptide (see, e.g., Pechan et al. (2009) *Gene Ther.* 16:10); pigment epithelium-derived factor (PEDF); a soluble Tie-2 receptor; etc.); tissue inhibitor of metalloproteinases-3 (TIMP-3); a light-responsive opsin, e.g., a rhodopsin; anti-apoptotic polypeptides (e.g., Bcl-2, Bcl-X1); and the like. Exemplary polypeptides include, but are not limited to, glial derived neurotrophic factor (GDNF); fibroblast growth factor 2; neurturin (NTN); ciliary neurotrophic factor (CNTF); nerve growth factor (NGF); neurotrophin-4 (NT4); brain derived neurotrophic factor (BDNF; epidermal growth factor; rhodopsin; X-linked inhibitor of apoptosis; and Sonic hedgehog.

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[0108] In some cases, a polypeptide can comprise retinoschisin, retinitis pigmentosa GTPase regulator (RGPR)-interacting protein-1 (see, e.g., GenBank Accession Nos. Q96KN7, Q9EPQ2, and Q9GLM3, peripherin-2 (Prph2) (see, e.g., GenBank Accession No. NP_000313, peripherin, a retinal pigment epithelium-specific protein (RPE65), (see, e.g., GenBank AAC39660; and Morimura et al. (1998) *Proc. Natl. Acad. Sci. USA* 95:3088), CHM (choroidermia (Rab escort protein 1)), a polypeptide that, when defective or missing, causes choroideremia (see, e.g., Donnelly et al. (1994) Hum. Mol. Genet. 3:1017; and van Bokhoven et al. (1994) *Hum. Mol. Genet.* 3:1041); and Crumbs homolog 1 (CRB1), a polypeptide that, when defective or missing, causes Leber congenital amaurosis and retinitis pigmentosa (see, e.g., den Hollander et al. (1999) *Nat. Genet.* 23:217; and GenBank Accession No. CAM23328). Suitable polypeptides also include polypeptides that, when defective or missing, lead to achromotopsia, where such polypeptides include, e.g., cone photoreceptor cGMP-gated channel subunit alpha (CNGA3) (see, *e.g.*, GenBank Accession No. NP_001289; and Booij et al. (2011) *Ophthalmology* 118:160-167); cone photoreceptor cGMP-gated cation channel beta-subunit (CNGB3) (see, e.g., Kohl et al. (2005) *Eur J Hum Genet.* 13(3):302); guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2 (GNAT2) (ACHM4); and ACHM5; and polypeptides that, when defective or lacking, lead to various forms of color blindness.

[0109] In some cases, a biologic comprises a protein or polypeptide coding for a site-specific endonuclease that provides for site-specific knockdown or knockout of gene function, *e.g.*, where the endonuclease knocks out an allele associated with a retinal disease. For example, where a dominant allele encodes a defective copy of a gene that, when wild-type, is a retinal structural protein and/or provides for normal retinal function, a site-specific endonuclease can be targeted to the defective allele and knock out the defective allele. In addition to knocking out a defective allele, a site-specific nuclease can also be used to stimulate homologous recombination with a donor DNA that encodes a functional copy of the protein encoded by the defective allele. Thus, a non-naturally occurring nucleic acid can be used to deliver both a site-specific endonuclease that knocks out a defective allele, and can be used to deliver a functional copy of the defective allele, resulting in repair of the defective allele, thereby providing for production of a functional retinal protein (e.g., functional retinoschisin, functional RPE65, functional peripherin, etc.). See, e.g., Li et al. (2011) Nature 475:217. In some embodiments, a non-naturally occurring nucleic acid comprises a transgene that encodes a site-specific endonuclease; and a heterologous nucleotide sequence that encodes a functional copy of a defective allele, where the functional copy encodes a functional retinal protein.

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[0110] Exemplary functional retinal proteins include, e.g., retinoschisin, RPE65, retinitis pigmentosa GTPase regulator (RGPR)-interacting protein-1, peripherin, peripherin-2, and the like. Site-specific endonucleases that are suitable for use include, e.g., CRISPR, zinc finger nucleases (ZFNs); and transcription activator-like effector nucleases (TALENs), where such site-specific endonucleases are non-naturally occurring and are modified to target a specific gene. Such site-specific nucleases can be engineered to cut specific locations within a genome, and non-homologous end joining can then repair the break while inserting or deleting several nucleotides. Such site-specific endonucleases (also referred to as "INDELs") then throw the protein out of frame and effectively knock out the gene. See, e.g., U.S. Patent Publication No. 2011/0301073. In some cases, a protein or polypeptide biologic is selected from: Lipoprotein Lipase, Retinoid Isomerohydrolase RPE65, or complement H. In some cases, a biologic is a polypeptide such as a fusion protein, such as aflibercept.

[0111] In an aspect, a biologic is aflibercept. Aflibercept is also known as VEGF Trap-eye (VTE) and EYLEA® and can be used interchangeably herein. Aflibercept is a recombinant fusion protein comprising extracellular domains of human VEGF receptors 1 and 2 fused to the Fc portion of human IgG. In contrast to the antibody-based VEGF binding strategy used by ranibizumab and bevacizumab, aflibercept incorporates the second binding domain of the VEGFR-1 receptor and the third domain of the VEGFR-2 receptor. Aflibercept acts as a soluble decoy receptor that binds VEGF-A and PDGF with greater affinity than the native receptors. **SEQ ID NO: 30** illustrates aflibercept amino acid sequence aligned with DNA coding sequence (**SEQ ID NO: 31**) for aflibercept. For **SEQ ID NO: 31**, the nucleic acid comprises: an Af signal peptide; and a high GC content coding sequence containing 16 Arg codon changes from AGA to AGG and 36 Ser codon changes from AGC to TCC as compared with **SEQ ID NO: 70**.

[0112] The approved dose of intravitreal aflibercept injection is 2.0 mg, the dosing of which varies according to indication. Aflibercept is indicated for the treatment of neovascular (wet) age-related macular degeneration, macular edema following retinal vein occlusion, diabetic macular edema, and diabetic retinopathy. In some cases, a signal peptide provided herein can be from aflibercept or derived from aflibercept. In an embodiment, a signal peptide comprises a percent homology from about 50%, about 60%, about 70%, about 80%, about 85%, about 90%, about 95%, about 97%, about 98%, about 99%, or about 100% identity to a sequence (e.g., a signal peptide sequence) from aflibercept. In an embodiment, a signal peptide is from the human antibody heavy chain from aflibercept. In an embodiment, a signal peptide is from the human antibody light chain from aflibercept.

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[0113] In some cases, a biologic is an aptamer. An aptamer can be a DNA aptamer or RNA aptamer. Aptamers are oligonucleotides (single-stranded or double-stranded) that fold into defined architectures and bind to targets such as proteins. Unlike some protein-based biologics, aptamers do not elicit or elicit reduced antibodies as compared to a protein-based biologic because aptamers generally contain sugars modified (for example at their 2′-positions). Additionally, Toll-like receptor-mediated innate immune responses are also abrogated or reduced. Aptamer therapeutics can be developed for intracellular, extracellular, or cell-surface targets. In some aspects, a biologic therapeutic is an aptamer. Exemplary aptamers of interest include an aptamer against vascular endothelial growth factor (VEGF). See, e.g., Ng et al. (2006) *Nat. Rev. Drug Discovery* 5:123; and Lee et al. (2005) *Proc. Natl. Acad. Sci. USA* 102:18902. For example, a VEGF aptamer can comprise the nucleotide sequence 5′-cgcaaucagugaaugcuuauacauccg-3′ (**SEQ ID NO: 69**). Also suitable for use is a PDGF-specific aptamer, e.g., E10030; see, e.g., Ni and Hui (2009) *Ophthalmologica* 223:401; and Akiyama et al. (2006) *J. Cell Physiol.* 207:407). Exemplary aptamers include Pegaptanib. Pagaptanib is a 50 kDa aptamer that is a specific nucleic acid ligand binding to VEGF165. Exemplary aptamers include but are not limited to Pegaptanib or Fovista.

[0114] In an embodiment, a biologic is a nucleic acid. Nucleic acids can include but are not limited to: non-translated RNAs, such as an antisense RNA, a ribozyme, an RNAi and/or an siRNA. In an embodiment, a biologic therapeutic is an interfering RNA (RNAi). Suitable RNAi include RNAi that can reduce a level of an apoptotic or angiogenic factor in a cell. For example, an RNAi can be an shRNA or siRNA that reduces the level of a gene product that induces or promotes apoptosis in a cell. Genes whose gene products induce or promote apoptosis are referred to herein as "pro-apoptotic genes" and the products of those genes (mRNA; protein) are referred to as "pro-apoptotic gene products." Pro-apoptotic gene products include, e.g., Bax, Bid, Bak, and Bad gene products. See, e.g., U.S. Pat. No. 7,846,730. Interfering RNAs could also be against an angiogenic product, for example VEGF (e.g., Cand5; see, e.g., U.S. Patent Publication No. 2011/0143400; U.S. Patent Publication No. 2008/0188437; and Reich et al. (2003) Mol. Vis. 9:210), VEGFR1 (e.g., Sirna-027; see, e.g., Kaiser et al. (2010) Am. J. Ophthalmol. 150:33; and Shen et al. (2006) Gene Ther. 13:225), or VEGFR2 (Kou et al. (2005) Biochem. 44:15064). See also, U.S. Pat. Nos. 6,649,596, 6,399,586, 5,661,135, 5,639,872, and 5,639,736; and U.S. Pat. Nos. 7,947,659 and 7,919,473.

[0115] In some cases, a biologic comprises a siRNA that targets VEGF-A, for example Bevasiranib.

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[0116] In some embodiments, cell type-specific or a tissue-specific promoter can be operably linked to a transgene encoding for a subject therapeutic, such that the gene product is produced selectively or preferentially in a particular cell type(s) or tissue(s). In some embodiments, an inducible promoter may be operably linked to a transgene sequence. In some cases, a promoter can be operably linked to a photoreceptor-specific regulatory element (e.g., a photoreceptor-specific promoter), e.g., a regulatory element that confers selective expression of the operably linked gene in a photoreceptor cell. Suitable photoreceptor-specific regulatory elements include, e.g., a rhodopsin promoter; a rhodopsin kinase promoter (Young et al. (2003) Ophthalmol. Vis. Sci. 44:4076); a beta phosphodiesterase gene promoter (Nicoud et al. (2007) J. Gene Med. 9:1015); a retinitis pigmentosa gene promoter (Nicoud et al. (2007) supra); an interphotoreceptor retinoid-binding protein (IRBP) gene enhancer (Nicoud et al. (2007) supra); an IRBP gene promoter (Yokoyama et al. (1992) Exp Eye Res. 55:225) and the like.

[0117] In some embodiments, a biologic delivered by a subject modified AAV can act to inhibit angiogenesis. In an embodiment, a biologic comprises an anti-angiogenic agent. Exemplary anti-angiogenic agents can comprise: a VEGF inhibitor, Multi-tyrosine kinase inhibitor, Receptor tyrosine kinase inhibitor, inhibitor of Akt phosphorylation, PDGF-1 inhibitor, PDGF-2 inhibitor, NP-1 inhibitor, NP-2 inhibitor, Del 1 inhibitor, or/or integrin inhibitor. In an embodiment, the anti-angiogenic agent comprises a VEGF inhibitor. A VEGF inhibitor can target VEGF or the VEGF receptor.

[0118] The VEGF family includes placental growth factor (PLGF), VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E. These agents are important regulators of angiogenesis and vascular permeability; VEGF-A in particular, plays a pivotal role in pathologic ocular angiogenesis. The VEGF-A gene has been localized to chromosome 6p12.3 and comprises of 8 exons and 8 intermediate introns. VEGF-A has 9 isoforms including VEGF121, VEGF145, VEGF148, VEGF162, VEGF165, VEGF165b, VEGF183, VEGF189 and VEGF206. These isoforms differ from each other by the number of amino acids and heparin-binding affinity. Anyone of the aforementioned VEGF family members and/or their receptors can be targeted with an inhibitor.

[0119] In certain preferred embodiments, the biologic delivered by a subject modified AAV can act to inhibit the activity of one or more mammalian VEGF proteins selected from the group consisting of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and/or PDGF. In particularly preferred embodiments, the biologic delivered by the subject AAV variants inhibit the activity of VEGF-A. VEGF-A has 9 isoforms generated by alternative splicing, the most physiologically relevant of which is VEGF 165. VEGF-A levels have been found to be elevated in the vitreous of patients with wet age-related macular degeneration, diabetic macular edema and retinal vein occlusion.

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Gene product(s) which inhibit the activity of VEGF-A in the eye and which are therefore effective to treat patients with elevated vitreous VEGF-A include, but are not limited to, aflibercept, Ranibizumab, Brolucizumab, Bevacizumab, and soluble fms-like tyrosine kinase 1 (sFLTl) (GenBank Acc. No. U01134). In some embodiments, an infectious AAV virion is provided comprising (i) a variant AAV capsid protein as herein described and (ii) a transgene comprising a VEGF inhibitor. In an embodiment, a transgene comprises multiple sequences, each of which encodes a distinct VEGF-A inhibitor. In an embodiment, an anti-angiogenic is Ranibizumab. Ranibizumab is a humanized monoclonal antibody Fab fragment that inhibits all human isoforms of VEGF-A. In an embodiment, the transgene can be aflibercept.

[0120] In yet another embodiment, an isolated, non-naturally occurring nucleic acid provided herein comprises a modification. Various modifications are contemplated and can be employed for improved introduction, expression, persistence, and/or functionality of biologics that comprise anti-angiogenic agents as compared to otherwise comparable biologics. In some cases, an otherwise comparable biologic can be aflibercept.

[0121] In an embodiment, an isolated, non-naturally occurring nucleic acid comprises a modification that confers enhanced expression of a biologic that comprises an anti-angiogenic agent. For example, some biologics known in the art are derived from natural gene sequences and contain unmodified sequences that are not optimized for introduction and expression in target cells. In an embodiment, an isolated, non-naturally occurring nucleic acid is codon optimized. Codon optimization can be specific for cell type-specific codon usage. Different organisms and cell types exhibit bias towards use of certain codons over others for the same amino acid. Some species are known to avoid certain codons almost entirely. Similarly, certain cell types are biased toward use of certain codons over others for the same amino acid. In an embodiment, a method of optimizing a codon of a non-naturally occurring nucleic acid can comprise reassigning codon usage based on the frequencies of each codon's usage in a target cell. In some cases, a target cell can be of a certain tissue or organ.

[0122] In some cases, a modification is performed to increase guanine and/or cytosine content in a sequence as provided in, Grzegorz Kudla, et al., High guanine and cytosine content increases mRNA levels in mammalian cells, PLoS Biol 4(6): e180. DOI: 10.1371/journal.pbio.0040180, herein incorporated by reference in its entirety.

Table 1. Non-limiting, exemplary codons that can be interchanged for modification of nucleic acids. Thymine can be replaced with uracil in the below codons.

AA	Codons	AA	Codons
Ala	GCT, GCC, GCA, GCG	**Leu**	TTA, TTG, CTT, CTC, CTA, CTG
Arg	CGT, CGC, CGA, CGG, AGA, AGG	**Lys**	AAA, AAG

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Asn	AAT, AAC	**Met**	ATG
Asp	GAT, GAC	**Phe**	TTT, TTC
Cys	TGT, TGC	**Pro**	CCT, CCC, CCA, CCU
Gln	CAA, CAG	**Ser**	TCT, TCC, TCA, TCG, AGT, AGC
Glu	GAA, GAG	**Thr**	ACT, ACC, ACA, ACG
Gly	GGT, GGC, GGA, GGG	**Trp**	TGG
His	CAT, CAC	**Tyr**	TAT, TAC
Ile	ATT, ATC, ATA	**Val**	GTT, GTC, GTA, GTG
Start	ATG	**Stop**	TAA, TGA, TAG

[0123] In an embodiment, a non-naturally occurring nucleic acid sequence can be modified to replace at least one codon with another codon coding for an identical amino acid. In some cases, a codon is modified within a coding region of a sequence. In some cases, a codon is modified within a non-coding region of a sequence. In some cases, a codon is modified within about 100, about 50, about 25, about 15, or about 5 bases from a termination codon. E-CAI can be utilized to estimate a value of a codon adaptation index as provided in: Puigbo, P., Bravo, I. G. & Garcia-Vallve, S. E-CAI: a novel server to estimate an expected value of Codon Adaptation Index (eCAI). BMC Bioinformatics 9, 65, doi:10.1186/1471-2105-9-65 (2008).

[0124] Various modifications are contemplated herein. In some cases, codons can be interchanged. For example, a sequence can be modified to replace AGA with AGG. In other cases, CCC is replaced with CCT. In other cases, AGC is replaced with TCC. In other cases, CCC is replaced with CCG. Any of the non-limiting replacements provided in **Table 1** can be applied to modify a nucleic acid. Any number of codons can be interchanged in a nucleic acid. In some cases, 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 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at least 32, at least 34, at least 36, at least 38, at least 40, at least 42, at least 44, at least 46, at least 48, or up to 50 codons can be replaced. In an embodiment, a non-naturally occurring nucleic acid comprises 3 codon modifications. In an embodiment, a non-naturally occurring nucleic acid comprises 16 codon modifications. In an embodiment, a non-naturally occurring nucleic acid comprises 3-5, 5-10, 5-15, 10-15, 10-20, 15-20, 1-20, 12-20, 12-25, 15-30, or 15-25 codon modifications. In an embodiment, a non-naturally occurring nucleic acid comprises two codon modifications that are: AGA to AGG and at least one of: CCT to CCC, AGC to TCC, or CCC to CCG. In an embodiment, a non-naturally occurring nucleic acid comprises three codon modifications that are: AGA to AGG and at least two of: CCT to CCC, AGC to TCC, or CCC to CCG. In an embodiment, a non-naturally

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occurring nucleic acid comprises four codon modifications that are: AGA to AGG, CCT to CCC, AGC to TCC, and CCC to CCG. Additional modifications can comprise any of the codon modifications provided in **Table 1** in combination with any of the above codons and/or any additional modifications possible from **Table 1**. In an embodiment, a nucleic acid is modified such that AGA is replaced with AGG and CCT is replaced with CCC. In an embodiment, a nucleic acid is modified such that AGA is replaced with AGG and AGC is replaced with TCC. In an embodiment, a nucleic acid is modified such that AGA is replaced with AGG and CCC is replaced with CCG.

[0125] In an embodiment, a non-naturally occurring nucleic acid sequence is modified to replace AGA with AGG in 16 codons of a sequence. In an embodiment, a non-naturally occurring nucleic acid sequence is modified to replace AGA with AGG in 16 codons of a coding sequence. In some cases, a sequence is modified to replace CCT with CCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or up to 30 codons of the coding region of a nucleic acid sequence. In some cases, a sequence is modified to replace CCT with CCC in at least 30 codons of the coding region of the sequence. In some cases, a sequence is modified to replace AGC with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30, at least 33, or up to 36 codons of a region of a nucleic acid sequence. In some cases, a sequence is modified to replace AGC with TCC in 36 codons of the coding region of a nucleic acid sequence. In some cases, a non-naturally occurring nucleic acid sequence is modified to replace CCC with CCG in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or up to 30 codons of the coding region of the sequence. In some cases, the non-naturally occurring nucleic acid sequence is modified to replace CCC with CCG in 29 codons of the coding region of the non-naturally occurring nucleic acid sequence.

[0126] In some embodiments, an isolated, non-naturally occurring nucleic acid described herein comprises a sequence encoding a biologic comprising an anti-angiogenic agent, said sequence comprises a modification in a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region, said modification comprises replacing at least one, at least two, at least three, or at least four non-AGG arginine codons with AGG. In some embodiments, the non-AGG arginine codon is AGA. In some embodiments, the sequence is modified to replace non-AGG arginine codon with AGG in at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, or at least 16 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace non-AGG arginine codon with AGG in 16 codons of the coding region of the

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sequence. In some embodiments, the sequence is modified to replace non-AGG arginine codon with AGG in any one of 16 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace non-AGG arginine codon with AGG in at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, or at least 16 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace non-AGG arginine codon with AGG in 16 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace non-AGG arginine codon with AGG in any one of 16 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the non-AGG arginine codon is AGA. In some embodiments, the sequence is modified to replace AGA with AGG in at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, or at least 16 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace AGA with AGG in 16 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace AGA with AGG in any one of 16 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace AGA with AGG in at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, or at least 16 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace AGA with AGG in 16 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace AGA with AGG in any one of 16 codon positions as compared to **SEQ ID NO: 70**.

[0127] In some embodiments, an isolated, non-naturally occurring nucleic acid described herein comprises a sequence encoding a biologic comprising an anti-angiogenic agent, said sequence comprises a modification in a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region, said modification comprises replacing at least one non-CCC proline codon with CCC. In some embodiments, the at least one non-CCC proline codon is CCT. the sequence is modified to replace non-CCC proline codon with CCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or up to 30 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace non-CCC proline codon with CCC in 30 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace non-CCC proline codon with CCC in any one of 30 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace non-CCC proline codon with CCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or up to 30 codon positions as compared to **SEQ ID NO: 70**. In some

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embodiments, the sequence is modified to replace non-CCC proline codon with CCC in 30 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace non-CCC proline codon with CCC in any one of 30 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the non-CCC proline codon is CCT. In some embodiments, the sequence is modified to replace CCT with CCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or up to 30 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace CCT with CCC in 30 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace CCT with CCC in any one of 30 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace CCT with CCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or up to 30 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace CCT with CCC in 30 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace CCT with CCC in any one of 30 codon positions as compared to **SEQ ID NO: 70**.

[0128] In some embodiments, an isolated, non-naturally occurring nucleic acid described herein comprises a sequence encoding a biologic comprising an anti-angiogenic agent, said sequence comprises a modification in a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region, said modification comprises replacing at least one non-TCC serine codon with TCC. In some embodiments, the sequence is modified to replace non-TCC serine codon with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30, at least 33, or up to 36 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace non-TCC serine codon with TCC in 36 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace non-TCC serine codon with TCC in any one of 36 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace non-TCC serine codon with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30, at least 33, or up to 36 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace non-TCC serine codon with TCC in 36 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace non-TCC serine codon with TCC in any one of 36 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the non-TCC serine codon is AGC. In some embodiments, the sequence is modified to replace AGC with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at

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least 27, at least 30, at least 33, or up to 36 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace AGC with TCC in 36 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace AGC with TCC in any one of 36 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace AGC with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30, at least 33, or up to 36 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace AGC with TCC in 36 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace AGC with TCC in any one of 36 codon positions as compared to **SEQ ID NO: 70**.

[0129] The isolated, non-naturally occurring nucleic acid of any one of claims 3-38, wherein the sequence is modified to replace non-CCG proline codon with CCG in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, or up to 30 codons of the coding region of the sequence.

[0130] In some embodiments, an isolated, non-naturally occurring nucleic acid described herein comprises a sequence encoding a biologic comprising an anti-angiogenic agent, said sequence comprises a modification in a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region, said modification comprises replacing at least one non-CCG proline codon with CCG. In some embodiments, the sequence is modified to replace non-CCG proline codon with CCG in 30 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace non-CCG proline codon with CCG in any one of 30 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace non-CCG proline codon with CCG in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, or up to 30 codons position as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace non-CCG proline codon with CCG in 30 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace non-CCG proline codon with CCG in any one of 30 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the non-CCG proline codon is CCC. In some embodiments, the sequence is modified to replace CCC with CCG in in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, or up to 30 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace CCC with CCG in 30 codons of the coding region of the sequence. In some embodiments, the sequence is modified to replace CCC with CCG in any one of 30 codons of the coding region of the sequence. In some embodiments, the

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sequence is modified to replace CCC with CCG in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, or up to 30 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace CCC with CCG in 30 codon positions as compared to **SEQ ID NO: 70**. In some embodiments, the sequence is modified to replace CCC with CCG in any one of 30 codon positions as compared to **SEQ ID NO: 70**.

[0131] In some embodiments, a non-naturally occurring nucleic acid sequence described herein comprises a nucleic acid sequence that is at least about 60% sequence identity or similarity with any one of **SEQ ID NOS: 13-19, 21-27, 31, 62, 64, 66,** or **68**. In some embodiments, the sequence identity is from about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100%. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises the sequence identity is from about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100% to **SEQ ID NO: 31**. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises a nucleic acid sequence of **SEQ ID NO: 31**. In some embodiments, the isolated, non-naturally occurring nucleic acid is 100% identical to a nucleic acid sequence of **SEQ ID NO: 31**. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises the sequence identity is from about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100% to **SEQ ID NO: 66**. In some embodiments, the isolated, non-naturally occurring nucleic acid comprises a nucleic acid sequence of **SEQ ID NO: 66**. In some embodiments, the isolated, non-naturally occurring nucleic acid is 100% identical to a nucleic acid sequence of **SEQ ID NO: 66**. In some embodiments, the isolated, non-naturally occurring nucleic acid is single stranded. In some embodiments, the isolated, non-naturally occurring nucleic acid is double stranded.

[0132] In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace AGA with AGG at positions: X1-X16 as compared to **SEQ ID NO: 70**. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace CCT with CCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or up to 30 codons of the coding region of the non-naturally occurring nucleic acid sequence. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace CCT with CCC in 30 codons of the coding region of the non-naturally occurring nucleic acid sequence. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace CCT with CCC at positions: X1-X30 as compared to **SEQ ID NO: 70**. In some embodiments, a non-naturally occurring nucleic acid sequence can be

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modified to replace AGC with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30, at least 33, or up to 36 codons of the coding region of the non-naturally occurring nucleic acid sequence. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace AGC with TCC in 36 codons of the coding region of the non-naturally occurring nucleic acid sequence. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace AGC with TCC at positions: X1-X36 as compared to **SEQ ID NO: 70**. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace CCC with CCG in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or up to 30 codons of the coding region of the non-naturally occurring nucleic acid sequence. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace CCC with CCG in 29 codons of the coding region of the non-naturally occurring nucleic acid sequence. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace CCC with CCG at positions: X1-X29 as compared to **SEQ ID NO: 70**. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace AGA with AGG at positions: X1-X16 as compared to **SEQ ID NO: 28**. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace CCT with CCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or up to 30 codons of the coding region of the non-naturally occurring nucleic acid sequence. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace CCT with CCC in 30 codons of the coding region of the non-naturally occurring nucleic acid sequence. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace CCT with CCC at positions: X1-X30 as compared to **SEQ ID NO: 28**. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace AGC with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30, at least 33, or up to 36 codons of the coding region of the non-naturally occurring nucleic acid sequence. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace AGC with TCC in 36 codons of the coding region of the non-naturally occurring nucleic acid sequence. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace AGC with TCC at positions: X1-X36 as compared to **SEQ ID NO: 28**. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace CCC with CCG in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or up to 30 codons of the coding region of the non-naturally

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occurring nucleic acid sequence. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace CCC with CCG in 29 codons of the coding region of the non-naturally occurring nucleic acid sequence. In some embodiments, a non-naturally occurring nucleic acid sequence can be modified to replace CCC with CCG at positions: X1-X29 as compared to **SEQ ID NO: 28**. In some embodiments, a non-naturally occurring nucleic acid sequence can comprise a viral vector sequence. In some embodiments, a viral vector sequence can be a scAAV vector sequence. In some embodiments, a AAV vector sequence can be of serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, or any combination thereof. In some embodiments, an AAV vector sequence can be of the AAV2 serotype. In some embodiments, a viral vector sequence can comprise sequences of at least 2 AAV serotypes. In some embodiments, at least two serotypes can be selected from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV8, AAV9, AAV11, and AAV12. In some embodiments, an isolated non-naturally occurring nucleic acid sequence can comprise a sequence having at least about 60% sequence identity or similarity with any one of **SEQ ID NOS: 13-19** or **21-27**. In some embodiments, the AAV vector sequence is single stranded. In some embodiments, the AAV vector sequence is double stranded.

[0133] In some cases, a modification can also comprise a chemical modification. Modified nucleic acids can comprise modifications of their backbones, sugars, or nucleobases, and even novel bases or base pairs. Modified nucleic acids can have improved chemical and/or biological stability. Decoration with diverse chemical substituents (e.g., hydrophobic groups) can also yield improved properties and functionalities such as new structural motifs and enhanced target binding.

[0134] Exemplary chemical modification include but are not limited to: 2′F, 2′-fluoro; 2′OMe, 2′-O-methyl; LNA, locked nucleic acid; FANA, 2′-fluoro arabinose nucleic acid; HNA, hexitol nucleic acid; 2′MOE, 2′-O-methoxyethyl; ribuloNA, (1′-3′)-β-L-ribulo nucleic acid; TNA, α-L-threose nucleic acid; tPhoNA, 3′-2′ phosphonomethyl-threosyl nucleic acid; dXNA, 2′-deoxyxylonucleic acid; PS, phosphorothioate; phNA, alkyl phosphonate nucleic acid; PNA, and peptide nucleic acid.

[0135] In some cases, a nucleic acid comprises additional features. Additional features can comprise sequences such as tags, signal peptides, intronic sequences, promoters, stuffer sequences, and the like.

[0136] In some cases, a nucleic acid comprises a signal peptide. A signal peptide is sometimes referred to as signal sequence, targeting signal, localization signal, localization sequence, transit peptide, leader sequence or leader peptide, is a short peptide present at the N-terminus of the

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majority of newly synthesized proteins that are destined toward the secretory pathway. These proteins include those that reside either inside certain organelles (the endoplasmic reticulum, Golgi or endosomes), secreted from the cell, or inserted into most cellular membranes. In some cases, nucleic acids provided herein can comprise signal peptides. A signal peptide can be of any length but typically from 15-30 amino acids long. A signal peptide can be from about: 10-15, 10-20, 10-30, 15-20, 15-25, 15-30, 20-30, or 25-30 amino acids long. Various signal peptides can be utilized and include but are not limited to: human antibody heavy chain (Vh), human antibody light chain (Vl), and aflibercept.

[0137] In some cases, a nucleic acid comprises an intronic sequence. An intron is any nucleotide sequence within a sequence that can be removed by RNA splicing during maturation of the final RNA product. In other words, introns are non-coding regions of an RNA transcript, or the DNA encoding it, that are eliminated by splicing before translation. While introns do not encode protein products, they are players in gene expression regulation. Some introns themselves encode functional RNAs through further processing after splicing to generate noncoding RNA molecules. Alternative splicing is widely used to generate multiple proteins from a single gene. Furthermore, some introns play essential roles in a wide range of gene expression regulatory functions such as nonsense-mediated decay and mRNA export. In an embodiment, an intronic sequence is included in a nucleic acid of the disclosure and can be selected from: hCMV intron A, adenovirus tripartite leader sequence intron, SV40 intron, hamster EF-1 alpha gene intron 1, intervening sequence intron, human growth hormone intron, and/or human beta globin intron. Any number of intronic sequences are contemplated. In an embodiment, the intronic sequence is SV40. In some cases, 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, or up to 10 intronic sequences can be included in a nucleic acid.

[0138] In an embodiment, an additional feature includes a promoter. Promoter is a sequence of DNA to which proteins bind that initiate transcription of a single RNA from the DNA downstream of it. This RNA may encode a protein, or can have a function in and of itself, such as tRNA, mRNA, or rRNA. Promoters are located near the transcription start sites of genes, upstream on the DNA (towards the 5' region of the sense strand). Promoters can be about 100–1000 base pairs long. Various promoters are contemplated and can be employed in the non-naturally occurring nucleic acids of the disclosure. In an embodiment, a promoter is: a cytomegalovirus (CMV) promoter, an elongation factor 1 alpha (EF1α) promoter, a simian vacuolating virus (SV40) promoter, a phosphoglycerate kinase (PGK1) promoter, a ubiquitin C (Ubc) promoter, a human beta actin promoter, a CAG promoter, a Tetracycline response element (TRE) promoter, a UAS promoter, an Actin 5c (Ac5) promoter, a polyhedron promoter, a

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Ca2+/calmodulin-dependent protein kinase II (CaMKIIa) promoter, a GAL1 promoter, a GAL 10 promoter, a TEF1 promoter, a glyceraldehyde 3-phosphage dehydrogenase (GDS) promoter, an ADH1 promoter, a CaMV35S promoter, a Ubi promoter, a human polymerase III RNA (H1) promoter, a U6 promoter, a polyadenylated construct thereof, and any combination thereof. In some cases, the promoter is the CMV promoter.

[0139] Any of the provided nucleic acid sequences can comprise viral vector sequences. A viral vector can be, without limitation, a lentivirus, a retrovirus, or an adeno-associated virus. A viral vector can be an adeno-associated viral (AAV) vector. In some cases, a viral vector is an adeno-associated viral vector. Many serotypes of AAV vectors are contemplated and include but are not limited to: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, and/or AAV12. Based on these initial serotypes, AAV capsid of each serotype can be engineered to make them better suited for biological functions, tissue or cell selection. In some embodiments, an AAV vector is AAV2 and variants AAV2.N53 and AAV2.N54 which are used in the examples of the invention. Chimeric AAV vectors are also contemplated that may contain at least 2 AAV serotypes. In some cases, at least 3, at least 4, at least 5, at least 6, at least 7, or up to 8 different serotypes are combined in a chimeric AAV vector. In some cases, only a portion of the AAV is chimeric. For example, suitable portions can include the capsid, VP1, VP2, or VP3 domains and/or Rep. In some cases, at least one of VP1, VP2, and VP3 has at least one amino acid substitution compared to an otherwise comparable wild-type AAV capsid protein. In some cases, a mutation can occur in VP1 and VP2, in VP1 and VP3, in VP2 and VP3, or in VP1, VP2, and VP3. In some embodiments, at least one of VP1, VP2, and VP3 has from one to about 25 amino acid substitutions compared to wild-type AAV VP1, VP2, and VP3, e.g., from about one to about 5, from about 5 to about 10, from about 10 to about 15, from about 15 to about 20, or from about 20 to about 25 amino acid substitutions compared to wild-type AAV VP1, VP2, and VP3. In some cases, a VP can be removed. For example, in some embodiments a mutant AAV does not comprise at least one of VP1, VP2, or VP3.

[0140] In some cases, an AAV vector can be modified. For example, an AAV vector can comprise a modification such as an insertion, deletion, chemical alteration, or synthetic modification. In some cases, a single nucleotide is inserted into an AAV vector. In other cases, multiple nucleotides are inserted into a vector. Nucleotides that can be inserted can range from about 1 nucleotide to about 5 kb.

[0141] In some cases, a mutation can occur at any of the previously mentioned AAV capsid positions and can include any number of mutations. In some cases, a mutation can be from one amino acid to another amino acid. Any combination or permutation of the canonical amino acids

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can be performed. Any of the following amino acid modifications can be made at any of VP1, VP2, and VP3: A to R, A to N, A to D, A to C, A to Q, A to E, A to G, A to H, A to I, A to L, A to K, A to M, A to F, A to P, A to S, A to T, A to W, A to Y, A to V, R to N, R to D, R to C, R to Q, R to E, R to G, R to H, R to I, R to L, R to K, R to M, R to F, R to P, R to S, R to T, R to W, R to Y, R to V, N to D, N to C, N to Q, N to E, N to G, N to H, N to I, N to L, N to K, N to M, N to F, N to P, N to S, N to T, N to W, N to Y, N to V, D to C, D to Q, D to E, D to G, D to H, D to I, D to L, D to K, D to M, D to F, D to P, D to S, D to T, D to W, D to Y, D to V, C to Q, C to E, C to G, C to H, C to I, C to L, C to K, C to M, C to F, C to P, C to S, C to T, C to W, C to Y, C to V, Q to E, Q to G, Q to H, Q to I, Q to L, Q to K, Q to M, Q to F, Q to P, Q to S, Q to T, Q to W, Q to Y, Q to V, E to G, E to H, E to I, E to L, E to K, E to M, E to F, E to P, E to S, E to T, E to W, E to Y, E to V, G to H, G to I, G to L, G to K, G to M, G to F, G to P, G to S, G to T, G to W, G to Y, G to V, H to I, H to L, H to K, H to M, H to F, H to P, H to S, H to T, H to W, H to Y, H to V, I to L, I to K, I to M, I to F, I to P, I to S, I to T, I to W, I to Y, I to V, L to K, L to M, L to F, L to P, L to S, L to T, L to W, L to Y, L to V, K to M, K to F, K to P, K to S, K to T, K to W, K to Y, K to V, M to F, M to P, M to S, M to T, M to W, M to Y, M to V, F to P, F to S, F to T, F to W, F to Y, F to V, P to S, P to T, P to W, P to Y, P to V, S to T, S to W, S to Y, S to V, T to W, T to Y, T to V, W to Y, W to V, Y to V, and any of the previously described mutations in reverse.

[0142] A mutation can be a conservative mutation or replacement. For example, 20 naturally occurring amino acids can share similar characteristics. Aliphatic amino acids can be glycine, alanine, valine, leucine, or isoleucine. Hydroxyl or sulfur/selenium-containing amino acids can be: Serine, cysteine, selenocysteine, threonine, or methionine. A cyclic amino acid can be proline. An aromatic amino acid can be phenylalanine, tyrosine, or tryptophan. A basic amino acid can be histidine, lysine, and arginine. An acidic amino acid can be aspartate, glutamate, asparagine, or glutamine. A conservative mutation can be, serine to glycine, serine to alanine, serine to serine, serine to threonine, serine to proline. A conservative mutation can be arginine to asparagine, arginine to lysine, arginine to glutamine, arginine to arginine, arginine to histidine. A conservative mutation can be Leucine to phenylalanine, leucine to isoleucine, leucine to valine, leucine to leucine, leucine to methionine. A conservative mutation can be proline to glycine, proline to alanine, proline to serine, proline to threonine, proline to proline. A conservative mutation can be threonine to glycine, threonine to alanine, threonine to serine, threonine to threonine, threonine to proline. A conservative mutation can be alanine to glycine, alanine to threonine, alanine to proline, alanine to alanine, alanine to serine. A conservative mutation can be valine to methionine, valine to phenylalanine, valine to isoleucine, valine to leucine, valine to

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valine. A conservative mutation can be glycine to alanine, glycine to threonine, glycine to proline, glycine to serine, glycine to glycine. A conservative mutation can be Isoleucine to phenylalanine, isoleucine to isoleucine, isoleucine to valine, isoleucine to leucine, isoleucine to methionine. A conservative mutation can be phenylalanine to tryptophan, phenylalanine to phenylalanine, phenylalanine to tyrosine. A conservative mutation can be tyrosine to tryptophan, tyrosine to phenylalanine, tyrosine to tyrosine. A conservative mutation can be cysteine to serine, cysteine to threonine, cysteine to cysteine. A conservative mutation can be histidine to asparagine, histidine to lysine, histidine to glutamine, histidine to arginine, histidine to histidine. A conservative mutation can be glutamine to glutamic acid, glutamine to asparagine, glutamine to aspartic acid, glutamine to glutamine. A conservative mutation can be asparagine to glutamic acid, asparagine to asparagine, asparagine to aspartic acid, asparagine to glutamine. A conservative mutation can be lysine to asparagine, lysine to lysine, lysine to glutamine, lysine to arginine, lysine to histidine. A conservative mutation can be aspartic acid to glutamic acid, aspartic acid to asparagine, aspartic acid to aspartic acid, aspartic acid to glutamine. A conservative mutation can be glutamine to glutamine, glutamine to asparagine, glutamine to aspartic acid, glutamine to glutamine. A conservative mutation can be methionine to phenylalanine, methionine to isoleucine, methionine to valine, methionine to leucine, methionine to methionine. A conservative mutation can be tryptophan to tryptophan, tryptophan to phenylalanine, tryptophan to tyrosine.

[0143] In some embodiments, the modified AAV vector comprises modified AAV2 serotype vector. In some embodiments, the modified AAV vector comprises modified VP1, VP2, VP3, or a combination thereof. In some embodiments, the modified AAV2 serotype vector comprises modified VP1, VP2, VP3, or a combination thereof.

[0144] In an aspect, the isolated non-naturally occurring nucleic acid comprises at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, and up to about 100% sequence identity or similarity with any one of **SEQ ID NOS: 13-19**. In an embodiment, the isolated non-naturally occurring nucleic acid comprises at least about 60% sequence identity or similarity with any one of **SEQ ID NOS: 13-19**. In some cases, the isolated non-naturally occurring nucleic acid is any one of **SEQ ID NOS: 13-19**. In an aspect, the isolated non-naturally occurring nucleic acid comprises at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, and up to about 100% sequence identity or similarity with any one of **SEQ ID NO: 21-SEQ ID NO: 27**. In an embodiment, the isolated

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non-naturally occurring nucleic acid comprises at least 60% sequence identity or similarity with any one of **SEQ ID NO: 21-SEQ ID NO: 27**. In some cases, the isolated non-naturally occurring nucleic acid is any one of **SEQ ID NO: 21-SEQ ID NO: 27**. In some cases, a nucleic acid encodes for a polypeptide having 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% identity with **SEQ ID NO: 12**. In some cases, a nucleic acid encodes for the polypeptide of **SEQ ID NO: 12**.

[0145] In an aspect, provided herein are also methods of modifying cells to thereby generate engineered cells. Cells can refer to primary cells, recombinant cells, or cell lines. In some cases, a cell is a packaging cell. A packaging cell can be any one of: HEK 293 cells, HeLa cells, and Vero cells to name a few. An engineered cell can be a primary cell. In some cases, an engineered cell can be an ocular cell. Suitable ocular cells include but are not limited to a: photoreceptor, ganglion cell, RPE cell, amacrine cell, horizontal cell, muller cell, and the like.

[0146] In some cases, a cell is a packaging cell utilized to generate viral particles. To general AAV virions or viral particles, an AAV expression vector is introduced into a suitable host cell using known techniques, such as by transfection. In some cases, transfection techniques are used, *e.g.*, $CaPO_4$ transfection or electroporation, and/or infection by hybrid adenovirus/AAV vectors into cell lines such as the human embryonic kidney cell line HEK 293 (a human kidney cell line containing functional adenovirus E1 genes which provides trans-acting E1 proteins). Transfection techniques are known in the art. See, e.g., Graham et al. (1973) Virology, 52:456, Sambrook et al. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Davis et al. (1986) Basic Methods in Molecular Biology, Elsevier, and Chu et al. (1981) Gene 13:197. Suitable transfection methods include calcium phosphate co-precipitation, direct micro-injection, electroporation, liposome mediated gene transfer, and nucleic acid delivery using high-velocity microprojectiles, which are known in the art.

[0147] To engineer a cell, a plurality of cells may be contacted with an isolated non-naturally occurring nucleic acid. A contacting can comprise any length of time and may include from about 5 min to about 5 days. Contacting can last from about 5, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 55, or about 60 minutes. In some cases, the contacting can last from 1 hour, 3 hours, 5 hours, 10 hours, 15 hours, 20 hours, 1 day, 2 days, 3 days, 4 days or up to about 5 days.

[0148] In some cases, supernatant of the packaging cell line is treated by PEG precipitation for concentrating the virus. In other cases, a centrifugation step can be used to concentrate a virus. For example, a column can be used to concentration a virus during a centrifugation. In some embodiments, a precipitation occurs at no more than about 4° C. (for example about 3° C., about

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2° C., about 1° C., or about 1° C.) for at least about 2 hours, at least about 3 hours, at least about 4 hours, at least about 6 hours, at least about 9 hours, at least about 12 hours, or at least about 24 hours. In some embodiments, the recombinant AAV is isolated from the PEG-precipitated supernatant by low-speed centrifugation followed by CsCl gradient. The low-speed centrifugation can be to can be about 4000 rpm, about 4500 rpm, about 5000 rpm, or about 6000 rpm for about 20 minutes, about 30 minutes, about 40 minutes, about 50 minutes or about 60 minutes. In some cases, recombinant AAV is isolated from the PEG-precipitated supernatant by centrifugation at about 5000 rpm for about 30 minutes followed by CsCl gradient. In some cases, CsCl purification can be replaced with IDX gradient ultracentrifugation. Supernatant can be collected at about 12 hours, about 24 hours, about 36 hours, about 48 hours, about 72 hours, about 96 hours, about 120 hours, or a time between any of these two time points after a transfection. Supernatant can also be purified, concentrated, or a combination thereof. For example, a concentration or viral titer can be determined by qPCR or silver stain.

[0149] In an aspect, provided is also a plurality of AAV particles isolated from an engineered cell. A viral titer can be from about 10^2 vp/mL, about 10^3 vp/mL, about 10^4 vp/mL, about 10^5 vp/mL, about 10^6 vp/mL, about 10^7 vp/mL, about 10^8 vp/mL, or up to about 10^9vp/mL. A viral titer can be from about 10^2 GC/mL, about 10^3 GC/mL, about 10^4 GC/mL, about 10^5 GC/mL, about 10^6 GC/mL, about 10^7 GC/mL, about 10^8 GC/mL, or up to about 10^9GC/mL. In some cases, a viral titer can be from about 10^2 TU/mL, about 10^3 TU/mL, about 10^4 TU/mL, about 10^5 TU/mL, about 10^6 TU/mL, about 10^7 TU/mL, 10^8 TU/mL, or up to about 10^9TU/mL. An optimal viral titer can vary depending on cell type to be transduced. A range of virus can be from about 1000 MOI to about 2000 MOI, from about 1500 MOI to about 2500 MOI, from about 2000 MOI to about 3000 MOI, from about 3000 MOI to about 4000 MOI, from about 4000 MOI to about 5000 MOI, from about 5000 MOI to about 6000 MOI, from about 6000 MOI to about 7000MOI, from about 7000 MOI to about 8000 MOI, from about 8000 MOI to about 9000 MOI, from about 9000 MOI to about 10,000 MOI. For example, to infect 1 million cells using a MOI of 10,000, one will need 10,000 x 1,000,000 = 10^{10} GC.

[0150] In some cases, a plurality of AAV particles can be formulated into unit dose form. Various formulations are contemplated for adult or pediatric delivery and include but are not limited to: 0.5 x 10^9 vg, 1.0 x 10^9 vg, 1.0 x 10^{10}, 1.0 x 10^{11} vg, 3.0 x 10^{11} vg, 6 x 10^{11} vg, 8.0 x 10^{11} vg, 1.0 x 10^{12} vg, 1.0 x 10^{13} vg, 1.0 x 10^{14} vg, 1.0 x 10^{15} vg, or up to 1.5 x 10^{15} vg. Compositions of viral particles can be cryopreserved or otherwise stored in suitable containers.

[0151] Provided compositions and methods herein can be sufficient to enhance delivery and/or expression of subject biologic by at least about 3%, about 5%, about 10%, about 15%, about

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20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or up to 100% more than an otherwise comparable unmodified nucleic acid. In some cases, the otherwise comparable unmodified nucleic acid is one that encodes VEGF-Trap. In some cases, modifications can be sufficient to enhance delivery and/or expression of subject biologics by at least about 1-fold, about 6-fold, about 11-fold, about 16-fold, about 21-fold, about 26-fold, about 31-fold, about 36-fold, about 41-fold, about 46-fold, about 51-fold, about 56-fold, about 61-fold, about 66-fold, about 71-fold, about 76-fold, about 81-fold, about 86-fold, about 91-fold, about 96-fold, about 101-fold, about 106-fold, about 111-fold, about 116-fold, about 121-fold, about 126-fold, about 131-fold, about 136-fold, about 141-fold, about 146-fold, about 151-fold, about 156-fold, about 161-fold, about 166-fold, about 171-fold, about 176-fold, about 181-fold, about 186-fold, about 191-fold, about 196-fold, about 201-fold, about 206-fold, about 211-fold, about 216-fold, about 221-fold, about 226-fold, about 231-fold, about 236-fold, about 241-fold, about 246-fold, about 251-fold, about 256-fold, about 261-fold, about 266-fold, about 271-fold, about 276-fold, about 281-fold, about 286-fold, about 291-fold, about 296-fold, about 301-fold, about 306-fold, about 311-fold, about 316-fold, about 321-fold, about 326-fold, about 331-fold, about 336-fold, about 341-fold, about 346-fold, or about 350-fold more than an otherwise comparable unmodified nucleic acid. In an embodiment, increased expression comprises at least a 5-fold, at least a 10-fold, at least a 20-fold, at least a 50-fold, at least a 100-fold, at least a 200-fold, or at least a 500-fold increase as determined by in in vitro assay. Suitable in vitro assays include ELISA, Western blot, Luminex, microscopy, imaging, and/or flow cytometry.

[0152] A subject AAV virion can exhibit at least 1-fold, at least 6-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, or more than 50-fold, increased infectivity of a retinal cell, compared to the infectivity of the retinal cell (photoreceptor, ganglion cell, RPE cell, amacrine cell, horizontal cell, muller cell, and the like) by an AAV virion comprising an otherwise comparable WT AAV capsid protein.

[0153] Provided herein are also methods of treating a disease or condition. A method of treatment can comprise introducing to a subject in need a virion or vector coding for a biologic provided herein. In some cases, a method of treatment comprises introducing a plurality of virions or vectors that code for the biologic that comprises an anti-angiogenic agent. Also provided is a method of treating disease that comprises administering a pharmaceutical composition to a subject in need thereof. A pharmaceutical composition can comprise a sequence

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that encodes a biologic that comprises an anti-angiogenic agent and/or virions that code for the anti-angiogenic agent.

[0154] In an embodiment, a method of treatment comprises administering a therapeutically effective amount of a pharmaceutical composition that comprises an isolated non-naturally occurring nucleic acid that comprises a sequence that encodes a biologic that comprises an anti-angiogenic agent. A sequence can be or can comprise any of the nucleic acids provided herein. For example, the sequence can comprises a nucleic acid sequence that is at least about 60% sequence identity or similarity with any one **SEQ ID NOS: 13-19, 21-27, 31, 62, 64, 66,** or **68**. In some embodiments, the sequence identity is from about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100%. In some embodiments, the sequence comprises the sequence identity is from about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100% to **SEQ ID NO: 31**. In some embodiments, the sequence comprises a nucleic acid sequence of **SEQ ID NO: 31**. In some embodiments, the sequence is 100% identical to a nucleic acid sequence of **SEQ ID NO: 31**. In some embodiments, the sequence comprises the sequence identity is from about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100% to **SEQ ID NO: 66**. In some embodiments, the iso sequence comprises a nucleic acid sequence of **SEQ ID NO: 66**. In some embodiments, the sequence is 100% identical to a nucleic acid sequence of **SEQ ID NO: 66**. In some embodiments, the sequence is single stranded. In some embodiments, the sequence is double stranded.

[0155] In some cases, a sequence is modified according to the disclosure, for example the sequence is modified to replace AGA with AGG in at least one codon of a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region. As provided herein, the modifications of the disclosure can have certain benefits such as increasing a level of the biologic in a subject as compared to an otherwise comparable subject administered an otherwise comparable isolated non-naturally occurring nucleic acid lacking a modification. Increasing levels of biologics in subjects can have therapeutic effects and can reduce or eliminate any of the diseases or conditions provided herein.

[0156] In some cases, an increased level of a biologic in a subject is at least a 5-fold, a 10-fold, a 20-fold, a 50-fold, a 100-fold, a 200-fold, or a 500-fold increased, as determined by a diagnostic assay.

[0157] Suitable diagnostic assays can include ocular diagnostic assays. Ocular diagnostic assays can include ophthalmic testing such as refraction testing, ocular scans, Ocular coherence

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tomography, Farnworth-Munsell 100 Hue Test, Computerized Optic Disc Imaging and Nerve Fiber Layer Analysis (GDX, HRT, OCT), Corneal Topography, Electroretinography (ERG), electro-oculography (EOG), visual evoked potentials (VEP), visual evoked response (VER), Fluorescein Angiography, Ocular Coherence Tomography (OCT), retinal photography, fundus photography, Specular Microscopy, Goldmann, Humphrey, FDT, Octopus, Biometry/IOL calculation, A-Scan, B-Scan, and combinations thereof.

[0158] In some cases, a retinal test can be utilized. Nonlimiting methods for assessing retinal function and changes thereof include assessing visual acuity (e.g. best-corrected visual acuity [BCVA], ambulation, navigation, object detection and discrimination), assessing visual field (e.g. static and kinetic visual field perimetry), performing a clinical examination (e.g. slit lamp examination of the anterior and posterior segments of the eye), assessing electrophysiological responsiveness to all wavelengths of light and dark (e.g. all forms of electroretinography (ERG) [full-field, multifocal and pattern], all forms of visual evoked potential (VEP), electrooculography (EOG), color vision, dark adaptation and/or contrast sensitivity). Nonlimiting methods for assessing anatomy and retinal health and changes thereof include Optical Coherence Tomography (OCT), fundus photography, adaptive optics scanning laser ophthalmoscopy (AO-SLO), fluorescence and/or autofluorescence; measuring ocular motility and eye movements (e.g. nystagmus, fixation preference, and stability), measuring reported outcomes (patient-reported changes in visual and non-visually-guided behaviors and activities, patient-reported outcomes [PRO], questionnaire-based assessments of quality-of-life, daily activities and measures of neurological function (e.g. functional Magnetic Resonance Imaging (MRI)).

[0159] Relevant ocular diseases and conditions can include but are not limited to: blindness, Achromatopsia, Age-related macular degeneration (AMD), Diabetic retinopathy (DR), Glaucoma, Bardet-Biedl Syndrome, Best Disease, Choroideremia, Leber Congenital Amaurosis, Macular degeneration, Polypoidal choroidal vasculopathy (PCV), Retinitis pigmentosa, Refsum disease, Stargardt disease, Usher syndrome, X-linked retinoschisis (XLRS), Rod-cone dystrophy, Cone-rod dystrophy, Oguchi disease, Malattia Leventinese (Familial Dominant Drusen), and Blue-cone monochromacy. In an embodiment, the ocular disease or condition is AMD. AMD can be wet AMD or dry AMD.

[0160] In some cases, an administration of a pharmaceutical is sufficient to reduce at least a symptom of a disease or condition, treat the disease or condition, and/or eliminate the disease or condition. In some cases, improvements of diseases or conditions can be ascertained by any of the provided diagnostic assays. In other cases, an improvement can be obtained via an interview with the treated subject. For example, a subject may be able to communicate to an attending

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physician that their vision is improved as compared to their vision prior to administration of a subject pharmaceutical. In other cases, an in vivo animal model may be used to ascertain reduction of a disease or condition after treatment. Suitable animal models include mouse models, primate models, rat models, canine models, and the like.

[0161] Pharmaceutical compositions can be administered to a subject using various techniques, such as: intravitreally, intramuscular, intravenous, subcutaneous, and/or intraperitoneal injection.

[0162] For in vivo delivery, subject nucleic acids and/or AAV virions can be formulated into pharmaceutical compositions and will generally be administered intravitreally or parenterally (e.g., administered via an intramuscular, subcutaneous, intratumoral, transdermal, intrathecal, etc., route of administration). In some aspects, a pharmaceutical composition can be used to treat a subject such as a human or mammal, in need thereof. In some cases, a subject can be diagnosed with a disease, *e.g.*, ocular disease. In some aspects, subject pharmaceutical compositions are co-administered with secondary therapies. A secondary therapy can comprise any therapy for ocular use. In some cases, a secondary therapy comprises nutritional therapy, vitamins, laser treatment, such as laser photocoagulation, photodynamic therapy, Visudyne, anti-VEGF therapy, eye-wear, eye drops, numbing agents, Orthoptic vision therapy, Behavioral/perceptual vision therapy, and the like. In some aspects, any of the previously described biologics can be considered a secondary therapy.

[0163] Any of the pharmaceutical compositions can also comprise an excipient. Such excipients, carriers, diluents, and buffers include any pharmaceutical agent that can be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. A wide variety of pharmaceutically acceptable excipients are known in the art and need not be discussed in detail herein. Pharmaceutically acceptable excipients have been amply described in a variety of publications, including, for example, A. Gennaro (2000) "Remington: The Science and Practice of Pharmacy," 20th edition, Lippincott, Williams, & Wilkins; Pharmaceutical Dosage Forms and Drug Delivery Systems (1999) H. C. Ansel et al., eds., 7th ed., Lippincott, Williams, & Wilkins; and Handbook of Pharmaceutical Excipients (2000) A. H. Kibbe et al., eds., 3rd ed. Amer. Pharmaceutical Assoc.

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[0164] In some embodiments, an effective amount of the subject rAAV virion results in a decrease in the rate of loss of retinal function, anatomical integrity, or retinal health, e.g. a 2-fold, 3-fold, 4-fold, or 5-fold or more decrease in the rate of loss and hence progression of disease, for example, a 10-fold decrease or more in the rate of loss and hence progression of disease. In some embodiments, the effective amount of the subject rAAV virion results in a gain in visual function, retinal function, an improvement in retinal anatomy or health, and/or an improvement in ocular motility and/or improvement in neurological function, e.g. a 2-fold, 3-fold, 4- fold or 5 - fold improvement or more in retinal function, retinal anatomy or health, and/or improvement in ocular motility, e.g. a 10-fold improvement or more in retinal function, retinal anatomy or health, and/or improvement in ocular motility. As will be readily appreciated by the ordinarily skilled artisan, the dose required to achieve the desired treatment effect will typically be in the range of 1 x 10^8 to about 1 x 10^{15} recombinant virions, typically referred to by the ordinarily skilled artisan as 1 x 10^8 to about 1 x 10^{15}"vector genomes".

[0165] In some aspects, compositions provided herein, such as pharmaceutical compositions are administered to a subject in need thereof. In some cases, an administration comprises delivering a dosage of an AAV vector of about vector 0.5 x 10^9 vg, 1.0 x 10^9 vg, 1.0 x 10^{10}, 1.0 x 10^{11} vg, 3.0 x 10^{11} vg, 6 x 10^{11} vg, 8.0 x 10^{11} vg, 1.0 x 10^{12} vg, 1.0 x 10^{13} vg, 1.0 x 10^{14} vg, 1.0 x 10^{15} vg, 1.5 x 10^{15} vg. For example, for in vivo injection, i.e., injection directly into the eye, a therapeutically effective dose can be on the order of from about 10^6 to about 10^{15} of subject AAV virions, e.g., from about 10^8 to 10^{12} engineered AAV virions. For in vitro transduction, an effective amount of engineered AAV virions to be delivered to cells will be on the order of from about 10^8 to about 10^{13} of the engineered AAV virions. Other effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves.

[0166] Administrations can be repeated for any amount of time. In some aspects, administering is performed: twice daily, every other day, twice a week, bimonthly, trimonthly, once a month, every other month, semiannually, annually, or biannually.

[0167] Dosage treatment may be a single dose schedule or a multiple dose schedule. Moreover, the subject may be administered as many doses as appropriate. One of skill in the art can readily determine an appropriate number of doses. In some aspects, a pharmaceutical composition is administered via intravitreal injection, subretinal injection, microinjection, or supraocular injection.

[0168] In some aspects, a subject can be screened via genetic testing for a mutation before, during, and/or after administration of a pharmaceutical composition provided herein. Relevant genes that can be screened for mutations comprise: RPE65, CRB1, AIPL1, CFH, or RPGRIP.

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[0169] Also provided are kits comprising any of the compositions provided herein. Provided is also a container that comprises a) a subject modified adeno-associated virus (AAV) capsid; b) a subject vector; or c) a subject engineered virion. In an aspect, the container is a vial, syringe, or needle. In some cases, the container is configured for ocular delivery.

[0170] Kits may comprise a suitably aliquoted composition. The components of the kits may be packaged either in aqueous media or in lyophilized form. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe, or another container means, into which a component may be placed, and preferably, suitably aliquoted. Where there is more than one component in the kit, the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial. The kits also will typically include a means for containing the components in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

[0171] In some instances, a packaged product comprising a composition described herein can be properly labeled. In some instances, the pharmaceutical composition described herein can be manufactured according to good manufacturing practice (cGMP) and labeling regulations. In some cases, a pharmaceutical composition disclosed herein can be aseptic.

[0172] While preferred embodiments of the present invention 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.

EXAMPLES

Example 1: Codon optimization and cloning of nucleic acid constructs

[0173] A DNA sequence containing SV40 intron-VEGF-Trap open reading frame (ORF)-synthetic poly A signal was synthesized by a service company (Twist Bioscience, South San Francisco, CA) to contain 40% GC-content in the VEGF-Trap ORF. The DNA sequence was PCR amplified with forward primer A024 and reverse primer A025 and cloned into the XbaI and SphI sites of pFB-scCMV-MIF using the NEBuilder HiFi DNA Assembly kit (New England Biolabs, Ipswich, MA) to create AMI059-pFB-scCMV-SV40-intron-Af- VEGF-Trap as shown in **Fig. 1**. The pFB-scCMV-MIF plasmid that contains one full AAV ITR and one truncated ITR

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for self-complementary AAV (scAAV) is a derivative of pFastBac shuttle plasmid from the Bac-to-Bac Baculovirus Expression System (Invitrogen). In the cloning process, MIF was replaced with VEGF-Trap gene. The VEGF-Trap gene contains its original signal peptide (Af). To replace the Af with the human antibody heavy chain signal peptide (Vh), the SV40-intron-Vh-fragment was PCR amplified with forward primer A024 and reverse primer A082, and the plasmid AMI060 that contains the SV40-intron-Vh sequence as template. The VEGF-Trap ORF was amplified with forward primer A085 and reverse primer A025 and plasmid AMI059-pFB-scCMV-SV40-intron-kozak- VEGF-Trap as template. These two PCR fragments were cloned into the XbaI and SphI sites of AMI059 using the NEBuilder HiFi DNA Assembly kit to create AMI066-pFB-scCMV-SV40-intron-Vh- VEGF-Trap as shown in **Fig. 1**.

[0174] To optimize the VEGF-Trap ORF, reverse translation of the VEGF-Trap protein sequence into DNA was performed using the SnapGene Software (GSL Biotech, San Diego, CA) by selecting the homo sapiens preferred codon output. This sequence was synthesized by Twist Bioscience and PCR amplified with forward primer A086 and reverse primer A087. The PCR fragment was cloned into the StuI and BstBI sites of AMI059 via HiFi reaction to create AMI067-pFB-scCMV-SV40-intron-kozak-Af-VEGF-Trap-GC as shown in **Fig. 1**. To replace the signal peptide Af in AMI067 with Vh, the SV40-intron-Vh-fragment was PCR amplified with forward primer A024 and reverse primer A089, and the plasmid AMI060 that contains the SV40-intron-Vh sequence as template. The Twist synthesized VEGF-Trap ORF was PCR amplified with forward primer A088 and reverse primer A090. These two PCR fragments were cloned into the XbaI and BstBI sites of AMI059 using the NEBuilder HiFi DNA Assembly kit to create AMI068-pFB-scCMV-SV40-intron-kozak-Vh- VEGF-Trap-GC as shown in **Fig.1** and **Fig. 6**.

[0175] To perform further optimization of the VEGF-Trap DNA codons, the DNA sequence was manually adjusted to make three more versions of variant VEGF-Trap DNA molecules. In Version 1, 16 Arg codons were changed from AGA to AGG and 29 Pro codons were changed from CCC to CCT to create AMI119-pFB-scCMV-SV40-intron-kozak-Af-VEGF-Trap-GCRP (CCT), see **Fig. 1** and **Fig. 7**. In Version 2, in addition to the 16 Arg codon changes, 36 Ser codons were further changed from AGC to TCC to create AMI120-pFB-scCMV-SV40-intron-kozak-Af-VEGF-Trap-GCRS (TCC), see **Fig. 1** and **Fig. 8**. In Version 3, in addition to the 16 Arg codon changes, 29 Pro codons were further changed from CCC to CCG to create AMI130-pFB-scCMV-SV40-intron-kozak-Af-VEGF-Trap-GCRP (CCG), see **Fig. 1** and **Fig. 9**. All the variant versions of VEGF-Trap DNA sequences were first synthesized by Twist Bioscience, then PCR amplified with forward and reverse primers, and finally cloned into the BstBI and StuI sites of AMI067 plasmid using the NEBuilder HiFi DNA Assembly kit. The plasmid constructs are

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listed in **Table 2** and the information of the primers used for the PCR amplifications are listed in **Table 3** and **Table 4**.

Table 2. List of pFB shuttle plasmids containing the VEGF-Trap gene with different codons

Clone No.	Clone Name	Codon changes
AMI059	pFB-scCMV-SV40-intron-kozak-Af-VEGF-Trap	Low GC
AMI066	pFB-scCMV-SV40-intron-kozak-Vh-VEGF-Trap	Low GC
AMI067	pFB-scCMV-SV40-intron-kozak-Af-VEGF-Trap-GC	High GC
AMI068	pFB-scCMV-SV40-intron-kozak-Vh-VEGF-Trap-GC	High GC
AMI119	pFB-scCMV-SV40-intron-kozak-Af-VEGF-Trap-GCRP(CCT)	16 Arg codons from AGA to AGG and 29 Pro codons from CCC to CCT
AMI120	pFB-scCMV-SV40-intron-kozak-Af-VEGF-Trap-GCRS(TCC)	16 Arg codons from AGA to AGG and 36 Ser codons from AGC to TCC
AMI130	pFB-scCMV-SV40-intron-kozak-Af-VEGF-Trap-GCRP	16 Arg codons from AGA to AGG and 29 Pro codons from CCC to CCG

Table 3. List of clone number and corresponding PCR primers used to generate the PCR fragments for the cloning process

Clone No.	5' PCR Fragment		3' PCR Fragment	
	F-primer	R-primer	F-primer	R-primer
AMI059	A024	A025	-	-
AMI066	A024	A082	A085	A025
AMI067	A086	A087	-	-
AMI068	A024	A089	A088	A090
AMI119	A086	A381	-	-
AMI120	A086	A382	-	-
AMI130	A086	A087	-	-

Table 4. Sequences of primers used for PCR reactions and DNA sequencing analysis

Primer ID	**SEQ ID NO**	**DNA sequence**
A024	1	5'-ATCCAGCCTCCGGACTCTAGAGTTAACTGGTAAGTTTAGT-3'
A025	2	5'-TGGGGTTGATCTCTCCCCAGCATGCCACACAAAAAACCAA-3'
A082	3	5'-TACATTTCTACAAAAGGTCTACCAGTATCGCACTGCACGCCCTTAAGGA-3'
A085	4	5'-GATACTGGTAGACCTTTTGTAG-3'

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Primer ID	SEQ ID NO	DNA sequence
A086	5	5'-GTTGCCTTTACTTCTAGGCCTGCCGCCACCATGGTGAGCTACTGGGAC-3'
A087	6	5'-TAATGAAAATAAAGATATTTTATTTTCGAATCACTTGCCGGGGCTCAGG-3'
A088	7	5'-GACACCGGCAGACCCTTCGTGG-3'
A089	8	5'-ACGAAGGGTCTGCCGGTGTCGCACTGCACGCCCTTAAGGA-3'
A090	9	5'-TAATGAAAATAAAGATATTTTATTTTCGAATCACTTGCCGGGGCTCAGG-3'
A381	10	5'-TAATGAAAATAAAGATATTTTATTTTCGAATCACTTGCCaggGCTCAGG-3'
A382	11	5'-TAATGAAAATAAAGATATTTTATTTTCGAATCACTTGCCGGGggaCAGg-3'

[0176] To determine the relationship between codon optimization and protein expression levels in the human cells, four plasmids were constructed containing the same expression cassette but with variations of VEGF-Trap DNA coding sequences. Two of the coding sequences have low GC-contents and two high GC-content. In one of each the low and high GC-content coding sequence, a human antibody heavy chain (Vh) secretion signal peptide was used to replace the VEGF-Trap secretion signal peptide (Af). All four plasmids have the identical DNA sequence except the VEGF-Trap open reading frame (ORF). The expression cassette contains a CMV promoter, a SV40 intron, a Kozak sequence located upstream of the start codon of the VEGF-Trap ORF and a synthetic polyadenylation signal located downstream the stop codon. In order to make more variations of the VEGF-Trap codons, the VEGF-Trap codons were changed and AMI119, AMI120, and AMI130 plasmids were cloned. The diagrams of these expression cassettes flanked by a full AAV2 ITR and a truncated AAV2 ITR are shown in **Fig. 1**. The full DNA sequences of each expression cassettes are **SEQ ID NO: 13-SEQ ID NO: 19** or **SEQ ID NO: 21-SEQ ID NO: 27** (with **SEQ ID NO: 20** being control plasmid sequence; and **SEQ ID NO: 28** or **SEQ ID NO: 70** being control coding sequence for refence VEGF-Trap).

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[0177] The estimated eCAI values of each ORF for human cells and its relationship to the GC content were determined. The Expected Codon Adaptation Index (eCAI) values were estimated based on the web-based free E-CAI server. Briefly, the target DNA coding sequences were respectively pasted on the dialog box. The human codon usage table was selected from the "Codon Usage Databases" in the same server and pasted on the next dialog box. The Poissen Method was chosen (The Markov Method gave very similar results) and "Standard" genetic code was selected. The eCAI value was estimated by pressing the "Accept" button. The results are shown in **Table 5**. The GC contents are positively correlated with the eCAI values, the higher the GC contents, the higher the eCAI values. AMI059 and AMI066 both contained low GC content of 40% and eCAI of 0.723 and 0.722, respectively. AMI067 and AMI068 both contained high GC of 62% and eCAI values of 0.867 and 0.864, respectively. A natural cDNA sequence of VEGF-Trap was compiled using the secretion signal peptide sequence and FLT1 domain from natural occurring mRNA sequence of NM_001160031, the natural occurring mRNA KDR domain from NM_002252.3, and the human antibody IgG1 Fc domain of natural occurring mRNA AK129809.1. This compiled natural occurring VEGF-Trap was estimated to contain 52% GC and have an eCAI of 0.790, well below the optimized codons of AMI067 and AMI068. Further changes of the VEGF-Trap codons slightly changed the GC contents and eCAI values as shown in **Table 5**. Since higher eCAI values indicate more commonly used codons are in the ORF, that can suggest higher protein expression levels in those expression cassettes.

Table 5. The correlation of GC content with Expected Codon Adaptation Index (eCAI) in human cells

Clone No.	Description	GC Content	Expected Codon Adaptation Index (eCAI)
N/A	Natural cDNA sequence	52%	0.790
AMI059	Af-VEGF-Trap	40%	0.723
AMI066	Vh-VEGF-Trap	40%	0.722
AMI067	Af-VEGF-Trap-GC	62%	0.867
AMI068	Vh-VEGF-Trap-GC	62%	0.864
AMI119	Af-VEGF-Trap-GCRP(CCT)	61%	0.857
AMI120	Af-VEGF-Trap-GCRS(TCC)	63%	0.874
AMI130	Af-VEGF-Trap-GCRP	63%	0.873

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Example 2: Increase of GC-content level enhances VEGF-Trap expression in transient transfected mammalian cells

[0178] To determine the impact of GC-content on the expression of VEGF-Trap in mammalian cells, the four plasmids (AMI059, AMI066, AMI067, and AMI068) were purified and transfected into HEK 293 cells. For the transfection, cells were seeded on 100 mm cell culture dish (Corning, NY) at about 2 x 10^6 cells/dish in 10 mL media overnight. Fourteen µg plasmid DNA and 22 µL of Lipofectamine 3000 (Thermo fisher) were each diluted in 0.5 mL of Opti-medium (Thermo fisher) and mixed together. The mixture was added to the cells drop-wise and cells were incubated at 37 °C in the CO_2 incubator for 48 hours. Media were harvested.

[0179] Forty-eight hours after transfection, media were harvested and loaded directly (non-reducing) or mixed with loading buffer and heated at 90 °C for 5 minutes (reducing) before loading to the SDS-gel. After blotting onto a PVDF membrane, the VEGF-Trap protein was detected with anti-human IgG Fc antibody.

[0180] As shown in **Fig. 2**, a high GC-content of a coding sequence had higher protein expression than a low GC-content coding sequence. Lanes 1 through 5 are shown on a non-reducing gel and lanes 6 through 10 are shown on a reducing gel. Lanes 3, 4, 5, 8, 9 and 10 show a high GC-content (plasmids AMI067, lanes 3 and 8 and AMI068, lanes 4, 5, 9 and 10) and lanes 1, 2, 6, and 7 show a low GC-content (plasmids AMI059, lanes 1 and 6, and AMI066 lanes 2 and 7). Based on the intensity of the Western blot image measured with ImageJ (free software, NIH), high GC-content ORF displayed a 9 to 11-fold increase in protein expression over the low GC-content ORF. Different secretion signal peptides seemed to have a minor impact on the secretion of protein into the media. In the low GC-content ORF, the original VEGF-Trap secretion peptide showed slightly higher expression than the human antibody heavy chain secretion signal peptide, whereas there was no difference in protein secretion between these two secretion peptides in the high GC-content ORF.

Example 3: GC-content level has no impact on AAV2.N53 and AAV2.N54 vector production

[0181] The expression cassettes were cloned into the scAAV shuttle plasmid backbone and rBVs were generated to produce AAV2, AAV2.53, and AAV2.N54 vectors by co-infection of Sf9 cells.

[0182] Sf9 cells (Expression Systems) were cultured in ESF AF media (Expression Systems) containing 100 units/mL penicillin and 100 µg/mL streptomycin (Thermo Fisher Scientific, Pleasanton, CA) in Corning bottle with gentle shaking at 150 rpm and 28 °C. Once cells grew to

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~1e+7cells/mL, they were split 1:4 in fresh media into a new bottle and continuously cultured for maintenance purpose.

[0183] Recombinant baculoviruses (rBV) were generated using the Bac-to-Bac Baculovirus Expression System according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). Briefly, the pFB shuttle plasmids containing the target genes were each diluted into 1 ng/uL in TE buffer, and 2 ng of each DNA was mixed with 20 μL of Δcath-DH10Bac competent bacteria containing a bacmid DNA molecule with the cathepsin gene deleted (Virovek, Hayward, CA) and incubated on ice for 30 minutes followed by heat-shock at 42 °C for 30 seconds. After incubating on ice for 2 minutes, the bacteria were cultured at 37 °C for 4 hours to recover and then plated on agar plates containing 50 μg/mL of kanamycin, 7 μg/mL of gentamycin, 10 μg/mL of tetracycline, 40 μg/mL of IPTG, and 100 μg/mL of X-gal. After 48 hours of incubation at 37 °C, white colonies containing the recombinant bacmid DNAs were picked and miniprep bacmid DNAs purified under sterile condition. About 5 μg of each bacmid DNA and 10 μL of GeneJet Reagent (SignaGen Laboratories, Fredrick, MD) were respectively diluted in 100 μL ESFAF media (Expression Systems, Davis, CA) and then mixed together for about 30min to form the transfection mixture. Sf9 cells were plated in the 6-well plate at 1.5e+6 cells/well in 2 mL ESFAF media at 28°C for about 30min. After removing the old media from the Sf9 cells, each transfection mixture was diluted in 800 uL ESFAF media and then added to the Sf9 cells. After incubation at 28°C overnight, each well was added with additional 1 mL ESFAF media. After a total incubation time of 4 days, media containing the rBVs were collected and amplified at 1:200 ratio to generate sufficient quantity of rBVs ready for use in the AAV production process.

[0184] To produce and purify the AAV vectors comprising the non-naturally occurring nucleic acid comprising a modification described herein (e.g., at least one codon modification), the rBV carrying the AAV2 Rep and capsid genes (rBV-Cap2-Rep, rBV-Cap2.N53-Rep, rBV-Cap2.N54-Rep), and the rBV carrying the VEGF-Trap expression cassettes (rBV-VEGF-Trap) were used to co-infect Sf9 cells for AAV production. Briefly, 10 multiplicity of infection (moi) of rBV-Cap2-Rep (or rBV-Cap2.N53-Rep or rBV-Cap2.N54-Rep) and 5 moi of rBV-VEGF-Trap were used to co-infect the Sf9 cell line at density of about 5e+6 cells/mL with 50% fresh ESFAF media for 3 days at 28 °C with shaking speed of 180 revolutions per minute (rpm) in a shaker incubator. At the end of infection, cell pellets were collected by centrifugation at 3,000 rpm for 10 minutes. The cells were lysed in Sf9 lysis buffer containing 50 mM Tris-HCl, pH 8.0, 2 mM MgCl2, 1% sarkosyl, 1% Triton X-100, and 125 units/mL benzonase with vigorous vortex followed by shaking at 350 rpm, 37°C for 1 hour. At the end of shaking, salt concentration was increased to

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500 millimolar (mM) by vortexing, and the lysates were cleared by centrifugation at 8,000 rpm for 20 min at 4 °C. The cleared lysates were transferred to ultraclear centrifuge tubes for SW28 swing bucket rotor which contain 5 mL of 1.50 g/cc and 10 mL of 1.30 g/cc cesium chloride solutions. After centrifugation at 28,000 rpm, 15 °C for about 18 hours, the AAV bands were collected with syringes and transferred to ultraclear centrifuge tubes for the 70 Ti centrifuge rotor. The centrifuge tubes were filled with 1.38 g/cc cesium chloride solution and heat-sealed. The AAV samples were subjected to a second round of ultracentrifugation at 65,000 rpm, 15 °C for about 18 hours and AAV bands were collected with syringes. The purified AAV samples were buffer-exchanged into PBS buffer containing 0.001% Pluronic F-68 and filter-sterilized with 0.22 µm syringe filters. The sterilized AAV samples were stored at 4 °C within a month and then transferred to -80 °C for long term storage. AAV titer was determined with real-time PCR method using the QuantStudio 7 Flex Real-Time PCR System (Invitrogen).

[0185] The AAV2, AAV2.53, and AAV2.N54 vector titers are listed below in the **Table 6**. The purity of AAV2.53 vectors is shown in **Fig. 3**, which shows an SDS-PAGE with Simply Blue Staining of low-GC and high-GC AAV vector production in Sf9 cells**.** M indicates a size marker for the gel. **Fig. 3A** shows the AAV5 vector loaded as the control (1e+11 viral genome (vg)/lane loaded) compared to AMI059 loaded into lane 2 (5e+10 vg/lane loaded), AMI066 loaded into lane 3 (5e+10 vg/lane loaded), AMI067 loaded into lane 4 (5e+10 vg/lane loaded), and AMI068 loaded into lane 5 (5e+10 vg/lane loaded). **Fig. 3B** shows the AAV2 vector loaded as the control (1e+11 vg/lane loaded) compared to AMI119 loaded into lane 2 (1e+11 vg/lane loaded), AMI120 loaded into lane 3 (1e+11 vg/lane loaded) and AMI130 loaded into lane 4 (1e+11 vg/lane loaded). All AAV vectors were produced at normal range and no GC-content related difference was observed.

Table 6. Comparison of AAV2.N53 production yields between constructs containing low and high-GC DNA content

Clone No.	AAV name (production Lot no.)	AAV Titer (vg/mL)	AAV Production Yield (vg/L)
AMI059	AAV2-CMV-VEGF-Trap (20-118)	1.32e+12	3.29e+13
AMI066	AAV2-CMV-VEGF-Trap (20-119)	2.02e+12	4.29e+13
AMI067	AAV2-CMV-VEGF-Trap (20-120)	0.99e+12	2.38e+13
AMI068	AAV2-CMV-VEGF-Trap (20-121)	1.85e+12	3.88e+13
AMI119	AAV2-CMV-VEGF-Trap (20-113)	4.59e+12	3.52e+13
AMI120	AAV2-CMV-VEGF-Trap (20-114)	5.01e+12	3.50e+13

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AMI130	AAV2-CMV-VEGF-Trap (20-115)	2.96e+12	1.97e+13
AMI120	AAV2.N53-CMV-VEGF-Trap (20-140)	1.92e+13	1.92e+14
AMI120	AAV2.N54-CMV-VEGF-Trap (21-004)	1.62e+13	1.45e+14

Example 4: Increase of GC-content level enhances VEGF-Trap expression in scAAV2.N53 transduced mammalian cells

[0186] scAAV2.N53 vectors harboring VEGF-Trap expression cassettes were produced and purified. They were used to transduce HEK293 cells and the expression levels of VEGF-Trap were determined with Western blot analysis and ELISA assay using the goat anti-human IgG-Fc antibody.

[0187] For cell culture, Human HEK293 cells were cultured in DMEM medium (Thermo fisher) with 5% FBS (ATCC, Manassas, VA) in a Series II Water Jacketed CO_2 incubator (Thermo Forma) at 37 °C. For maintenance, cells were split 1:10 once a week.

[0188] For AAV vector transduction, HEK293 cells (**Fig. 10**) or hARPE-19 cells (**Fig. 11**) were seeded at a density of 2×10^6 cells in 100 mm dish and grown overnight to about 80% confluency in the CO_2 incubator at 37 °C. The next morning AAV vectors were added to the cells at MOI of 1.0×10^4 vg/cell and transduction was carried out for 48 hours at 37 °C. Supernatants were collected without the cells and analyzed for protein expression by Western Blot or protein levels by ELISA. All transductions were performed at least three independent experiments.

[0189] For the ELISA, media harvested from transient transfection or AAV transduction were centrifuged at 10,000 rpm for 5 minutes to remove any particulates and used for the assay. A 96-well plate was first coated with recombinant human endothelial growth factor-A (rhVEGF-A) at 100µL/well in coating buffer at room temperature overnight. After removing the coating buffer, the plate was washed 3 times with 200µL/well of washing buffer (0.05% Tween-20 in PBS, pH 7.3 ± 0.1), and blocked with 200µL/well of Blocking buffer (1% BSA in PBS, pH 7.3 ± 0.1) for a minimum 1 hour at room temperature. After removing the Blocking buffer, the plate was washed 3 times with the washing buffer. After removing the residue of washing buffer, 100 µL/well of testing samples diluted in the Blocking buffer was added to the plate for 2 hours at room temperature. After removing the testing samples, the plate was washed again 3 times with the washing buffer. After removing the residue of washing buffer, 100 µL/well of the Biotinylated Mouse monoclonal (H2) anti-Human IgG Fc (Biotin) Detection Antibody (Abcam, Cambridge, MA) was added to the plate and incubated at room temperature for one hour protected from direct light. At the end of incubation, the plate was washed 3 times, and 100µL/well of 1X HRP-

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Streptavidin solution was added to the plate and incubated at room temperature for 45 min. At the end of incubation, the plate was washed 3 times and 200µL/well of Substrate Solution (Tetramethylbenzidine) was added to the plate. After incubating at room temperature for about 10 min to about 15 min to develop the color, 50µL/well of the Stop solution (2 N sulfuric acid) was added to the plate and optical density values were determined at wavelength 450 nm with the Multimode Plate Reader EnVision (PerkinElmer, Santa Clara, CA).

[0190] For the Western blot, HEK293 cell supernatants at 48 hours post-transfection were collected. A total of 20 µl of the supernatant was mixed with loading buffer, heated at 95 °C for 5 minutes, and loaded onto NuPAGE 10% Tris-Glycine gels (Invitrogen) for electrophoresis. For non-reducing gel, the supernatant was mixed with a non-reducing loading buffer and then loaded on the gel. Proteins on the gels were transferred onto PVDF membranes using the X Cell II™ Blot Module (Invitrogen, Carlsbad, CA, USA). The membranes were treated with casein blocker in PBS (Thermo Scientific, Waltham, MA, USA) for at least one hour at room temperature and probed with the appropriate primary antibody, followed by incubation with the appropriate anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Amersham Biosciences, Uppsala, Sweden). Proteins were detected using ECL™ Western blotting reagents (Amersham).

[0191] The results are shown in **Fig. 4** and **Table 7**. In **Fig. 4A,** lanes 1-4 were loaded with AMI059, AMI066, AMI067, and AMI068, respectively. In **Fig. 4B**, lanes 1-4 were loaded with AMI120, AMI119, AMI067, and AMI130, respectively. As shown, in **Fig. 4A** in lanes 3 and 4 corresponding to AMI067, and AMI068, respectively, the increase of GC content in the VEGF-Trap ORF increased the expression of the VEGF-Trap proteins. In the low-GC content of VEGF-Trap ORFs, the Af signal peptide yielded higher expression than the human Vh signal peptide (7 folds difference) as indicated in **Table 6**. However, in the high-GC content of VEGF-Trap ORFs, there is no difference between the Af and the human Vh signal peptide as shown in **Table 7** and by comparing **Fig. 4A** lane 3 (Af signal peptide) with **Fig. 4A** lane 4 and **Fig. 4B** lanes 1, 2, 3, and 4 (Vh signal peptide).

Table 7. ELISA measurement of VEGF-Trap expression level in media of AAV2 vector transduced HEK293 cells

Clone No.	scAAV2.N53 vectors	VEGF-Trap (ng/mL)	Fold
AMI059	scCMV-SV40-intron-kozak-Af-VEGF-Trap	456	7
AMI066	scCMV-SV40-intron-kozak-Vh-VEGF-Trap	64	1

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Clone No.	scAAV2.N53 vectors	VEGF-Trap (ng/mL)	Fold
AMI067	scCMV-SV40-intron-kozak-Af-VEGF-Trap-GC	19993	312
AMI068	scCMV-SV40-intron-kozak-Vh-VEGF-Trap-GC	19383	303
AMI119	scCMV-SV40-intron-kozak-Vh-VEGF-Trap-GC	19019	297
AMI120	scCMV-SV40-intron-kozak-Vh-VEGF-Trap-GC	18995	297
AMI130	scCMV-SV40-intron-kozak-Vh-VEGF-Trap-GC	18507	289

Example 5: High purity of VEGF-Trap purified from HEK293 cell media

[0192] A 500 mL culture supernatant was harvested from an AAV2-VEGF-Trap (AMI067) transduced HEK293 cells and filtered through 0.2 μm OptiScale filter (Millipore) by peristaltic pump. The HiTrapTM 1mL MabSelectTM PrismA Protein A column (GE Health Care Lifesciences) was first equilibrated with equilibration buffer (Tris-HCl pH 7.2, 150 mM NaCl) at flow rate of 1 mL/min with 5 column volume (CV). Afterwards the filtered supernatant was loaded into the column at the same flow rate using the AKTA explorer 100 system according to instruction manual. The column was washed with equilibration buffer for 10 CV at same flow rate before elution. AAV-Eylea protein was eluted with elution buffer (0.1 M Sodium Citrate Dihydrate pH 3.0) (Fisher Chemical) 6CV at 0.5mL/min flow rate. Fractions were collected into tubes containing 0.1 volume of neutralization buffer (1 M Tris/HCl buffer pH 9.0). The eluted collection was desalted with 1xPBS using centrifugal filter (Millipore, Amico Ultra-4).

[0193] Media from HEK293 cells transduced with AAV2.53-AMI067 were subjected to column chromatography purification. A total of about 4.3 mg pure VEGF-Trap was obtained from about 500 mL of media harvested from scAAV2.53-AMI067 transduced HEK293 cells. SDS-PAGE and SimplyBlue staining show that the protein was pure and had the correct molecular weight of 66.5 kDa as the same size in gel as the commercial product (Regeneron Tarrytown, NY) shown in **Fig. 5**.

[0194] Purified AAV2.VEGF-Trap vector was used to transduce HEK293 cells at MOI of 500,000 vector genome (vg) per cells. VEGF-Trap produced and released into cell culture supernatant was analyzed daily using an operational protocol using commercial VEGF-A_{165} as a coating antigen. VEGF-A_{165} was expressed in HEK293 cells and purified to homogeneity

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(GeneScript, Cat# Z03073) and reconstituted into sterile Milli-Q water at 100 µg/mL. The ELISA procedures were: coating 96-well plate with 0.1 µg/mL VEGF final concentration per well in the coating buffer with a 50 µL volume; covering the plate with sealing; and putting the plate at 4 °C overnight. Next day, the ELISA procedures were: removing the plate; discarding the solution; tapping the plate on the paper towel to remove excess solution; and washing the plate thrice with wash buffer (300 µL). After last wash, the plate was tapped again on a fresh paper towel to remove as much buffer as possible. 300 µL of blocking buffer was added to each well using multichannel pipette. The plate was covered with sealing cover into 37 °C incubator for 2 hours. After incubation, the blocking buffer was discarded, and the plate was tapped on paper towel to remove excess buffer. Sample dilutions were prepared in blocking buffer by adding 50 µL into each well according to the scheme of the experiment. The plate was covered with sealing cover and placed back into the incubator for 1 hour (hr). After 1 hour, the solution was discarded, and the plate was washed with 300 µL of wash buffer 6 times. Excess solution was removed by tapping on paper towel as described above. Capture antibody was added at 1:40,000 dilution in blocking buffer and incubate at 37 °C for 1 hour. Solution was discarded, and washing procedure was repeated as necessary. Detection antibody was added at 1:40,000 dilution in blocking buffer at 37 °C for 1 hour. The solution was discarded, and the plate was washed with additional incubation (5 minutes in wash buffer) for last wash. The wash buffer was then discarded and one additional wash was performed with PBS. 50 µL TMB was added. The plate was kept from direct light source for 15-20 min (it could be for fewer than 15 minutes) or until saturation of the signal was observed at highest concentration wells. 50 µL stop solution was then added, and the plate was read at 450 nm with 620 nm as reference within 15 minutes.

[0195] As seen from **Fig. 10**, the cell culture supernatant harvested from HEK293 cells transduced with AAV2.054-AMI120 secreted VEGF-Trap to approximately 800 ng/mL, while the HEK93 cells without transduction did not secrete VEGF-Trap. The production continues with increase of date of cultivation before the cells aged (**Fig. 11**).

[0196] VEGF-Trap expressed by transduction of HEK293 cells with AAV2.N53-VEGF-Trap vector was purified by the protein A affinity column chromatography. The column eluate was neutralized and buffer changed into 10 mM phosphate buffer (pH 7.0; 150 mM NaCl). The purity of the vector derived VEGF-Trap was determined by loading 1.5 µg/lane into the SDS-PAGE gel (10%) and stained with Coomassie blue R-250 and a single band at 66.5 kDa is seen in gel. The size was identical to that of commercial VEGF-Trap (**Fig. 5A**).

[0197] AVMX-110 derived VEGF-Trap binding affinity was measured by two methods for analysis of AVMX-110 derived VEGF-Trap binding to VEGF- A_{165}: ELISA and surface plasmon

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resonance (SPR). For the ELISA, binding of AVMX-110 derived VEGF-Trap and commercial aflibercept (Regeneron, Tarrytown, NY) to VEGF-A_{165} was performed in parallel under the same conditions. The results showed both were the same in binding, with IC_{50} of 159.1 ng/mL and 158.8 ng/mL respectively for AVMX-110 derived VEGF-Trap (A) and aflibercept (B) as shown in **Fig. 5B** and **Fig. 5C**. The binding constant (Kd) was calculated in nanomolar concentration using molecular weight of 115000 Da. The affinity of AVMX-110 derived VEGF-Trap was identical to that of commercial aflibercept (1.38 nM for both AVMX-110 derived VEGF-Trap and commercial aflibercept). Accordingly, the purified product functioned as the same as those from AAV2.VEGF-Trap.

[0198] Based on the biochemical and biological characterization, AVMX-110 is a qualified AAV vector carrying a VEGF-Trap gene for transduction of human cells including HEK293 and retina cell ARPE-19. The product of AAV2.N54-VEGF-Trap can produce VEGF-Trap *in vivo* similar to that of currently commercial aflibercept. Therefore, AVMX-110 (AAV2.N54-VEGF-Trap) can be used for long term expression for the treatment of neovasculogenic retina disorders *in vivo*.

[0199] Interaction between AVMX-110 derived VEGF-Trap and human VEGF-A_{165} was analyzed by the surface plasmon resonance (SPR) method. 15 µl of 200 nM of AVMX-110 derived VEGF-Trap or aflibercept in phosphate buffered saline with 0.01% Tween 20 (PBS-T) was bound to a sensor chip protein A. AVMX-110 VEGF-Trap and aflibercept were bound to HEK293 expressed human VEGF-A_{165} tightly with a Kd at 33 pM and 55 pM respectively (**Fig. 13** and **Table 8**).

[0200] Proliferation inhibition of HUVEC cells was used to determine the potency AVMX-110 *in vitro*. Briefly, AVMX-110 was firstly used to transduce HEK293 cells, which were cultivated at 37 ºC for 4 days. The cell culture supernatant was harvested and purified via affinity column chromatography. The purified VEGF-Trap was compared to the commercial aflibercept (Eylea, Regeneron Pharmaceuticals, Tarrytown, NY) for blocking human VEGF-A_{165} induced HUVEC cell proliferation. The assay was repeated in duplicates. Briefly, in a 96-well microplate, HUVEC cells (ATCC, Cat#. CRL-1730, Manassas, VA) was seeded at 5000 cells/well in 100 µL cell culture medium and incubated in a CO_2 incubator with 5% CO_2 and 90% humidity. Cells were allowed to adhere for 24 hours, and cell culture medium was removed and replaced with 100 mL/well fresh assay medium (vascular cell basal medium with 1% dFBS) containing 20 ng/mL human VEGF-A_{165} and followed by the purified VEGF-Trap at molar ratio (VEGF-A_{165}/VEGF-Trap) of 1/0, 1:2, 1:5, 1:10 and 1:100 individually or 0, 101, 253, 506 and 5060 ng/mL respectively. Both the commercial aflibercept (40 mg/mL) and AAV2.VEGF-Trap were diluted

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to the same ratio. The plate was incubated for 72 hours followed by measurement procedures. The assay media in each well were removed and fresh assay medium (90 µL) along with 10 µL of WST-8 (Dojindo Molecular Technologies, Inc., Cat. # CK04-11, Rockville, MD) was added. The plate was read every hour post addition. Data demonstrated that AAV2.VEGF-Trap inhibition of HUVEC cell proliferation was VEGF-Trap concentration dependent. The potency is equally effective as aflibercept (**Fig. 12**).

Example 6: Determination of glycosylation of AAV2 derived VEGF-Trap

[0201] AAV2.N54-AMI120 derived VEGF-Trap protein purified from cell culture supernatant of HEK293 transduced with AVMX-110 at a MOI of 100,000 was purified *via* affinity column chromatography. The purified VEGF-Trap was treated with recombinant *F. meningosepticum* PNGase F enzyme which was ideal for removing all *N*-linked carbohydrates from glycoproteins. The treatment was carried out with the commercial aflibercept. The treatment mix was separated by SDS-PAGE gel shift analysis. In the presence of NPGase F, the carbohydrates were removed from the glycoprotein *via* N-linkage, then the deglycosylated protein migrated faster because of decreased molecular weight (**Fig. 12**).

Example 7: Biacore binding assay for binding affinity (KD) of VEGF-A_{165} with vector expressed VEGF-Trap and Afllibercept (Elyea)

[0202] VEGF-Trap protein was expressed by HEK293 cell transfected with expression vector, AAV2.N54-AMI120, and purified by a protein A affinity column chromatography. The eluate was neutralized and concentrated to the concentration of 4.31mg/ml. Aflibercept was commercial product, which was diluted into 4 mg/ml. VEGF-A_{165} was obtained from Genescript (GeneScript, Cat# Z03073, Nanjing China), which was reconstituted into 1 mg/ml. Working solution of each stock protein was prepared at 200 nM prior to experimentation. Molarity concentrations were calculated using MW: 115 kDa for VEGF-Trap and aflibercept, 45 kDa for VEGF-A_{165}. Biacore operation was started with coating a protein A sensor chip with VEGF-Trap or aflibercept at 200 nM by injecting 15 µL at 5 µL/min, followed by injection of 90 µL of serially diluted VEGF-A_{165} solutions of 200, 100, 50, 25, 12.5, and 0 nM at a flow rate of 30 µL/min. Binding kinetic simulation was analyzed with Biacore evaluation software. The KD of AAV2.N54-AMI120 derived VEGF-Trap is 33 pM for its binding to VEGF-A_{165}. The KD of aflibercept was 55 pM for its binding to VEGF-A_{165}. Binding kinetic parameters shown in **Table 8** and **Fig. 13** demonstrate that the AAV2.N54-AMI120 derived VEGF-Trap are similar to those of aflibercept.

Table 8. Kinetic binding affinity determined by Biacore assay

	VEGF-Trap	Aflibercept

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Sources	AAV2.N54-AMI120 expressed in HEK293 cells	Commercial Product (Regeneron Pharmaceuticals)
Ligand	VEGF-Trap	aflibercept
Analyte	VEGF-A_{165}	VEGF-A_{165}
KD(M)	3.3e-11	5.45e-11
KA(1/M)	3e+10	1.83e+10
Kd(1/s)	2.02e+04	1.88e-04
Ka(1/Ms)	6.07E+06	3.45E+06
Rmax(RU)	1.43E+03	1.41E+03

Example 8. Binding affinity of vector expressed VEGF-Trap with rhVEGF-A_{165}

[0203] The assay for determining binding affinity was performed. Briefly, purified from AAV2.N54-VEGF-Trap expressed VEGF-Trap was assayed for its binding affinity to rhVEGF. A 96-well plate was coated with rhVEGF-A_{165} at 0.1 µg/mL at 2-8 ºC overnight. The plate was washed 3x 300 µL with washing buffer (0.05% Tween-20 in PBS, pH 7.3 ± 0.1) and blocked with blocking buffer containing 1% casein for 120 min. Serially diluted VEGF-Trap or aflibercept (the same lot as mentioned above) at 200, 100, 50, 25 , 12.5, 6.25, and 3.1 ng/mL, was added individually to each well at 100 µL/well and incubated at 37±1 ºC for 60 minutes. Diluted (1:40,000) biotinylated rabbit anti-human IgG1 Fc antibody solution was added at 100 µL/well after 3 x 300 µL/well of washing buffer. Further incubation at 37±1 ºC for 60 minutes and washing with 3 x 300 µL/well of washing buffer were performed. Finally, to each well, streptavidin-HRP, 1:40,000, was added at 100 µL/well and incubated at 37±1ºC for 60 minutes. The substrate TMB solution after 3x300 µL/well washing, was added and incubated at 37±1 ºC for 15 minutes followed by stopping with stop solution 100 µL/well. The plate was read at 600 nm wavelength in a microplate reader (Molecular Device, Sunnyvale, CA). Results from assay showed that the binding affinity of VEGF-Trap and aflibercept to rhVEGF-A_{165} was 75 pM and 60 pM separately, demonstrating similar both proteins binding to rhVEGF-A_{165} (**Fig. 14** and **Table 9**).

Table 9. Binding affinity analysis using Biacore assay

Vmax	0.2999	0.2724
Kd (ng/mL)	8.633	6.856
Kd (nM)	0.075	0.060
pM	75	60

Example 9. Inhibition of HUVEC cell proliferation stimulated by VEGF-A_{165}

[0204] The purified VEGF-Trap was compared to the commercial aflibercept (Eylea) (Regeneron Pharmaceuticals, Tarrytown, NY) for blocking human VEGF-A induced HUVEC

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cell proliferation. The assay was repeated in duplicates. Briefly, in a 96-well microplate, HUVEC cells (ATCC, Cat#. CRL-1730, Manassas, VA) were seeded at 5000 cells/well in 100 µL cell culture medium and incubated in a CO_2 incubator with 5% CO_2 and 90% humidity. After cultivation for 24 hours, cell culture medium was removed and replaced with 100 mL/well fresh assay medium containing 20 ng/mL human VEGF-A_{165} and following by the purified VEGF-Trap at molar ratio (VEGF-A_{165}/VEGF-Trap) of 1:0, 1:2, 1:5, 1:10, and 1:100 individually or 0, 101, 253, 506 and 5060 ng/mL respectively. The commercial aflibercept (40 mg/mL) and AAV.VEGF-Trap were diluted in the same ratio. The assay medium was vascular basal medium with 1 % dFBS, and the plate was incubated for 72 hours followed by measurement procedures. The assay media in each well was removed, and fresh assay medium (90 µL) along with 10 µL of WST-8 (Dojindo Molecular Technologies, Inc., Cat. # CK04-11, Rockville, MD) was added. The plate was read every hour post addition. Data demonstrated that AAV.VEGF-Trap inhibited HUVEC cell proliferation a VEGF-Trap concentration dependent. The potency was equally effective as aflibercept (**Fig. 15**).

Example 10. *In vivo* evaluation of AAV2.N54-AMI120VEGF-Trap protection of choroidal damage caused by laser induced neovascularization

[0205] Efficacy and pharmacokinetics of AAV2.N54-AMI120-VEGF-Trap were tested using mouse LCNV model. The study of the cohorts is shown in **Table 10**.

Table 10. Experimental design of AAV2.N54-AMI120-VEGF-Trap in mouse laser choroidal neovascularization

Group	No. of Animals	OU Treatment/ Dose	Volume/ Route	Treatment Day	CNV Induction	Experimental Endpoints
1	8	AAV2-GFP 4e+8 vg/eye	1.0 µL IVT	Day -3	OU: CNV Laser Day 0	**DAY 7:** • Fluorescein angiography • Flatmount analysis and necropsy (n=6 mice/group or 12 eyes per group) • Dissect eye cups and homogenize for ELISA (n=2
2	8	Aflibercept (Eylea®) 200 µg				
3	8	AVMX110 Low Dose 2e+7 vg/eye		Day -28		

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4	8	AVMX110 Medium Dose 4e+8 vg/eye				mice/group for Groups 1-5). • Collect serum of each animal for ELISA
5	8	AVMX110 High Dose 1.6e+10 vg/eye				
6	2	NA	NA	NA	NA	**DAY 7:** Dissect eye cups and homogenize for ELISA (n=2 mice/4 eyes)

[0206] Mouse (*Mus Musculus*) / C57BL/6 (Charles River or Tacomic Farms) of 8-12 weeks old, 15-25 grams each, are used in the study. The animals are randomly divided into groups, 5 animals per group. Each animal is injected bilaterally, 10 eyes per group, with the test article intravitreally (IVT) 28 days prior to lasering. The test article of AAV2.N54AMI120 is tested at three doses, a high (1.6e+10), medium (4e+8) and low dose (2e+7) per microliter (μL). As a control, AAV2.N54-GFP is used as a vector transmission control. AAV2.N54-AMI120 formulation buffer (vehicle) is used as a negative control. The mice are dosed (IVT administration) with test articles and controls 28 days prior to laser injury control animals are dosed 3 days prior to lasering for aflibercept and vehicle. Seven (7) days post lasering, all groups are analyzed for fluorescent angiography, VEGF-Trap level serum and eye cups. VEGF-Trap concentration are detected using ELISA. According to *in vitro* potency results mentioned above, the animals dosed with AAV2.N54-AMI120 can gain protection from inflammation and wound healing responsive neovascularization, while the AAV2.N54-GFP and vehicle controls may not have any protection.

Example 11. Efficacy of modified adeno-associated Virus (AAV2) encoding aflibercept (AVMX-110) in a laser-induced choroidal neovascularization (CNV) model in mice

[0207] The objective of this study was to evaluate the inhibition of neovascularization in a laser-induced model of choroidal neovascularization (CNV) in the mouse using the adeno-associated viral (AAV2) vectors. **Table 11** illustrates the experimental design for this study.

Table 11. Experimental design for examining CNV in mice

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Group	No. of Animals	OU Treatment/ Dose	Volume/ Route	Treatment Day	CNV Induction	Experimental Endpoints
1	8	Formulation Buffer (Vehicle) N/A	1.0 µL IVT	Day -3	OU: CNV Laser Day 0	**DAY 7:** • Color/Cobalt Blue Fundus Imaging (**Group 3 only**): Day 7,
2	8	Aflibercept (Eylea)) 40 µg				
3	8	AAV2-GFP 1.6e10 vg/eye		Day -28		• Fluorescein angiography: n=8 mice/group • Terminal blood collection (serum): n=8 mice/group • Flatmount analysis: n=6 mice (12 eyes)/group • Dissect and homogenize eye cups for ELISA: n=2 mice (4 eyes)/group
4	8	AVMX110 2e7 vg/eye				
5	8	AVMX110 4e8 vg/eye				
6	8	AVMX110 1.6e10 vg/eye				
7	2	N/A	N/A	N/A	N/A	**DAY 7:** • Terminal blood collection (serum): n=2 mice • Dissect and homogenize eye cups for

Abbreviations: CNV — choroidal neovascularization; IVT — Intravitreal; OU — both eyes; N/A — not applicable.

[0208] This laser choroidal neovascularization (CNV) study was conducted in mice (Mus Musculus) C57BL/6 strain. The study met the acceptance criteria for a valid, non-GLP status study, because average CNV lesion size evaluated via fluorescein angiography (FA) and Flatmount immunofluorescence was greatly reduced in aflibercept treated animals when compared to formulation buffer or a control vector (GFP-AAV2) following IVT injections.

[0209] Test articles were provided in a ready-to-inject format and stored at < -70°C until use. Aflibercept, which was stored refrigerated (2-8°C) and injected neat without dilution. Thirty minutes prior to injection, each test article was thawed in a 37°C water bath for 20 minutes and vortexed to reduce virus aggregation.

[0210] On Days -28 (Groups 3-6) and -3 (Groups 1 and 2) prior to injection, mice were given buprenorphine 0.01-0.05 mg/kg subcutaneously (SQ). Animals were then tranquilized for the intravitreal injections with inhaled isoflurane and one drop of 0.5% proparacaine HCL was applied to both eyes. The conjunctiva was gently grasped with Dumont #4 forceps, and the injection was made using a 33 G needle and a Hamilton Syringe. After dispensing the syringe contents, the syringe needle was slowly withdrawn. Following the injection procedure, 1 drop of Ofloxacin ophthalmic solution was applied topically to the ocular surface with eye lube.

[0211] On day 0, mice were given buprenorphine 0.01-0.05 mg/kg SQ. A topical mydriatic (1.0% Tropicamide HCL, and 2.5% phenylephrine HCL) was applied at least 15 minutes prior to the laser procedure. The mice were tranquilized with an intraperitoneal (IP) injection of

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ketamine/xylazine. The cornea was kept moistened using topical eyewash, and body temperature was maintained using hot pads. A 532 nm diode laser delivered through a slit-lamp was used to create 4 single laser spots surrounding the optic nerve. Both mouse eyes received laser treatment at predetermined time intervals. Eye lube was placed (OU) after laser.

[0212] Morbidity and mortality were observed daily along with cage-side observations, with particular attention paid to both eyes. Four (4) animals died post-dosing but before lasering in Groups 2 (#'s 210, 212, and 216) and 4 (# 432). Two (2) additional animals died prior to final tissue collections but after lasering in Groups 4 (#430) and 6 (#644).

Fundus imaging

[0213] Both color and cobalt blue (EGFP expression) fundus imaging were performed on both eyes of Group 3 (AAV2-GFP; 1.6e10 vg/eye) animals on Day 7 after laser and prior to fluorescein angiography (FA). Animals were given a cocktail of tropicamide (1.0%) and phenylephrine (2.5%) topically to dilate and proptose the eyes, and topical eye anesthetic was applied to the eyes (proparacaine 0.5% or similar). Color fundus photography was followed by cobalt blue photography. Intensity settings were kept constant between animals during acquisition. As shown in **Fig. 16**, GFP was observed in all eyes, with the exception of 318 OS, which had a cataract that prevented imaging of the fundus. The observed cataract may have been caused by trauma to the lens during the IVT injection.

Fluorescein angiography (FA)

[0214] FA was performed on both eyes on Day 7 post-laser. Mydriasis for FA was achieved using a topical drop in each eye 15 minutes prior to examination (1.0 % tropicamide HCL, and 2.5% phenylephrine HCL). The mice were tranquilized with an IP injection of ketamine/xylazine. Retinal photography was performed approximately 1 minute after intravenous sodium fluorescein injection (1% stock solution; 12 mg/kg). Fluorescein leakage was analyzed with ImageJ software; representative images are shown in **Fig. 17**, which illustrates day 7 fluorescein angiography. Representative images from each group are shown. Measurement of lesions in Group 3 was not feasible as the GFP expressed by the AAV precluded analysis. Angiograms were used to quantify lesion area as pixels ± standard deviation (SD) and the results are displayed in **Fig. 18**. On study day 7 post-laser, Group 6 (AVMX110, 1.6e10 vg/eye) had the smallest average lesion area while the vehicle group had the largest lesion area.

Ocular tissue collection and processing

[0215] On Day 7, animals were humanely euthanized, and eyes were enucleated and immediately fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4°C. Using a dissecting microscope, each eye was trimmed of extraneous tissue and the anterior segment and

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lens were removed. The retina was detached and removed from the optic nerve head with fine curved scissors. Eye cups were rinsed with cold immunocytochemistry (ICC) buffer (PBS + 0.5% BSA and 0.2% Tween 20) and blocked for at least 6 hours in ICC buffer. Eye cups were then placed in cold ICC buffer containing the 4',6-diamidino-2-phenylindole (DAPI; nucleus), Phalloidin DyLight 550 (actin cytoskeleton) and Isolectin IB4-DyLight 649 (blood vessels) and incubated at 4°C with gentle rotation for 2.5 hours. Tissues were washed, and radial cuts were made toward the optic nerve head, avoiding lesions, and tissue was flat-mounted. Two-dimensional (2D) fluorescent microscopy images were acquired on an Olympus Bx63 fluorescent scope and neoformed vessels were quantified using the Isolectin IB4 signal area (µm2) and CellSens software. Representative images are shown in **Fig. 19**. Quantification of isolectin area is shown in **Fig 20**. Injection of aflibercept reduced the lesion area. Group 3 eyes had the largest lesion area, though this may have been impacted in part by the GFP driven by the AAV2 construct. When compared to the AAV2-GFP from Group 3, AVMX-110 reduced lesion size at all doses tested (Groups 4-6).

[0216] This study was conducted to determine the inhibition of retinal vascular leakage after IVT administration of test articles (Groups 3-6) and a positive control (aflibercept, Group 2) in mice. AAV2 vectors (Groups 3-6) were injected on day 28 to ensure adequate expression prior to induction of laser CNV; vehicle and aflibercept (Groups 1 and 2) were injected on day 3. Based on the tests performed and the objectives of this study, the aflibercept control resulted in decreased lesion size at Day 7 post-laser when compared to vehicle. In comparison to Aflibercept (positive control; 40.5% reduction in fluorescein area), the test articles in Groups 5 and 6 demonstrated equivalence or superiority by at least one endpoint analysis, with the highest dose of AVMX-110 (1.6e10 vg/eye) having a 68% reduction in vascular leakage for the angiography endpoint. Analysis of lesion size in Flatmount revealed that all AAV2-preparation injected eyes had larger lesions than vehicle or aflibercept-injected eyes. However, when AVMX-110 was compared to AAV2-GFP, lesion sized was reduced at all three AVMX-110 dose levels, with the highest dose of AVMX-100 leading to a 40% reduction in isolectin area.

Example 12. Analysis of AVMX-110 and AVMX-116

[0217] Example 12 illustrates a comprehensive analysis of data that lead to the candidate selection for AVMX-110 and AVMX-116. The strategy involved construct design, cloning, sequencing, production, purification and bioanalytics. The data was analyzed using one-way ANOVA method. Candidates that had best expression, potency and penetrability (*in vivo*) were selected on following criteria: Gene of Interest (GOI); capsid; and AAV serotype.

Capsid Engineering and Selection

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[0218] Wild type AAV2 capsid had been engineered methodologically to introduce mutations at specific sites to increase the penetrability or expression of GOI. The following list of mutations were introduced and screened in mouse and/or pig models to check for expression and penetrability (**Table 12**). The sequences and positions were inserted with a short peptide fragment in the loop 4 of AAV2-VP1 (**Table 12**), and the plasmid names are listed in **Table 13**.

Table 12. AAV construct with modified capsid region

Construct ID	Modified Capsid Region	SEQ ID NO:	HEK293 Cell	ARPE19 Cell
Wild type (Wt)	No insertion	N/A	++++	++++
V466	TPSG(453):LALGETTRPA:TTTQ	40	++	++
V467	QRGN(587):LALGETTKPA:RQAA	41	++++	++++
V468	NTPS(452):- ALGETTKP:GTTT	42	++	++
V471	NTPS(452):- ALGETTKP:GTTT; QRGN(587):LALGETTKPA:RQAA	43 41	++	++
AMI051	QRGN(587):LKLGQTTKPK:RQAA	44	+	ND
AMI052	QRGN(587):LALGQTTKPK:RQAA	45		
AMI053	QRGN(587):LKLGQTTKPA:RQAA	46	++++	++++
AMI054	QRGN(587):LALGQTTKPA:RQAA	47	++++	++++
AMI055	R585_588A	N/A	+	ND
AMI056	R585A	N/A	++	ND
AMI057	R588A	N/A	++	ND
AMI097	TPSG(453):LALGQTTKPA:TTTQ	48	ND	ND
AMI098	QRGN(587):LALGQTTEPA:RQAA	49	ND	ND
AMI099	RGNR(588):LALGQTTKPA:QAAT	50	ND	ND
AMI100	NLQR(585):LALGQTTKPA:GNRQ	51	ND	ND
AMI101	QRGN(587):VALGQTTKPA:RQAA	52	ND	ND
AMI102	LQRG(586):LALGESTARG:NRQA	53	ND	ND
AMI103	LQRG(586):LALGETSKRA:NRQA	54	ND	ND
AMI104	LQRG(586):LALGQSTKPA:NRQA	55	ND	ND
AMI105	NTPS(452):LALGQTTKPA:GTTT	56	ND	ND
AMI106	TPSG(453):LALGQTTKPA:TTTQ QRGN(587):LALGQTTKPA:RQAA	57 47	ND	ND

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Construct ID	Modified Capsid Region	SEQ ID NO:	HEK293 Cell	ARPE19 Cell
AMI107	QRGN(587):LALGQTTKPA LALGQTTKPA:RQAA	58	ND	ND
AMI110	QRGN(587):VKLGQTTKPA:RQAA	59	ND	ND

Table 13. List of different AAV2 capsid mutations screened in animal models

Construct	Description	Study/Animal
AMI051	pFB-inCap2-587-KQKK-inRep-kozak-hr2	20-AVI-002/Mouse
AMI052	pFB-inCap2-587-QKK-inRep-kozak-hr2	20-AVI-002/Mouse
AMI053	pFB-inCap2-587-KQK-inRep-kozak-hr2	20-AVI-002 & 005/Mouse & Pig
AMI054	pFB-inCap2-587-QK-inRep-kozak-hr2	20-AVI-002 & 005/Mouse & Pig
AMI055	pFB-inCap2-R585_588A-inRep-kozak-hr2	20-AVI-002/Mouse
AMI056	pFB-inCap2-R585A-inRep-kozak-hr2	20-AVI-002/Mouse
AMI057	pFB-inCap2-R588A-inRep-kozak-hr2	20-AVI-002/Mouse
AMI097	pFB-inCap2-453QK-inRep-hr2	20-AVI-003/Mouse
AMI099	pFB-inCap2-588QK-inRep-hr2	20-AVI-003/Mouse
AMI101	pFB-inCap2-587VQK-inRep-hr2	20-AVI-003 & -005/Mouse & Pig
AMI102	pFB-inCap2-586SARG-inRep-hr2	20-AVI-003/Mouse
AMI103	pFB-inCap2-586SKR-inRep-hr2	20-AVI-003/Mouse
AMI104	pFB-inCap2-586QSK-inRep-hr2	20-AVI-003 & -005/Mouse
AMI105	pFB-inCap2-452QK-inRep-hr2	20-AVI-003 & -005/Mouse & Pig
AMI106	pFB-inCap2-453_587QK-inRep-hr2	20-AVI-003/Mouse
AMI107	pFB-inCap2-587QKx2-inRep-kozak-hr2	20-AVI-003/Mouse
AMI110	pFB-inCap2-587VKQK-inRep-hr2	20-AVI-003/Mouse
V104 (wt)	pFB-inCap2-inRepOpt-Kan	20-AVI-002 & -003/Mouse
V226 (+)	pFB-inCap2-7m8-GFP WT	20-AVI-002, 003 & 005/Mouse & Pig
V467	pFB-inCap2-587RtoK -GFP WT	20-AVI-002/Mouse

[0219] 2E+10 vg/eye of different constructs were injected in the mouse model. **Fig. 21** shows the fundus images all the groups. These images were noted on day 23 after injection of constructs. V226 was the positive control and showed good expression. One eye from each group was further analyzed by immunohistochemistry (**Fig. 22**), and eyes injected with construct AMI-053, AMI-054, AMI104 and V226 showed bright GFP expression. AMI054 particularly showed uniform expression in Ganglion cell layer (GCL), inner plexiform layer (IPL), and outer nuclear layer (ONL). In the other mouse study, the rest of the constructs mentioned previously were analyzed. Similar to the 20-AVI-002, 2E+10 vg/eye construct was injected into the mouse eyes. The fundus imaging was done on day 24 (**Fig. 23**).

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[0220] AMI101, AMI104 and AMI105 were subjected to the immunohistochemistry (**Fig. 24**) on day 28, and AMI104 showed uniform distribution of GFP expression in GCL, IPL, inner nuclear layer (INL), outer plexiform layer (OPL), ONL, inner segment (IS), and outer segment (OS). From the mouse studies, several candidates (AMI053, AMI054, AMI101, AMI104, AMI105 and V226) were picked up for additional pig study. Each pig eye was injected with 1E+11 vg/eye. **Fig. 25** shows the fundus images of the animals in the pig study. AMI053 showed faint GFP expression similar to the results observed in the mouse study. AMI054 showed uniform and bright expression similar to V226. To further confirm the expression and penetrability of the constructs, immunohistochemistry (IHC) was done on the cryosections generated on day 28 after injection (**Fig. 6**) similar to the mouse studies. According to the IHC data, AMI054 and V226 showed brightest, broadest and consistent GFP expression. V226 showed intense GFP expression mainly in the GCL and AMI054 showed intense GFP signal at INL. From the mouse and pig studies it can be concluded that AMI054 had brightest, broadest and consistent results. Therefore, AMI054 was selected and finalized as the AAV capsid for inserting and screening for AVMX-110. Confocal microscopic images were also documented for better visualization of different layers of eye. These images illustrated penetrability of capsid into different ocular layers (**Fig. 27**). AMI054, even though expressed lower GFP, showed deeper penetrability into INL. V226 showed more expression in RGC layer.

GOI screening

[0221] Several constructs were designed and screened before finalizing the candidate. Expression profile of all the constructs was checked in human embryonic kidney cells (HEK293) cells. Some of the constructs were also checked in ARPE-19 cells (Human retinal pigment epithelial cells) and hTERT-rpe 1 (human Telomerase reverse transcriptase – retinal pigmental cells). hTERT-rpe 1 cells were derived by transfecting the RPE-340 cell line (primary human RPE cell line) with the pGRN145 hTERT-expressing plasmid. This was used to mimic primary RPE cells. **Table 14** shows the expression of these constructs in different cell lines. For example, AAV2.N54.120 viral particle (also named AVMX-110) comprises the non-naturally occurring nucleic acid packaged inside the AAV2.N54 capsid comprising: high GC content (denoted by "GC" in the "GCRS"); codon AGG for encoding arginine (denoted by "R" in the "GCRS"); and codon TCC for encoding serine (denoted by "S" in the "GCRS"). For another example, AAV6.N54.120 virus particle (also named AVMX-116) comprises the non-naturally occurring nucleic acid packaged inside the AAV6.N54 capsids comprising: high GC content (denoted by "GC" in the "GCRS"); codon AGG for encoding arginine (denoted by "R" in the "GCRS"); and codon TCC for encoding serine (denoted by "S" in the "GCRS").

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Table 14. Constructs screened for the AVMX-110/116 project

Construct	Lot#	Description	Self-Complimentary	Promoter	Production date	Aflibercept Expression (µg/mL)	
						HEK293	ARPE-19
N54.120	**21-004**	**scCMV-Aflibercept-GCRS(TCC)**	**ds**	**CMV**	**02/01/2021**	**20.9 ± 3.1**	**1.4 ± 0.2**
N54.120	21-077	scCBA-Aflibercept-GCRS(TCC)	ds	CBA	08/09/2021	9.7 ± 2.2	ND
N54.164	21-088	pFB-CMV-SV40in-Aflibercept-pA-Stuffer-GFP	ss	CMV	09/10/2021	1.0	0.07
N54-190	**21-097**	**pFB-scCMV-SV40-intron-kozak-Aflibercept-rev**	**ds**	**CMV**	**10/06/2021**	**19.6 ± 6.3**	**2.1**
N54-191	21-093	pFB-scAd-enhancer-CMV-SV40in-Aflibercept-rev	ds	CMV	09/27/2021	9.8	1.4
N54.192	21-094	pFB-scCBA-SV40-intron-kozak-Aflibercept-rev	ds	CBA	10/06/2021	3.3 ± 1.4	1.1
N54.221	**21-114**	**scCMV-SV40-intron-kozak-VEGF-Trap-GCRS(TCC)-WPREa**	**ds**	**CMV**	**10/25/2021**	**22.9 ± 2.0**	**1.6**
N54.222	21-115	scCMV-TPL-kozak-VEGF-Trap-GCRS(TCC)	ds	CMV	10/25/2021	11.1	0.8
N54.223	21-116	scCMV-SV40-intron-kozak-VEGF-Trap-geneious opt	ds	CMV	10/25/2021	7.5	0.5
N54.224	21-117	scCMV-SV40-intron-kozak-VEGF-Trap-	ds	CMV	10/25/2021	5.0	0.7

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Construct	Lot#	Description	Self-Complimentary	Promoter	Production date	Aflibercept Expression (µg/mL)	
						HEK293	ARPE-19
		GenScript optimized					
N54.225	21-118	scmCMV-SV40-intron-kozak-VEGF-Trap-GCRS(TCC)	ds	Murine CMV	10/25/2021	1.7	1.1
N54.226	21-119	scmCMV-SV40-intron-kozak-VEGF-Trap-GCRS(TCC)-WPREa	ds	Murine CMV	10/25/2021	3.1	1.3
N54.228	21-126	scmCMV-SV40-intron-kozak-Aflibercept-geneious opt-WPREa	ds	Murine CMV	11/10/2021	0.6	
N54.229	21-127	scmCMV-SV40-intron-kozak-Aflibercept-GS opt-WPREa	ds	Murine CMV	11/10/2021	1.5	
N54.230	21-128	scmCMV-SV40-intron-kozak-VEGF-Trap-GCRS(TCC)-rev	ds	Murine CMV	11/10/2021	1.6	
N54.231	21-129	scmCMV-SV40-intron-kozak-VEGF-Trap-GCRS(TCC)-WPREa-rev	ds	Murine CMV	11/10/2021	1.4	
N54.232	21-130	scmCMV-SV40-intron-kozak-Geneious opt-Aflibercept-WPREa-rev	ds	Murine CMV	11/10/2021	0.6	
N54.233	21-131	scmCMV-SV40-intron-kozak-	ds	Murine CMV	11/10/2021	1.4	

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Construct	Lot#	Description	Self-Complimentary	Promoter	Production date	Aflibercept Expression (µg/mL)	
						HEK293	ARPE-19
		GenScript opt-Aflibercept-WPREa-rev					
N54.200	21-155	pFB-CMV-TPL-enh-Intron-Aflibercept-structure-pA	ss	CMV	1/5/2022	27.4 ± 7.2	2.3 ± 0.4
N54.204	21-156	pFB-scCMV-Vh-Leader-Fc-4xGGGGS-VEGF-Trap	ds	CMV	1/5/2022	8.4	0.1
N54.296	22-019	pFB-CMV-TPL-Full-enh-Aflibercept-structure-hGHpA (fusion)	ss	CMV	2/23/2022	2.0	0.1
N54.298	22-020	pFB-CMV-TPL-Full-enh-intron-Aflibercept-structure-hGHpA	ss	CMV	3/2/2022	5.2	0.5
N54.261	22-017	pFB-scCMV-VEGF-TrapGCRS(TCC)-1xGGGGS-Endostatin-IgG4Fc-WPREa	ds	CMV	2/23/2022	3.5	0.1
N54.268	22-018	pFB-scCMV-SV40in-kozak-VEGF-Trap-Fc4-1xGGGGS-Endostatin-WPREa	ds	CMV	2/23/2022	3.9	0.1
N54.243	22-026	pFB-scCMV-SV40-intron-kozak-FLT1-KDR-Fc4-WPREa	ds	CMV	3/10/2022		
N54.244	22-027	pFB-scCBA-SV40-intron-kozak-FLT1-	ds	CBA	3/10/2022		

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Construct	Lot#	Description	Self-Complimentary	Promoter	Production date	Aflibercept Expression (µg/mL)	
						HEK293	ARPE-19
		KDR-Fc4-WPREa					
N54.248	22-036	pFB-scCMV-TPL-AdEnh-Intron-AfliberceptGCRS-WPREmini-pA	ss	CMV	3/17/2022		
N54.290	22-028	pFB-scCMV-SV40in-kozak-VEGF-Trap-GCRS(TCC)-Albumin 3'-UTR-pA	ds	CMV	3/10/2022		
N54 (184)-311	HEK030920 22-17	Jcat optmized-VEGF-Trap hGHPA	ds	CMV	3/17/2022		

[0222] Three constructs listed in **Table 1**4 (N54.120, N54.190 and N54.221) exhibited higher aflibercept expression compared to others. The AAV construct back bone for these three candidates are shown in the **Fig. 28A**. During the manufacturing process, samples were taken at various points of process steps for various purposes of testing in order to achieve accurate control of processes. Exemplary sample process chromatograms for two manufacturing runs are shown in **Fig. 28B**. Through the capture step, a single and sharp fraction of the AVMX-110 vectors was obtained during the process (arrow). Almost 100% particles of AVMX-110 were captured by our capturing process and with more than 80% eluted in process intermediate pool. **Fig. 28C** analysis of the eluted AVMX-110 process intermediate purity by SDS-PAGE. AAV VP1, VP2 and VP3 were clearly visualized, and no other major impurity protein was visible. From the capsid charge difference, the performance profile of empty and full AAV was established in the column process. Under this condition, empty capsid ran inside column faster than the full capsid. The differential retention time was sufficient for separation of empty from the full capsid (**Fig. 28D**). The eluate pool for the capture step was processed for separation of a portion of empty capsid. The separation curves are shown in **Fig. 28E**. Using this separation model, AVMX-110 was purified and enriched in full capsid content. In the separation chromatograms, two separate peaks were obtained. The full capacity enriched fraction post-column chromatography was succeeded with concentration and buffer exchange to make a drug substance for further evaluation. AVMX-110 manufacturing intermediate and drug substance samples were analyzed for specify detected

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with AAV2 antibody Western blot, for each development lot. The antibody to AAV2 VP1, VP2, and VP3 were the only protein bands seen in gel and the Western blot (**Fig. 28F**). AVMX-110 purity was analyzed by gel electrophoresis stained by silver stain method. The first peak was empty, and the second peak was full capsid. The product was AAV particles containing VP1, VP2, and VP3. The purity of AVMX-110 process intermediates was analyzed by SDS-PAGE stained with silver stain gel (**Fig. 28G**). AAV VP1, VP2, and VP3 were clearly visualized, and no single impurity high than 4% was found.

Host cell contaminants

[0223] Sf9 host cell protein contaminants in AVMX-110 developmental product was measured by ELISA. Exemplary measurements of the host cell protein contaminant is shown in **Table 15**. The host protein level was very low (≤170 ng/10^{12} vg). The purification process was extremely specific for GOI as it removed 1.5+10-fold of host protein from starting cell culture lysate. The level of host protein had exceeded the requirement of ≤100 ng/dose for product release. For this lot, if a dose was at 2e+11 vg, the host cell protein level was 4 ng/dose. The PCNA of host cell DNA detection the limits of another batch was ≤46.12pg/1e+12 vg. The host cell protein and DNA testing indicated that AVMX-110 was highly purified and that exceeds regulatory requirements.

Table 15. Host cell protein and host cell DNA quantification

Sample	AAV2.N54-VEGF-Trap	Host Cell Protein			Host Cell DNA	
Capture eluate	3.06e+10	204	67e+3	6.1E-09	≤10	≤10 is the LoD at pg/reaction
Polish column	6.82e+10	11.6	170	1.5E-10	≤10	

[0224] Sf9 cell genomic DNA was purified with the Qiagen genomic DNA purification kit, and the DNA was diluted to 1000, 250, 63, 16, 4, and 1 ng/mL and used as standards. AVMX-110 process samples were diluted 2 folds, and 10 folds in QPCR dilution buffer and used for qPCR primers and probes to Sf9 cells. All tests performed with AVMX-110 process samples were non-detectable with Sf9 cell DNA at ≤10 pg/reaction, which was equivalent to 1 ng/mL ≤300 pg/dose.

Determination of baculoviral and other plasmid DNA contaminants

[0225] Baculoviral DNA was purified according to the protocol provided by Qiagen genomic DNA purification kit with few modifications. Briefly, 7.5 mL baculovirus in medium was mixed

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with equal volume of cold 20% PEG 8000 in 1 N NaCl and let stand at room temperature for 30 minutes. The baculoviral particles were pelleted by centrifugation at 10,000 rpm for 10 minutes and dissolved in 5 mL G2 buffer. 95 µL proteinase K was added to digest the protein capsids at 50 ℃ for 60 minutes. The column was equilibrated, and the sample was loaded. After washing, the baculoviral DNA was eluted and qPCR tested with primers and probe complementary to GP64 gene of baculovirus genome. The DNA was diluted to 1000, 250, 63, 16, 4, and 1 ng/mL and used as standards. AVMX-110 samples were diluted 2 and 10 folds in qPCR dilution buffer (without DNase digestion) and used for qPCR assay. The baculovirus DNA level was 8.58e+5 per 10^{12} vg of AVMX-110 vector as shown in **Table 16**.

Table 16. Level of Baculovirus and plasmid DNA in copies/per 1e+12 vg of plasmid genes

Vector copy* (vg)	Gentamicin	Ampicillin	PCNA (pg)	Bac IE1	GP64	Rep
1E+12	5.48 e+ 09	4.17e+ 09	≤46.12	4.45 e+ 07	8.58 e+ 05	1.57 e+ 07

AVMX-110 vector copy number. PCNA was the house keeping gene of SF9 cells. Bac IE1 was the DNA fragment cloned in the SF9 cell. GP64 was a gene of baculovirus DNA. Rep was DNA fragment in helper baculovirus.

[0226] The other plasmid selection marker genes such as gentlemen, ampicillin (AMP), Rep, host cell DNA marker, PCNA, BacIE1 DNA contaminants were below the regulatory requirements, indicating AVMX-110 was pure and met regulatory safety requirements. The levels of these evaluated DNA markers were approximately 10-fold lower than regulatory requirements.

Endotoxin level

[0227] Endotoxin level in AVMX-110 sample was measured by a chromogenic limulus amebocyte lysate (LAL) release assay using commercial kit. In the presence of endotoxin, proteolytic cleavage of the colorless chromogenic substrate in Pyrochrome LAL releases *p*NA, which produces yellow color solution and has absorption at 405 nm. The result of endotoxin level in AVMX-110 preparation are shown in the **Table 17**. The endotoxin level in AVMX-110 sample was 0.0154 EU/mL or 0.005 EU/dose.

Table 17. Endotoxin level of AVMX-110 sample

I.D.	undiluted	1:2	1:4	1:8	1:16	1:32
Unspiked sample (21-004)	0.0132	0.0077	0.0059	0.0051	0.0048	0.0045

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Spiked sample (0.04EU/ml)	n/a	0.0295	0.0275	0.0370	0.0436	0.0408
Recovery (spike-unspike)	n/a	0.0219	0.0216	0.0318	0.0388	0.0363
% Recovery	n/a	55	54	80	97	91

Sterility

[0228] Each batch of AVMX-110 was assayed for bioburden, and no microbiological contamination was detected for drug substance used for animal test.

Replication competent AAV Level

[0229] Traditional methods of using mammalian cells to produce AAV vectors with plasmid transfection or virus infection generally produces some replication competent AAV (rcAAV) virus particles due to the non-homologous recombination between AAV genome and AAV rep-cap plasmids. Contamination of rcAAV is not desirable in GMP manufacturing and the amount of contamination is required to be reported to the FDA. The AAV system described herein removed and disrupted the AAV promoters and replaced them with insect cell promoters which were not active in mammalian cells. No rcAAV is able to be generated in the Bac-to-AAV system during the manufacturing process. To establish the assay to monitor the rcAAV, 100 wild type (wt) AAV particles were spiked into 1e+12 vg AAV sample. The rcAAV assay was performed. Briefly, 1e+12 vg AAV sample was mixed with 100 vg of wtAAV2 particles in the presence of adenovirus type helper and transduced into HEK293 cells for 3 days. The cells were harvested, and lysate was prepared. After heat inactivation at 56 °C for 30 minutes to inactivate the adenovirus, 50% of the lysate was used to transduce the HEK293 cells in the presence of adenovirus type 5 to amplify the rcAAV or spiked wtAAV for 3 days. The cells were harvested, and lysate was prepared for qPCR detection for the rcAAV signal. The results showed that, without spike in wtAAV2, there was no positive signal of rcAAV. When spiked with 100 vg wtAAV in 1e+12 vg AAV sample, positive signal was detected (**Table 18**).

Table 18. Detection of replication competent AAV in AVMX-110 drug substance

Virus	Description	Lot #	vg/ml	rcAAV (vg)
AVMX-110	AAV2.N54-VEGF-Trap	20-140	1.92E+13	<1E-10

Empty/full Capsid Estimation

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[0230] Ratio of full and empty capsid of each lot was estimated using vector specific qPCR and AAV2 specific antibody ELISA. The product fractions of the column chromatography were analyzed for vector copy numbers and protein level. The empty capsid had no or very low vector DNA level but the capsid protein only, while the full vectors had both vector DNA and protein contents. The data showed that the elution 1 of the polish step had no or extremely low gene of interests. The eluate of peak 1 was extremely low or even to non-detectable for AAV2.N54-VEGF-Trap DNA but the protein. The second peak (Peak 2) in the profile showed both vector DNA and protein. The results demonstrated that the purified AVMX-110 and UFDF pool were all full particles **(Fig. 10A).**

AVMX-110 Infectivity

[0231] AVMX-110 infectivity was determined by $TCID_{50}$ assay (Tissue Culture Infectious Dose 50%/mL), which was the concentration of infectious organisms in the inoculum determined from the dilution at which the inoculum infects 50% of the target cultures (i.e., when the starting sample was diluted by an amount equal to the $TCID_{50}$/mL, 1 mL aliquots added to multiple target cultures for infection, on average, 50% of the cultures). The $TCID_{50}$ assay was used to determine the potency of AAV vectors based on manufacturer's protocol (ATCC). Briefly, a series of diluted AAV samples were used to transduce the HelaRC32 cells in the presence of adenovirus 5 helper for 3 days in a 96-well plate. Cell lysates were prepared and positive signals were detected with qPCR method. $TCID_{50}$ value was calculated based on the Spearman-Karber formula. The result is shown in **Table 19**.

Table 19. Detection of AVMX-110 $TCID_{50}$

Virus	Description	Lot #	vg/ml	$TCID_{50}$	vg/$TCID_{50}$
AVMX-110	AAV2.N54-VEGF-Trap	20-140	1.92E+13	2.00E+11	96.23
ATCC reference	AAV2-GFP				

AVMX-110 Expression of VEGF-Trap (AAV2.VEGF-Trap) in HEK293 Cells

[0232] Purified AAV2-VEGF-Trap vector was used to transduce HEK293 cells at MOI of 100,000 vector genome (vg) per cell. VEGF-Trap produced and released into cell culture supernatant was analyzed daily using an internal standard operational protocol (21-AD-VEGF-ELISA.01) using commercial VEGF-A_{165} as a coating antigen. VEGF-A_{165} was expressed in HEK293 cells, purified to homogeneity (GeneScript, Cat# Z03073), and reconstituted with sterile Milli-Q water at 100 µg/mL. The ELISA procedures were as followed. VGEF-A_{165} was coated

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onto the 96-well ELISA plate and incubated O/N at 4 °C. Next day the plate was washed with wash buffer thrice, and blocking buffer was added to each well. The plate was covered and incubated at 37 °C for 2 hours. Plate was covered and put back into the incubator for 1 hour. Thereafter, the plate was washed, and anti-human Fc antibody was added to the plate and again incubated for 1 hour at 37 °C. Plate was washed, and streptavidin-HRP was added for 45 minutes of incubation. Plate was washed, and TMB substrate was added. After about 15 minutes, stop solution was added. The wells were read at 450 nm in a microplate reader. As seen from **Fig. 10B**, the cell culture supernatant harvested from HEK293 cells transduced with AMVX-110 (AAV2•N53-VEGF-Trap) secreted VEGF-Trap to approximately 800 ng/mL, while the HEK293 cells without transduction did not secrete VEGF-Trap. The production continued with daily increase during cultivation before the cells aged.

Durability of AAV2.VEGF-Trap expression *in vitro*

[0233] HEK293 and ARPE-19 (human retina pigment epithelium) cells were cultured with 10% FBS and transduced with AAV2•N53-VEGF-Trap and AAV2•N54-VEGF-Trap at MOI of 100,000 per cell. Samples were taken for ELISA quantification of VEGE-trap expression. AAV2•N54-VEGF-Trap showed better expression when compared to AAV2•N53-VEGF-Trap (**Fig. 11**). This variation was observed significantly lower in ARPE19 cells than in HEK293 cells. In ARPE19 cells, after day 20 the VEGF-Trap expression stemmed from transduction of N53-VEGF-Trap construct was negligible. In contrary, VEGF-Trap expression stemmed from transduction of N54-VEGF-Trap construct was still present in quantifiable amounts. VEGF-Trap expression was higher in HEK293 cells compared to ARPE19 cells.

Mouse efficacy study

[0234] Construct AAV2.N54.120 (AVMX-110) was selected to be administered into the mouse at three different doses to check for the efficacy. Briefly, the study had 6 groups, i.e., vehicle (formulation buffer), commercial Eylea (protein), AAV2.N54-GFP (-) dosed at 1.6E+10 vg/eye, AVMX-110 low dose (2.0E+07 vg/eye), medium dose (4.8E+08 vg/eye) and high dose (1.6E+10 vg/eye). **Fig. 29** shows the fluorescence angiography (FA) data. The medium dose of the AAV2.N54-120 had similar efficacy compared to the commercial aflibercept (Eylea) protein administered at 40 µg/eye.

[0235] Aflibercept ELISA was used to quantify the expression level of aflibercept in the ocular samples (local expression) and serum samples (systemic level). Aflibercept expression in ocular samples injected with high dose of construct showed detectable expression. However, the other groups showed very low or no detectable aflibercept expression.

Serotype Selection

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[0236] It is well known that AAV (especially AAV2) neutralizing antibodies are widely present in humans. Another challenge in using AAV2 is lack of tropism. Therefore, AAV1 and AAV6 serotypes were screened *in vitro* (**Fig. 30** and **Table 30**) for expression level and *in vivo* for efficacy (FA data, **Fig. 31**) and aflibercept expression in ocular tissue (**Fig. 32** and **Table 20**).

Table 20. Tabular representation of aflibercept expression in ocular and serum samples

Serotype	Dose/eye (IVT)	Eye Cup (Retina)		Serum	
		ng/mL	ng/eye cup	ng/mL	ng/animal
AAV1.N54-Aflibercept	4.8e+8 vg	7.2 ± 5.3	≤LoQ	≤LoQ	≤LoQ
AAV2.N54-Aflibercept	4.8e+8 vg	≤LoQ	≤LoQ	≤LoQ	≤LoQ
AAV6.N54-Aflibercept	4.8e+8 vg	6.6 ± 8.1	≤LoQ	≤LoQ	≤LoQ

LoQ = Limits of Quantification

[0237] Aflibercept ELISA method had been performed, and LoQ for that assay was determined to be 2 ng/mL. The values that were under 2 ng/mL were denoted as >LoQ. From **Table 20**, it could be concluded that serum samples did not have any detectable aflibercept. However, AAV6 and AAV1 had detectable level of aflibercept in ocular samples.

[0238] A dose response study was designed and conducted, where AAV6.N54-Aflibercept was dosed in mice at 2.0E+07 (low dose) , 4.8E+08 (medium dose), and 1.6E+10 high dose. **Fig. 33** shows the FA data as a bar graph for comparing different groups.

[0239] Animals were injected with 5E+09 vg/eye of AAV2, AAV1, and AAV6-GFP to check for the expression and penetrability. Those animals didn't show any supremacy over AAV2.N54-GFP (**Fig. 34**).

[0240] In order to compare different serotypes, pig study was performed where pigs were injected with 1E+11 vg/eye with AAV1, AAV2, and AAV6-GFP. Similar to the previous pig study, V226 (+ control) had also been included. **Fig. 35** shows the representative fundus and IHC images. The fundus imaging was done on day 21, 28, and 34 due relatively low GFP signal in AAV1, AAV2, and AAV6-GFP injected animals. Finally, on day 34 the animals were sacrificed, and IHC was performed on the selected eyes. The *in vitro* ELISA assays for GFP did not show higher expression of GFP in AAV2.N54-GFP when compared to AAV2.wt-GFP (**Fig. 36**).

[0241] During the GOI screening, lot to lot variability in the AAV constructs was detected. For example, N54.120 construct was purified multiple times and stored at -80 ºC. The purification process varied from using different volumes of starting cell culture, purifying using either 2 cycles of ultracentrifugation (UC) or AAVx affinity column, cell viability and other culture condition variation. **Table 21** shows the summary of few lots of N54.120.

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Table 21. Comparison of lot to lot variability in N54.120 construct

Lot#	Culture Volume	Purification method	Aflibercept expression		
			HEK293	ARPE-19	hTERT-rpe1
21-004	200 mL	UC	20.9 ± 3.1	1.4 ± 0.2	ND
21-030	200 mL	UC	10.4 ± 0.6	0.6 ± 0.04	0.4
21-038	2 L	AAVx + UC	31.9 ± 2.1	ND	ND
21-045	2 L	AAVx + UC	33.5 ± 2.6	1.2	1.2
21-050	2 L	AAVx + UC	31.6	ND	ND
21-096	50L	AAVx + UC	16.6	1.3	0.8

[0242] Below is exemplary GFP expression captured in fluorescence microscope images (**Fig. 37A**) and also analyzed using GFP quantification ELISA (**Fig. 37B** and **Table 22**) showing lot to lot variability. Although intensity of GFP lot#20-147 was lower than 20-06-30-N54 lot, ELISA did not show significant difference in GFP expression *in vitro*.

Table 22. GFP concentration determined by GFP ELISA for different N54-GFP lots

N54.GFP lot#	GFP (µg/mL)
20-06-30-N54	6.0 ± 2.6
20-147	4.9 ± 2.2

[0243] After mouse and pig study capsid N54 was selected and AMI120 optimized GOI was selected. Animal efficacy study showed significant laser injury recovery stemmed from the AAV vector treatment that was comparable to commercial aflibercept. AAV2 serotype appeared to be more consistent in mouse efficacy CNV model than AAV6 serotype.

Example 13.Bioanalytical comparison results for animal studies

[0244] Example 13 illustrates the bioanalytical comparison of mouse ocular Choroidal Neovascularization (CNV) or macular neovascularization (MNV) efficacy model resulted from two different studies to check for the reproducibility of the model. Both studies used the same titer and volume of non-GLP, AAV2 construct, for intravitreal (IVT) injection. The study protocols were also similar (**Table 23**). AAV2 capsid quantification was performed using commercial AAV2 capsid protein kit (Progen, Cat#: PRAAV2R and Lot#: A20008). For the statistical analysis GraphPad Prism (v9.0.1) was used.

Table 23. Protocol comparison for Example 13

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Study code	No. of animals	OU treatment/dose	Vol./Route	Treatment day	MNV induction
21-AVI-AX110.01	8	Vehicle[1]	1.0 µL/eye IVT	Day -3	OU: CNV Laser Day 0
	8	AVMX-110 4E+08 vg/eye		Day -28	
21-AVI-AX112.01	8	Vehicle[2]	1.0 µL/eye IVT	Day -28	OU: CNV Laser Day 0
	8	AVMX-110 4E+08 vg/eye			

Vehicle = AAV formulation buffer; AVMX-110 = AAV2.N54-120.
[1]: 1X PBS pH 8.0, 0.1mM Sodium citrate, 0.001% Pluronic F-68
[2]: 150mM NaCl, 20 mM Sodium phosphate, pH 7.3, 0.01% Pluronic F-68

Material and methods

[0245] Table 24 shows the number of serum and ocular samples obtained from the mouse studies.

Table 24. Summary of number of serum and ocular samples obtained after euthanasia

Study Code	Treatment	Serum samples (no.)	Ocular samples (no.)
21-AVI-AX110.01	Vehicle	8	4
	AVMX-110	8	4
21-AVI-AX112.01	Vehicle	7	14
	AVMX-110	8	16

[0246] A 532 nm diode laser was used to create 4 single laser spots surrounding the optic nerve. If the construct injection or vehicle caused any inflammation reaction, then the lasering would not make a lesion (bubble). If there was no bubble, then this spot would not be used for analysis. For every eye, 4 laser spots were induced, and the size of the lesions were estimated after 7 days post laser treatment. The lesions corresponded to the wound healing due to the effects of the gene product of various constructs. Eight animals were used in every group, corresponding to 16 eyes with 4 laser spots per each eye resulting in a total of 64 laser spots. **Table 25** summarizes the number of no bubbles formed by each group injected with AVMX-110 or vehicle control.

Table 25. Number of no bubbles formed out of total 64 laser spots created

Study Code	Treatment	Number of no bubbles/total bubbles
21-AVI-AX110.01	Vehicle	13/64
	AVMX-110	4/64
21-AVI-AX112.01	Vehicle	4/56
	AVMX-110	8/64

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[0247] Eye cup samples consisting of Retina/RPE/Choroid/Sclera were homogenized, as described in the study protocol for both the studies, using 1X PBS and BSA without protease inhibitors. Each ocular sample was further homogenized using a sonicator with a pulse of 21 seconds and an interval of 30 second with 5-6 repeats. When a clear supernatant, visible to naked eye, was obtained, the samples were centrifuged at 13,000 rpm for 3 minutes. The supernatant was then collected and used to determine the VEGF-Trap levels.

FA analysis comparison

[0248] Seven days after laser injury, both eyes for each mouse were analyzed by FA for lesion area. The gene product of AVMX-110 in treated mice was expected to reduce the laser injury as compared to the vehicle control treated mice. **Fig. 38** and **Table 26** show the comparison of efficacy of AVMX-110 in laser lesion recovery. In both studies, AVMX-110 treated mice showed equal laser wound recovery. There was a variation in the baseline of area for the two studies, but the AVMX-110 treated animals showed almost identical lesion recovery. Representative images from FA image analysis also shows similar reduction in the lesion area **Fig. 39**.

Table 26. Tabular comparison of the lesion area

Study Code	Treatment	Lesion Area (pixel^2) ± S.D.
21-AVI-AX110.01	Vehicle	3930 ± 1052
	AVMX-110	2145 ± 1368
21-AVI-AX112.01	Vehicle	4411 ± 1083
	AVMX-110	2852 ± 1444

Comparing VEGF-Trap levels in serum samples

[0249] Serum samples were directly placed into 96-well plates without dilution. **Fig. 39A** shows the VEGF-Trap levels in serum samples. VEGF-Trap levels in the serum samples were below the limit of quantification of the qualified VEGF-Trap ELISA.

VEGF-Trap levels in ocular samples

[0250] VEGF-Trap in ocular samples was quantified by ELISA and directly plotted as ng/mL. VEGF-Trap concentrations per mg of tissue weight were calculated by determining the absolute amount of VEGF-Trap determined with ELISA, divided by the volume in which the ocular tissue was homogenized. The amount calculated was then divided by the tissue weight to determine the pg of VEGF-trap per eye cup. The VEGF-trap data was plotted **(Fig. 39B),** and the VEGF-Trap levels for serum and ocular samples were documented in **Table 31** (vehicle samples were excluded from the table since the values were below the LoQ). Overall, the VEGF-Trap

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expression in all serum and ocular samples were very low, and most of the animals were below limits of quantification of VEGF-Trap ELISA.

Table 31. VEGF-Trap levels in Ocular samples

Study code	Dose/eye (IVT)	Eye Cup (Retina/RPE/Choroid/Sclera)		Serum	
		ng/mL	ng/eye cup	ng/mL	ng/animal
21-AVI-AX110.01	4.8e+8 vg	≤ LoQ	≤ LoQ	≤ LoQ	≤ LoQ
21-AVI-AX112.01	4.8e+8 vg	0.1 ± 2.2	0.01 ± 0.1	≤ LoQ	≤ LoQ

Correlation analysis between VEGF-Trap concentration and area recovered after laser treatment

[0251] Presence of lesion was measured as area in pixels. The lesion pixel data was plotted against pg of VEGF-Trap per eye cup, and the correlation was calculated using Pearson correlation with a two-tailed p value estimation at 95% confidence interval using Graphpad Prism software. **Fig. 40A** shows a comparison between the two experiments. Both analysis showed negative correlation of VEGF-Trap concentration with the lesion pixel area. This data indicates that lower lesion areas (pixels) are associated with higher VEGF-Trap concentrations. Correlation coefficients and p values for both the studies are documented in **Table 32**.

Table 32. Correlation coefficients and p values

Study Code	Correlation coefficient	p value
21-AVI-AX110.01	-0.55	0.21
21-AVI-AX112.01	-0.36	0.057

Comparing the AAV2 capsid protein levels in both studies

[0252] The AAV2 capsid assay was performed using the AAV2 capsid ELISA kit. The AAV2 capsid protein levels also in both studies were comparable (**Fig. 40B**). However, it was important to note that the volume of ocular samples (in both studies) varied significantly from sample to sample, impacted the overall capsid protein estimation.

[0253] FA images and lesion area analysis results showed consistency between the two studies. There were more "no bubbles" in the vehicle group. For VEGF-Trap levels in ocular samples, many data points were below the limit of quantification. The assay had a LoQ of ~2 ng/mL. This LoQ value was lower than the commercially available kit (~5-6 ng/mL). In these studies, the ocular samples consisted of an eye cup and not the entire eye, which might be effecting the volume and level of VEGF-Trap in the samples. Hence, it was decided to include whole eyes in future studies. Similar to VEGF-Trap, AAV2 capsid protein ELISA was also affected by the volume of the samples. Commercial aflibercept (40 μg/eye) was injected as a reference and

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positive control. 4.0E+08 vg/eye of AVMX-110 showed similar lesion recovery as commercial aflibercept. Since the results of these two studies were similar, it could be concluded that this AVMX-110 dose showed similar results as commercial aflibercept. Overall, the mouse MNV model was a relevant model for initial construct screening before proceeding to larger animal models, such as NHP.

Example 14. Efficacy of Modified Adeno-Associated Virus (AAV6.N54-120) encoding aflibercept (AVMX-116) in a laser-induced choroidal neovascularization (CNV) model

[0254] Example 14 illustrates evaluation of inhibition of neovascularization in a laser-induced model of choroidal neovascularization (LCNV) in the mouse using different serotypes of adeno-associated viral vectors in Groups 1-5 and to evaluate the distribution of AAV6-N54-GFP, AAV1-N54-GFP, and AAV2.N54-GFP in mouse retina tissue in Groups 6-8; two naïve animals (Group 9) were included for the method development and bioanalysis efforts. **Table 33** illustrates the experimental design.

Table 33. Experimental design

Gp	No. of Animals	OU Treatment/ Dose	Volume/ Route	Treatment Day	CNV Induction	Experimental Endpoints
1	**10** CNV: 8 PK: 2	AAV6.N54-Δ120 1.6e+10 vg/eye **Sham Vector*	1.0 µL IVT	Day -28	OU: CNV Laser Day 0 (n=8/group)	**DAY 7:** • Fluorescein angiography (CNV animals only): n=8 mice/group • Terminal blood collection (serum): n=10 mice/group • Flatmount analysis (CNV animals only): n=8 mice/group • Non-CNV animals (n=2 animals/group).
2	**10** CNV: 8 PK: 2	AVMX-110 4e+8 vg/eye *CNV positive Control*				
3	**10** CNV: 8 PK: 2	AVMX-116 2e7 vg/eye				
4	**10** CNV: 8 PK: 2	AVMX-116 4e8 vg/eye				

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5	**10** CNV: 8 PK: 2	AVMX-116 1.6e10 vg/eye				
6	**2** IHC	AAV1.N54-GFP 5e9 vg/eye			N/A	**DAY 0:** • Color/Cobalt Blue Fundus Imaging • IHC for GFP: n=2 mice/group
7	**2** IHC	AAV2.N54-GFP 5e9 vg/eye *GFP Positive Control*				
8	**2** IHC	AAV6.N54-GFP 5e9 vg/eye				
9	**2** PK	N/A	N/A	N/A	N/A	**DAY 7:** • Terminal blood collection (serum): n=2 mice

Abbreviations: CNV – choroidal neovascularization; IVT – Intravitreal; OU – both eyes. For Groups 1-5, n=8 mice/group will receive an IVT injection of test material on Day -28 and then undergo laser-induced choroidal neovascularization; n=2 animals/group will only receive the IVT injection and will be processed for pharmacokinetic ELISA analyses. Sham vector is the non-expression vector of AVMX-110 whose open reading frame (ORF) of GOI (aflibercept) was disrupted.

Methods

[0255] On day 28, mice were given buprenorphine 0.01-0.05 mg/kg subcutaneously (SQ). Animals were then tranquilized for the intravitreal injections with inhaled isoflurane and one drop of 0.5% proparacaine HCl was applied to both eyes. The conjunctiva was gently grasped with Dumont #4 forceps, and the injection was made using a 33 G needle and a Hamilton Syringe. After dispensing the syringe contents, the syringe needle was slowly withdrawn. Following the injection procedure, 1 drop of Ofloxacin ophthalmic solution was applied topically to the ocular surface with eye lube.

[0256] On day 0, mice were given buprenorphine 0.01-0.05 mg/kg SQ. A topical mydriatic (1.0% tropicamide HCl, and 2.5% phenylephrine HCl) was applied at least 15 minutes prior to the laser procedure. The mice were tranquilized with an intraperitoneal (IP) injection of ketamine/xylazine. The cornea was kept moistened using topical eyewash, and body temperature

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was maintained using hot pads. A 532 nm diode laser delivered through a slit-lamp was used to create 4 single laser spots surrounding the optic nerve. Both mouse eyes received laser treatment according to the schedule in the Experimental Design. Eye lube was placed (OU) after laser. Morbidity and mortality were observed daily along with cage-side observations, with particular attention paid to both eyes. Animal 436 (Group 4) died on day 7 prior to imaging procedures. The baseline body weight of all animals was 25.1 ± 1.5 g. Animals in all groups gained a normal amount of body weight over the course of the study.

[0257] Samples from Groups 1-5 and 9 were shipped or stored on dry ice. Animals allocated for pharmacokinetic analysis (n=2/group) did not undergo LCNV induction for aflibercept quantification. Ocular samples (i.e. whole eye globes) were enucleated, frozen after isolation, and weighed. **Table 34** shows the detailed documentation of tissue weight and the volume of 1X PBS with protease inhibitor (PI) added to each sample ahead of homogenization. Samples were placed on ice and homogenized using a sonicator with a 20 second pulse followed by a 20 second rest for three cycles. Following sonication, the samples were rested on ice.

Table 34. Tissue Weight and PBS Volume

Animal #	Group #	Eye	Pre-weight (g)	Post-weight (g)	Tissue Weight (g)	µL of PBS + PI
109	1	OS	1.5858	1.6089	0.0231	231
		OD	1.6161	1.6385	0.0224	224
110	1	OS	1.5831	1.6083	0.0252	252
		OD	1.5922	1.6177	0.0255	255
219	2	OS	1.6117	1.6355	0.0238	238
		OD	1.5975	1.6209	0.0234	234
220	2	OS	1.5967	1.6213	0.0246	246
		OD	1.5802	1.6062	0.0260	260
329	3	OS	1.5930	1.6194	0.0264	264
		OD	1.6011	1.6278	0.0267	267
330	3	OS	1.5784	1.6018	0.0234	234
		OD	1.5927	1.6201	0.0274	274
439	4	OS	1.5940	1.6188	0.0248	248
		OD	1.5802	1.6038	0.0236	236
440	4	OS	1.5970	1.6204	0.0234	234
		OD	1.5973	1.6226	0.0253	253

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Animal #	Group #	Eye	Pre-weight (g)	Post-weight (g)	Tissue Weight (g)	µL of PBS + PI
549	5	OS	1.5916	1.6147	0.0231	231
		OD	1.5819	1.6036	0.0217	217
550	5	OS	1.6051	1.6302	0.0251	251
		OD	1.6137	1.6371	0.0234	234
957	9	OS	1.5959	1.6194	0.0235	235
		OD	1.5962	1.6200	0.0238	238
958	9	OS	1.5772	1.6027	0.0255	255
		OD	1.5960	1.6207	0.0247	247

[0258] The aflibercept expression in ocular (whole eye), and serum samples was quantified using the aflibercept ELISA. Briefly, 0.1 µg/mL VEGF was coated onto the 96-well plate and incubated O/N at 4 °C. The plate was washed with wash buffer and blocked with blocking buffer. Ocular and serum samples were delivered to the specified wells directly without any dilution with the exception of Group 5 samples (1:5, 1:10, and 1:100 dilution). Samples were incubated for 1 hour. Plates were washed and the detection antibody was added into each well at a 1:40,000 dilution. After a 1 hour incubation, plates were washed and Streptavidin-horse radish peroxidase (HRP) was added at a 1:40,000 dilution. After a 45 minutes of incubation, plates were washed, and aflibercept was detected by the addition of 3,3',5,5'-tetramethylbenzidine (TMB) substrate. The reaction was stopped by the addition of stop solution, and the plate was read immediately at 450 nm along with the reference wavelength of 600 nm.

[0259] Aflibercept expression in the animals treated with the sham vector control and untreated animals was similar. In Group 3, animals that were injected with AAV6.N54-Aflibercept, about 2.3 pg aflibercept/mg of ocular homogenate was measured (**Fig. 41A**). AAV2 and the mid-range level for AAV6.N54-Aflibercept had about 8.8 and about 25 pg aflibercept/mg of ocular homogenate respectively, which was a threefold-elevated expression for the AAV6-treated animals. High dose-AAV6 treated animals showed the highest level of aflibercept, with about 400 pg aflibercept/mg of ocular homogenate. Serum samples followed a similar trend as the ocular tissue samples. Group 1 (Sham), 2 (AAV2-Aflibercept) and 3 (AAV6-low dose) had no detectable aflibercept. Both the medium and high dose AAV6-Aflibercept treated animals showed 2-3 ng/mL aflibercept in the serum (**Fig. 41B**). The data were analyzed in GraphPad Prism software using a one-way ANOVA followed by Dunnett's multiple comparison (** = 0.005 and **** = <0.0001). Aflibercept expression is located in **Table 35** (showing that

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AAV1.N54-Aflibercept and AAV6.N54-Aflibercept expressed 30-fold higher Aflibercept than AAV2.N54-Aflibercept) and **Table 36**.

Table 35. Aflibercept Expression Level in Mice by 3 AAV Vectors

Serotype	Dose/eye (IVT)	Eye Cup (Retina)			Serum		
		ng/mL	pg/eye cup	-fold	ng/mL	pg/animal	-fold
AAV2.N54-Δ120 Sham vector	1.6e+10 vg/eye	<0.01	<0.01	NA	<0.01	<0.01	NA
AAV1.N54-Aflibercept	4.8e+8 vg	7.2 ± 5.3	0.3 ± 0.3	30	2.4 ± 0.5	0.6 ± 0.1	60
AAV2.N54-Aflibercept	4.8e+8 vg	0.1 ± 2.2	0.01 ± 0.1	1.0	0.0 ± 0.12	0.0 ± 0.03	1.0
AAV6.N54-Aflibercept	4.8e+8 vg	6.6 ± 8.1	0.31 ± 0.3	31	1.3 ± 0.95	0.3 ± 0.2	30

Table 36. Aflibercept Expression in Ocular Homogenate and Serum Samples

Product	Entity	Dose/eye (IVT)	Eye Cup (Retina)/Eyeball			Serum	
			ng/mL	pg/eye cup	pg/eye ball	ng/mL	ng/animal
Vehicle (FB-01)			≤LoQ *	≤LoQ	ND	≤LoQ	-0.01 ± 0.02
Sham vector control	AAV2.N54.Δ120	4.8E+08 vg/eye	≤LoQ	≤LoQ	ND	≤LoQ	-0.1 ± 0.0
		1.6E+10 vg/eye	0.2 ± 0.1 ≤LoQ		1.8 ± 1.2 ≤LoQ	≤LoQ	-0.01 ± 0.01
Aflibercept (Protein)	Commercial Eylea	40 µg/eye	≤LoQ	≤LoQ	ND	48.5 ± 10.8	9.71 ± 2.2

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AVMX-110 Study Number 21-AVI-002	AAV2.N54-Aflibercept	2E+07 vg/eye	≤LoQ	≤LoQ	ND	0.4 ± 0.5 ≤LoQ	0.06 ± 0.08
		4.8E+08 vg/eye	0.03 ± 2.3 (eye cup); 0.9 ± 0.9 (globe) ≤LoQ	2.4 ± 20	8.8 ± 8.6**	0.1 ± 0.4 ≤LoQ	0.005 ± 0.02
		1.6E+10 vg/eye	4 ± 2.7	36.4 ± 24	ND	1.3 ± 1.6 ≤LoQ	0.191 ± 0.22
AVMX-116 Study Number 21-AVI-004/-007	AAV6.N54-Aflibercept	2E+07 vg/eye	0.2 ± 0.1 ≤LoQ	ND	2.3 ± 1.0	≤LoQ	≤LoQ
		4.8E+08 vg/eye	5.1 ± 5.2 (eye cup); 2.5 ± 2.0 (globe)	51.3 ± 51.5**	25.2 ± 19.6	2.4 ± 2.9	0.17 ± 0.2
		1.6E+10 vg/eye	39 ± 19.2		389.7 ± 192.4	1.7 ± 2.8	0.17 ± 0.3

*≤LoQ=2ng/mL

Fundus Imaging (Groups 6-8, Day 0)

[0260] Color and cobalt blue fundus imaging for EGFP was performed on both eyes from the animals enrolled in Groups 6-8. Animals were given topical mydriatic (1.0% tropicamide HCl and 2.5% phenylephrine HCl) to dilate and proptose the eyes, and topical anesthetic was applied to the eyes (proparacaine 0.5%). Color fundus photography was followed by cobalt blue photography. Acquisition settings were set to Group 7 (positive control) and held consistent across Groups 6 and 8. Representative images are located in **Fig. 42**. GFP signal was observed in all three groups. It was strongest and brightest in Group 7 (positive control), then Group 8, then Group 6. Expression was more restricted than observed in previous studies despite holding the exposure time consistent across all studies.

Fluorescein Angiography (FA)

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[0261] FA was performed on both eyes on day 7 post-laser. Mydriasis for FA was achieved using a topical drop in each eye 15 minutes prior to examination (1.0 % tropicamide HCl and 2.5% phenylephrine HCl). The mice were tranquilized with an IP injection of ketamine/xylazine. Retinal photography was performed approximately 1 minute after intravenous sodium fluorescein injection (1% stock solution; 12 mg/kg). Fluorescein leakage was analyzed with ImageJ software; representative images showing the lesions are shown below in **Fig. 43**. Angiograms were used to quantify lesion area as pixels, 2.5 standard deviations (SD), and the results are displayed in **Fig. 44.** When analyzing Isolectin area size, lesions where Bruch's membrane did not rupture, where vitreal hemorrhages were noted, or where data points more than 2.5 standard deviations from the mean were excluded. The Group 5 (highest dose of AVMX-116) test material outperformed the Group 2 AVMX-110 (positive control), having the lowest lesion area of all groups. Statistical analysis was performed by one-way ANOVA followed by Dunnett multiple comparison on GraphPad Prism software (* = 0.0138).

[0262] When the data from this study were compared to a previous study, homogenization of whole eyes was superior for the estimation of aflibercept expression when compared to eye cups only, increasing the accuracy of quantification. Ocular samples from AAV6.Aflibercept showed about 10-fold increase in protein expression with a 20-fold increase in viral vector dose, and when compared to AAV2, the AAV6 constructs showed a 3-fold enhancement in aflibercept expression. Animals receiving the medium- or high-dose AAV6.Aflibercept demonstrated considerable aflibercept expression in serum samples. The study showed the average CNV lesion size evaluated via fluorescein angiography (FA) was greatly reduced in animals receiving IVT injections of their positive control, AVMX-110, consistent with a other study utilizing the same construct.

Ocular Tissue Collection and Processing

[0263] On Day 7, animals were humanely euthanized via carbon dioxide asphyxiation and death was confirmed by cervical dislocation.

Tissue Collection for Flatmount Analysis (Groups 1-5 only)

[0264] Eyes were enucleated and immediately fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and stored overnight at 4 °C. The following day, the eyes were transferred to cold immunocytochemistry (ICC) buffer (PBS containing 0.5% BSA and 0.2% Tween 20) until processing. Using a dissecting microscope, the eye was carefully trimmed of extraneous tissue and the anterior segment and lens were removed. The retina was detached and removed from the optic nerve head with fine curved scissors. Eye cups were rinsed with cold ICC buffer. Eye cups were placed in cold ICC containing 4',6-diamidino-2-phenylindole (DAPI; nuclear

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stain) isolectin IB4 AlexaFluor 649 (blood vessels; Vector Labs cat# DL-1208-.5), and phalloidin AlexaFluor 555 (f-actin; ThermoFisher A34055). Eye cups were incubated at 4°C with gentle rotation for 4 hours and washed with cold ICC buffer. Radial cuts were then made toward the optic nerve head avoiding lesions, and the sclera-choroid/RPE complexes were, flat mounted, covered, and sealed. Two-dimensional (2D) fluorescent microscopy images were acquired, digitized, and analyzed using an Olympus Bx63 upright fluorescent microscope and CellSens (Olympus) software. Post-acquisition analysis was performed with CellSens software. To quantify neoformed vessels, the isolectin IB4 area was measured in μm2. Representative images from the isolectin lesion area measurements are located in **Fig. 45**.

[0265] When analyzing isolectin area size (**Fig. 46**), lesions where Bruch's membrane did not rupture, where vitreal hemorrhages were noted, or where data points more than 2.5 standard deviations from the mean were excluded. Group 1 animals had the largest lesion area at 60,900 ± 38,500 μm^2, while Group 5 had the smallest lesion area at 18,700 ± 7,200 μm^2, a 70% reduction in lesion area. The positive control performed similarly to other studies, with a lesion area 30% smaller than sham vector controls. Statistical analysis was done using one-way ANOVA followed by Dunnett multiple comparison (*** = 0.0001 and **** = <0.0001).

Tissue Collection for Immunohistochemistry (Groups 6-8 Only)

[0266] All eyes designated for immunohistochemistry (IHC) were enucleated, the approximate site of injection was marked, and the tissue was then placed into 1x PBS. Eyes were fixed in 4% paraformaldehyde in separately labeled vials overnight at 4°C. Eyes were then transferred into 0.1M phosphate buffer (PB), brought through a sequential sucrose gradient (10-30%, 1 hour each) followed by embedding in OCT medium and freezing on dry ice. The entire eye was cryosectioned (14 μm sections) and stained with the following antibodies: 1/250 chicken anti-GFP, 1/250 rabbit anti-RPE65, and followed by 1/200 anti-chicken Cy2, 1/200 anti-rabbit Cy3, 1/100 PNA Lectin Cy5, and 1/1,000 DAPI.

[0267] Groups 6 and 7 had the brightest and broadest GFP signal, but Group 8 had GFP signal as well. In all groups, GFP expression tended to be localized in the central part of the retina near the optic nerve, but there was some geographic variability. Expression was consistently observed from the ganglion cell layer through the inner segment and was less consistently seen in the outer segment and retinal pigment epithelium. The GFP signal seen in the stained cryosections matched the GFP signal seen in the fundus imaging. The RPE staining seen in 856 OD was due to transduction of the AAV into the RPE following a standard IVT injection while the GFP expression observed in the RPE of mouse 652 OS may have been enhanced due to the unintentional delivery of the AAV to the subretinal space, rather than the intravitreal space.

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Representative images of day 0 IHC are located in **Fig. 47**. **Fig. 48A** illustrates Flatmount analysis of the images obtained from the dose response study of Example 14. **Fig. 48B** illustrates fluorescence angiograph (FA) analysis of the images obtained from the dose response study of Example 14.

Example 15. AVMX formulation and efficacy study

[0268] AVMX-110 was formulated into a liquid compotation sterile vials. AVMX-110 drug product concentrate for solution for IVT injection was manufactured as a sterile frozen liquid formulation stored in a 1 mL of 13 mm cyclic olefin copolymer (COC) vials with a 13 mm gray bromobutyl stopper and a 13 mm flip-off aluminum seal. Each vial was filled to a target weight of 100 µL to allow for the withdrawal volume of at least 50 µL (density 1.01 g/mL). The product was supplied at a concentration of ≥4.0 x 1012 vg/mL. For clinical use, drug product was thawed and aseptically withdraw with provided filter needle and delivered to patient via IVT by retina specialist.

[0269] The efficacy studying with AVMX-110: AAV2•N54-VEGF-Trap was performed based on the protocol shown in **Table 37**. Efficacy of AVMX-110 in mice was evaluated by using a Laser-Induced Choroidal Neovascularization (LCNV) Model.

Table 37. Experimental design of mouse LCNV

Group	No. of Animals	OU Treatment/ Dose	Volume/ Route	Treatment Day	CNV Induction
	8	Vehicle Formulation buffer	1.0 µL IVT	Day -3	OU: CNV Laser Day 0
2	8	AAV2.N54-GFP 1.6e+10 vg/eye		Day -28	
3	8	Aflibercept (Eylea®) 40 µg		Day -3	
4	8	AVMX110 Low Dose 2e+7 vg/eye		Day -28	

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5	8	AVMX110 Medium Dose 4e+8 vg/eye			
6	8	AVMX110 High Dose 1.6e+10 vg/eye			
7	2	NA	NA	NA	NA

[0270] Animals were acclimated to the study environment for a minimum of 3 days. At the completion of the acclimation period, each animal was physically examined by a laboratory animal technician for determination of suitability for study participation. Examinations included, but not be limited to, the skin and external ears, eyes, abdomen, behavior, and general body condition. Animals determined to be in good health were released to the study. The animal randomization, intravitreal injection, laser-induced CNV procedure, animal examination, fluorescein angiography (FA), ocular tissue collection and flat mount (n=6 mice/group) were described in the protocol. In addition, all mice were terminally bled to collect serum and retina tissues after observation by fluorescent angiography (FA) and collection of flat mounts.

[0271] In FA analysis, animals received aflibercept at 40 µg/eye or AVMX-110 IVT-injection showed significant reduction of lesion area (**Fig. 49A**). For AVMX-110 groups, the lesion area reduction values were correlated inversely with dose of vector genome copies (vg) ($P<0.01$ for 4e+8 vg/eye and $P<0.001$ at 1.6e+10 vg/eye), the higher vector injected, the smaller damaged area observed 7 days post laser shinning (**Table 38**). In the case of 1.6e+10 vg/eye, complete cure of laser injury was achieved. **Fig. 49B** illustrates FA analysis of the images from the AVMX-110 dosing study.

Table 38. Statistical analysis of AVMX-110 efficacy in LCNM

Dunnett's multiple comparisons test	**Adjusted P Value**
Group 1: Vehicle vs. Group 2: Aflibercept	0.055
Group 1: Vehicle vs. Group 4: AVMX110 2e7 vg	0.999
Group 1: Vehicle vs. Group 5. AVMX110 4e8 vg	0.0113
Group 1: Vehicle vs. Group 6: AVMX110 1.6e10 vg	0.0002

[0272] Following euthanasia, animals in Groups 1-5 and Group 9 had blood collected for serum isolation and eyes were enucleated and snap frozen. The eyes were placed into appropriate pre-weighed labeled analytical vials, immediately reweighed to determine sample weight, and placed on dry ice until being transferred to a freezer. Samples were weighed on a balance capable of

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measuring out to 4 decimal places. Following snap freezing and weighing, samples were returned to the -80°C freezer. VEFG-Trap concentration in serum and retina tissues were tested by ELISA assay. The eyes were enucleated and the retina and RPE/choroid segments were dissected from fresh eyes and snap frozen. The tissues were placed into appropriate pre-weighed labeled analytical vials, immediately reweighed to determine sample weight, and placed on dry ice until being transferred to a freezer. Samples were weighed on a balance capable of measuring out to 4 decimal places.

[0273] The samples were assayed using ELISA. VEGF-Trap was ~50 ng/mL for the aflibercept control group, low level of VEGF-Trap at ~2 ng/mL was detected in sera of high dose group, 1.6e10 vg/eye and was not detectable in the low and medium dose groups (**Fig. 51A**). VEGF-Trap level in retina tissue was also measured in the retina homogenates using ELISA (**Fig. 51B**). In the retina tissue suspension, the expression of VEGF-Trap is in good dose dependent manner to the AVMX-110 injected intravitreally. On average of 13 ng/mL of VEGF-Trap was detected for the high dose group of 1.6e10 vg/eye. The highest expression level reached over 40 ng/mL.

[0274] While the foregoing disclosure has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the disclosure. For example, all the techniques and apparatus described above can be used in various combinations. All publications, patents, patent applications, and/or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, and/or other document were individually and separately indicated to be incorporated by reference for all purposes.

Sequence Table

SEQ ID NO:	Annotation	Sequence
12	VEGF-Trap	MVSYWDTGVLLCALLSCLLLTGSSSGSDTGRPFVEMYSEIPEIIH MTEGRELVIPCRVTSPNITVTLKKFPLDTLIPDGKRIIWDSRKGFII SNATYKEIGLLTCEATVNGHLYKTNYLTHRQTNTIIDVVLSPSH GIELSVGEKLVLNCTARTELNVGIDFNWEYPSSKHQHKKLVNR DLKTQSGSEMKKFLSTLTIDGVTRSDQGLYTCAASSGLMTKKN STFVRVHEKDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKG QPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNG QPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPGK

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SEQ ID NO:	Annotation	Sequence
13	AMI059 plasmid	CATTCGCCATTCAGGCTGCAAATAAGCGTTGATATTCAGTCA ATTACAAACATTAATAACGAAGAGATGACAGAAAAATTTTC ATTCTGTGACAGAGAAAAAGTAGCCGAAGATGACGGTTTGTC ACATGGAGTTGGCAGGATGTTTGATTAAAAACATAACAGGA AGAAAAATGCCCCGCTGTGGGCGGACAAAATAGTTGGGAAC TGGGAGGGGTGGAAATGGAGTTTTTAAGGATTATTTAGGGAA GAGTGACAAAATAGATGGGAACTGGGTGTAGCGTCGTAAGC TAATACGAAAATTAAAAATGACAAAATAGTTTGGAACTAGA TTTCACTTATCTGGTTCGGATCTCCTAGGCTCAAGCAGTGATC AGATCCAGACATGATAAGATACATTGATGAGTTTGGACAAAC CACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAAT TTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAAT AAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAG GTTCAGGGGGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAA CCTCTACAAATGTGGTATGGCTGATTATGATCCTCTAGTACTT CTCGACAAGCTCGGATCCTGGCGCGCTCGCTCGCTCACTGAG GCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGC CCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGC CAACTCCATCACTAGGGGTTCCTAGGAAGCTGATCTGAATTC GGTACCCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTG ACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTA TGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACA TCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAA TGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGAC CTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTC ATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAAT GGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTC CACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAAT CAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTG ACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATAT AAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACG CCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCG ATCCAGCCTCCGGACTCTAGAGTTAACTGGTAAGTTTAGTCT TTTTGTCTTTTATTTCAGGTCCCGGATCCGGTGGTGGTGCAAA TCAAAGAACTGCTCCTCAGTGGATGTTGCCTTTACTTCTAGGC CTGCCGCCACCATGGTATCCTATTGGGATACGGGTGTTCTCTT GTGTGCACTCCTTTCCTGTCTCCTGCTCACTGGATCTTCTTCT GGGTCTGATACTGGTAGACCTTTTGTAGAAATGTATTCAGAA ATTCCGGAAATAATTCATATGACAGAAGGACGAGAACTCGTT ATACCATGTCGCGTCACGTCCCCTAATATTACTGTTACGCTCA AGAAGTTTCCTCTCGATACACTTATTCCAGATGGGAAACGCA TAATTTGGGATTCACGCAAAGGGTTTATTATTAGTAACGCAA CGTATAAAGAAATTGGGCTGCTCACATGTGAAGCTACGGTAA ATGGGCATCTTTATAAAACAAATTATTTGACTCATCGGCAAA CTAATACTATTATCGATGTAGTACTCTCCCCATCCCATGGTAT TGAATTGTCAGTTGGGGAGAAGTTGGTATTGAATTGTACTGC ACGGACAGAACTCAACGTTGGTATTGATTTTAATTGGGAATA

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SEQ ID NO:	Annotation	Sequence
		GAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGA CCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCG CCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGT TCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGA TCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCA GCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACA GGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACAC GGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTG AAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATT TGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCAC ATTTCCCCGAAAAGTGCCACCTGAAATTGTAAACGTTAATAT TTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTT TTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCA AAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGG AACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAA AGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTG AACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTA AAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGA GCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGG GAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTG TAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTA ATGCGCCGCTACAGGGCGCGTC

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SEQ ID NO:	Annotation	Sequence
15	AMI067 plasmid	CATTCGCCATTCAGGCTGCAAATAAGCGTTGATATTCAGTCA ATTACAAACATTAATAACGAAGAGATGACAGAAAAATTTTC ATTCTGTGACAGAGAAAAAGTAGCCGAAGATGACGGTTTGTC ACATGGAGTTGGCAGGATGTTTGATTAAAAACATAACAGGA AGAAAAATGCCCCGCTGTGGGCGGACAAAATAGTTGGGAAC TGGGAGGGGTGGAAATGGAGTTTTTAAGGATTATTTAGGGAA GAGTGACAAAATAGATGGGAACTGGGTGTAGCGTCGTAAGC TAATACGAAAATTAAAAATGACAAAATAGTTTGGAACTAGA TTTCACTTATCTGGTTCGGATCTCCTAGGCTCAAGCAGTGATC AGATCCAGACATGATAAGATACATTGATGAGTTTGGACAAAC CACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAAT TTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAAT AAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAG GTTCAGGGGGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAA CCTCTACAAATGTGGTATGGCTGATTATGATCCTCTAGTACTT CTCGACAAGCTCGGATCCTGGCGCGCTCGCTCGCTCACTGAG GCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGC CCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGC CAACTCCATCACTAGGGGTTCCTAGGAAGCTGATCTGAATTC GGTACCCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTG ACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTA TGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACA TCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAA TGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGAC CTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTC ATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAAT GGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTC CACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAAT CAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTG ACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATAT AAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACG CCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCG ATCCAGCCTCCGGACTCTAGAGTTAACTGGTAAGTTTAGTCT TTTTGTCTTTTATTTCAGGTCCCGGATCCGGTGGTGGTGCAAA TCAAAGAACTGCTCCTCAGTGGATGTTGCCTTTACTTCTAGGC CTGCCGCCACCATGGTGAGCTACTGGGACACCGGCGTGCTGC TGTGCGCCCTGCTGAGCTGCCTGCTGCTGACCGGCAGCAGCA GCGGCAGCGACACCGGCAGACCCTTCGTGGAGATGTACAGC GAGATCCCCGAGATCATCCACATGACCGAGGGCAGAGAGCT GGTGATCCCCTGCAGAGTGACCAGCCCCAACATCACCGTGAC CCTGAAGAAGTTCCCCCTGGACACCCTGATCCCCGACGGCAA GAGAATCATCTGGGACAGCAGAAAGGGCTTCATCATCAGCA ACGCCACCTACAAGGAGATCGGCCTGCTGACCTGCGAGGCC ACCGTGAACGGCCACCTGTACAAGACCAACTACCTGACCCAC AGACAGACCAACACCATCATCGACGTGGTGCTGAGCCCCAG CCACGGCATCGAGCTGAGCGTGGGCGAGAAGCTGGTGCTGA ACTGCACCGCCAGAACCGAGCTGAACGTGGGCATCGACTTCA

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		ACTGGGAGTACCCCAGCAGCAAGCACCAGCACAAGAAGCTG GTGAACAGAGACCTGAAGACCCAGAGCGGCAGCGAGATGAA GAAGTTCCTGAGCACCCTGACCATCGACGGCGTGACCAGAA GCGACCAGGGCCTGTACACCTGCGCCGCCAGCAGCGGCCTG ATGACCAAGAAGAACAGCACCTTCGTGAGAGTGCACGAGAA GGACAAGACCCACACCTGCCCCCCCTGCCCCGCCCCCGAGCT GCTGGGCGGCCCCAGCGTGTTCCTGTTCCCCCCCAAGCCCAA GGACACCCTGATGATCAGCAGAACCCCCGAGGTGACCTGCGT GGTGGTGGACGTGAGCCACGAGGACCCCGAGGTGAAGTTCA ACTGGTACGTGGACGGCGTGGAGGTGCACAACGCCAAGACC AAGCCCAGAGAGGAGCAGTACAACAGCACCTACAGAGTGGT GAGCGTGCTGACCGTGCTGCACCAGGACTGGCTGAACGGCA AGGAGTACAAGTGCAAGGTGAGCAACAAGGCCCTGCCCGCC CCCATCGAGAAGACCATCAGCAAGGCCAAGGGCCAGCCCAG AGAGCCCCAGGTGTACACCCTGCCCCCCAGCAGAGACGAGC TGACCAAGAACCAGGTGAGCCTGACCTGCCTGGTGAAGGGC TTCTACCCCAGCGACATCGCCGTGGAGTGGGAGAGCAACGG CCAGCCCGAGAACAACTACAAGACCACCCCCCCCGTGCTGG ACAGCGACGGCAGCTTCTTCCTGTACAGCAAGCTGACCGTGG ACAAGAGCAGATGGCAGCAGGGCAACGTGTTCAGCTGCAGC GTGATGCACGAGGCCCTGCACAACCACTACACCCAGAAGAG CCTGAGCCTGAGCCCCGGCAAGTGATTCGAAAATAAAATATC TTTATTTTCATTACATCTGTGTGTTGGTTTTTTGTGTGGCATGC TGGGGAGAGATCAACCCCACTCCCTCTCTGCGCGCTCGCTCG CTCACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCGGG CTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGCA AGCTGTAGCCAACCACTAGAACTATAGCTAGAGTCCTGGGCG AACAAACGATGCTCGCCTTCCAGAAAACCGAGGATGCGAAC CACTTCATCCGGGGTCAGCACCACCGGCAAGCGCCGCGACG GCCGAGGTCTTCCGATCTCCTGAAGCCAGGGCAGATCCGTGC ACAGCACCTTGCCGTAGAAGAACAGCAAGGCCGCCAATGCC TGACGATGCGTGGAGACCGAAACCTTGCGCTCGTTCGCCAGC CAGGACAGAAATGCCTCGACTTCGCTGCTGCCCAAGGTTGCC GGGTGACGCACACCGTGGAAACGGATGAAGGCACGAACCCA GTTGACATAAGCCTGTTCGGTTCGTAAACTGTAATGCAAGTA GCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACG CAGCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTA TGACTGTTTTTTTGTACAGTCTATGCCTCGGGCATCCAAGCAG CAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC AGCAACGATGTTACGCAGCAGCAACGATGTTACGCAGCAGG GCAGTCGCCCTAAAACAAAGTTAGGTGGCTCAAGTATGGGC ATCATTCGCACATGTAGGCTCGGCCCTGACCAAGTCAAATCC ATGCGGGCTGCTCTTGATCTTTTCGGTCGTGAGTTCGGAGAC GTAGCCACCTACTCCCAACATCAGCCGGACTCCGATTACCTC GGGAACTTGCTCCGTAGTAAGACATTCATCGCGCTTGCTGCC TTCGACCAAGAAGCGGTTGTTGGCGCTCTCGCGGCTTACGTT CTGCCCAGGTTTGAGCAGCCGCGTAGTGAGATCTATATCTAT GATCTCGCAGTCTCCGGCGAGCACCGGAGGCAGGGCATTGCC

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		ACCGCGCTCATCAATCTCCTCAAGCATGAGGCCAACGCGCTT GGTGCTTATGTGATCTACGTGCAAGCAGATTACGGTGACGAT CCCGCAGTGGCTCTCTATACAAAGTTGGGCATACGGGAAGAA GTGATGCACTTTGATATCGACCCAAGTACCGCCACCTAACAA TTCGTTCAAGCCGAGATCGGCTTCCCGGCCGCGGAGTTGTTC GGTAAATTGTCACAACGCCGCGAATATAGTCTTTACCATGCC CTTGGCCACGCCCCTCTTTAATACGACGGGCAATTTGCACTTC AGAAAATGAAGAGTTTGCTTTAGCCATAACAAAAGTCCAGTA TGCTTTTTCACAGCATAACTGGACTGATTTCAGTTTACAACTA TTCTGTCTAGTTTAAGACTTTATTGTCATAGTTTAGATCTATT TTGTTCAGTTTAAGACTTTATTGTCCGCCCACACCCGCTTACG CAGGGCATCCATTTATTACTCAACCGTAACCGATTTTGCCAG GTTACGCGGCTGGTCTGCGGTGTGAAATACCGCACAGATGCG TAAGGAGAAAATACCGCATCAGGCGCTCTTCCGCTTCCTCGC TCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGG TATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAAT CAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAG CAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTT TTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCG ACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAA GATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTC CTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCT CCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAG GTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTG TGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATC CGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTT ATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAG CGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGC CTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCG CTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCT CTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTT TTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTC AAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGT GGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTAT CAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAA GTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTG ACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGA TCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGT GTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAG TGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGA TTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCA GAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAA TTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAG TTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTC ACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAA CGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAA GCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAG TTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCAT

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		AATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGA CTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGC GGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATA CCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAA AACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGT TGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGAT CTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAA AACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCG ACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATT ATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACA TATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGC GCACATTTCCCCGAAAAGTGCCACCTGAAATTGTAAACGTTA ATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTC ATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAA ATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGT TTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGT CAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTAC GTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCC GTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTT AGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGA AGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCA AGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCG CTTAATGCGCCGCTACAGGGCGCGTC

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SEQ ID NO:	Annotation	Sequence
16	AMI068 plasmid	CATTCGCCATTCAGGCTGCAAATAAGCGTTGATATTCAGTCA ATTACAAACATTAATAACGAAGAGATGACAGAAAAATTTTC ATTCTGTGACAGAGAAAAAGTAGCCGAAGATGACGGTTTGTC ACATGGAGTTGGCAGGATGTTTGATTAAAAACATAACAGGA AGAAAAATGCCCCGCTGTGGGCGGACAAAATAGTTGGGAAC TGGGAGGGGTGGAAATGGAGTTTTTAAGGATTATTTAGGGAA GAGTGACAAAATAGATGGGAACTGGGTGTAGCGTCGTAAGC TAATACGAAAATTAAAAATGACAAAATAGTTTGGAACTAGA TTTCACTTATCTGGTTCGGATCTCCTAGGCTCAAGCAGTGATC AGATCCAGACATGATAAGATACATTGATGAGTTTGGACAAAC CACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAAT TTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAAT AAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAG GTTCAGGGGGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAA CCTCTACAAATGTGGTATGGCTGATTATGATCCTCTAGTACTT CTCGACAAGCTCGGATCCTGGCGCGCTCGCTCGCTCACTGAG GCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGC CCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGC CAACTCCATCACTAGGGGTTCCTAGGAAGCTGATCTGAATTC GGTACCCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTG ACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTA TGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACA TCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAA TGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGAC CTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTC ATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAAT GGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTC CACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAAT CAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTG ACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATAT AAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACG CCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCG ATCCAGCCTCCGGACTCTAGAGTTAACTGGTAAGTTTAGTCT TTTTGTCTTTTATTTCAGGTCCCGGATCCGGTGGTGGTGCAAA TCAAAGAACTGCTCCTCAGTGGATGTTGCCTTTACTTCTAGGC CTGCCGCCACCATGGAGTTCGGCCTGAGCTGGCTGTTCCTGG TGGCCATCCTTAAGGGCGTGCAGTGCGACACCGGCAGACCCT TCGTGGAGATGTACAGCGAGATCCCCGAGATCATCCACATGA CCGAGGGCAGAGAGCTGGTGATCCCCTGCAGAGTGACCAGC CCCAACATCACCGTGACCCTGAAGAAGTTCCCCCTGGACACC CTGATCCCCGACGGCAAGAGAATCATCTGGGACAGCAGAAA GGGCTTCATCATCAGCAACGCCACCTACAAGGAGATCGGCCT GCTGACCTGCGAGGCCACCGTGAACGGCCACCTGTACAAGA CCAACTACCTGACCCACAGACAGACCAACACCATCATCGACG TGGTGCTGAGCCCCAGCCACGGCATCGAGCTGAGCGTGGGC GAGAAGCTGGTGCTGAACTGCACCGCCAGAACCGAGCTGAA CGTGGGCATCGACTTCAACTGGGAGTACCCCAGCAGCAAGC

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		ACCAGCACAAGAAGCTGGTGAACAGAGACCTGAAGACCCAG AGCGGCAGCGAGATGAAGAAGTTCCTGAGCACCCTGACCAT CGACGGCGTGACCAGAAGCGACCAGGGCCTGTACACCTGCG CCGCCAGCAGCGGCCTGATGACCAAGAAGAACAGCACCTTC GTGAGAGTGCACGAGAAGGACAAGACCCACACCTGCCCCCC CTGCCCCGCCCCCGAGCTGCTGGGCGGCCCCAGCGTGTTCCT GTTCCCCCCCAAGCCCAAGGACACCCTGATGATCAGCAGAAC CCCCGAGGTGACCTGCGTGGTGGTGGACGTGAGCCACGAGG ACCCCGAGGTGAAGTTCAACTGGTACGTGGACGGCGTGGAG GTGCACAACGCCAAGACCAAGCCCAGAGAGGAGCAGTACAA CAGCACCTACAGAGTGGTGAGCGTGCTGACCGTGCTGCACCA GGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTGAGCA ACAAGGCCCTGCCCGCCCCCATCGAGAAGACCATCAGCAAG GCCAAGGGCCAGCCCAGAGAGCCCCAGGTGTACACCCTGCC CCCCAGCAGAGACGAGCTGACCAAGAACCAGGTGAGCCTGA CCTGCCTGGTGAAGGGCTTCTACCCCAGCGACATCGCCGTGG AGTGGGAGAGCAACGGCCAGCCCGAGAACAACTACAAGACC ACCCCCCCCGTGCTGGACAGCGACGGCAGCTTCTTCCTGTAC AGCAAGCTGACCGTGGACAAGAGCAGATGGCAGCAGGGCAA CGTGTTCAGCTGCAGCGTGATGCACGAGGCCCTGCACAACCA CTACACCCAGAAGAGCCTGAGCCTGAGCCCCGGCAAGTGATT CGAAAATAAAATATCTTTATTTTCATTACATCTGTGTGTTGGT TTTTTGTGTGGCATGCTGGGGAGAGATCAACCCCACTCCCTC TCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGT CGCCCGACGCCCGGGCTTTGCCCGGGCGGCCTCAGTGAGCGA GCGAGCGCGCAGCAAGCTGTAGCCAACCACTAGAACTATAG CTAGAGTCCTGGGCGAACAAACGATGCTCGCCTTCCAGAAAA CCGAGGATGCGAACCACTTCATCCGGGGTCAGCACCACCGGC AAGCGCCGCGACGGCCGAGGTCTTCCGATCTCCTGAAGCCAG GGCAGATCCGTGCACAGCACCTTGCCGTAGAAGAACAGCAA GGCCGCCAATGCCTGACGATGCGTGGAGACCGAAACCTTGC GCTCGTTCGCCAGCCAGGACAGAAATGCCTCGACTTCGCTGC TGCCCAAGGTTGCCGGGTGACGCACACCGTGGAAACGGATG AAGGCACGAACCCAGTTGACATAAGCCTGTTCGGTTCGTAAA CTGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCCAGA ACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGT TTTCATGGCTTGTTATGACTGTTTTTTTGTACAGTCTATGCCTC GGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTT TGATGTTATGGAGCAGCAACGATGTTACGCAGCAGCAACGAT GTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGTGG CTCAAGTATGGGCATCATTCGCACATGTAGGCTCGGCCCTGA CCAAGTCAAATCCATGCGGGCTGCTCTTGATCTTTTCGGTCGT GAGTTCGGAGACGTAGCCACCTACTCCCAACATCAGCCGGAC TCCGATTACCTCGGGAACTTGCTCCGTAGTAAGACATTCATC GCGCTTGCTGCCTTCGACCAAGAAGCGGTTGTTGGCGCTCTC GCGGCTTACGTTCTGCCCAGGTTTGAGCAGCCGCGTAGTGAG ATCTATATCTATGATCTCGCAGTCTCCGGCGAGCACCGGAGG CAGGGCATTGCCACCGCGCTCATCAATCTCCTCAAGCATGAG

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		GCCAACGCGCTTGGTGCTTATGTGATCTACGTGCAAGCAGAT TACGGTGACGATCCCGCAGTGGCTCTCTATACAAAGTTGGGC ATACGGGAAGAAGTGATGCACTTTGATATCGACCCAAGTACC GCCACCTAACAATTCGTTCAAGCCGAGATCGGCTTCCCGGCC GCGGAGTTGTTCGGTAAATTGTCACAACGCCGCGAATATAGT CTTTACCATGCCCTTGGCCACGCCCCTCTTTAATACGACGGGC AATTTGCACTTCAGAAAATGAAGAGTTTGCTTTAGCCATAAC AAAAGTCCAGTATGCTTTTTCACAGCATAACTGGACTGATTT CAGTTTACAACTATTCTGTCTAGTTTAAGACTTTATTGTCATA GTTTAGATCTATTTTGTTCAGTTTAAGACTTTATTGTCCGCCC ACACCCGCTTACGCAGGGCATCCATTTATTACTCAACCGTAA CCGATTTTGCCAGGTTACGCGGCTGGTCTGCGGTGTGAAATA CCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCTC TTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGG CTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACG GTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGT GAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGC CGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGA GCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACC CGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCT CCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATA CCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAA TGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCT CCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACC GCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGG TAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACA GGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCT TGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTAT TTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAA GAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTA GCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAA AAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGT CTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGG TCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAA ATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGT AAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCAC CTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTG ACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACC ATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTC ACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAA GGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCA TCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTT CGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAG GCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAG CTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCAT GTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGT TGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTAT GGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAG

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		ATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGA GAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCA ATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGT GCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAG GATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCG TGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTT TCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAA GGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTT CCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTC ATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAA ATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGAA ATTGTAAACGTTAATATTTTGTTAAAATTCGCGTTAAATTTTT GTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCA AAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTG AGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAAC GTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGG CGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTT GGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAG GGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAAC GTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCG CTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCA CCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTC

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17	AMI119 plasmid	CATTCGCCATTCAGGCTGCAAATAAGCGTTGATATTCAGTCA ATTACAAACATTAATAACGAAGAGATGACAGAAAAATTTTC ATTCTGTGACAGAGAAAAAGTAGCCGAAGATGACGGTTTGTC ACATGGAGTTGGCAGGATGTTTGATTAAAAACATAACAGGA AGAAAAATGCCCCGCTGTGGGCGGACAAAATAGTTGGGAAC TGGGAGGGGTGGAAATGGAGTTTTTAAGGATTATTTAGGGAA GAGTGACAAAATAGATGGGAACTGGGTGTAGCGTCGTAAGC TAATACGAAAATTAAAAATGACAAAATAGTTTGGAACTAGA TTTCACTTATCTGGTTCGGATCTCCTAGGCTCAAGCAGTGATC AGATCCAGACATGATAAGATACATTGATGAGTTTGGACAAAC CACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAAT TTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAAT AAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAG GTTCAGGGGGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAA CCTCTACAAATGTGGTATGGCTGATTATGATCCTCTAGTACTT CTCGACAAGCTCGGATCCTGGCGCGCTCGCTCGCTCACTGAG GCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGC CCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGC CAACTCCATCACTAGGGGTTCCTAGGAAGCTGATCTGAATTC GGTACCCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTG ACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTA TGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACA TCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAA TGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGAC CTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTC ATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAAT GGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTC CACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAAT CAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTG ACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATAT AAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACG CCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCG ATCCAGCCTCCGGACTCTAGAGTTAACTGGTAAGTTTAGTCT TTTTGTCTTTTATTTCAGGTCCCGGATCCGGTGGTGGTGCAAA TCAAAGAACTGCTCCTCAGTGGATGTTGCCTTTACTTCTAGGC CTGCCGCCACCATGGTGAGCTACTGGGACACCGGCGTGCTGC TGTGCGCCCTGCTGAGCTGCCTGCTGCTGACCGGCAGCAGCA GCGGCAGCGACACCGGCAGGCCTTTCGTGGAGATGTACAGC GAGATCCCTGAGATCATCCACATGACCGAGGGCAGGGAGCT GGTGATCCCTTGCAGGGTGACCAGCCCTAACATCACCGTGAC CCTGAAGAAGTTCCCTCTGGACACCCTGATCCCTGACGGCAA GAGGATCATCTGGGACAGCAGGAAGGGCTTCATCATCAGCA ACGCCACCTACAAGGAGATCGGCCTGCTGACCTGCGAGGCC ACCGTGAACGGCCACCTGTACAAGACCAACTACCTGACCCAC AGGCAGACCAACACCATCATCGACGTGGTGCTGAGCCCTAGC CACGGCATCGAGCTGAGCGTGGGCGAGAAGCTGGTGCTGAA CTGCACCGCCAGGACCGAGCTGAACGTGGGCATCGACTTCAA

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		CTGGGAGTACCCTAGCAGCAAGCACCAGCACAAGAAGCTGG TGAACAGGGACCTGAAGACCCAGAGCGGCAGCGAGATGAAG AAGTTCCTGAGCACCCTGACCATCGACGGCGTGACCAGGAGC GACCAGGGCCTGTACACCTGCGCCGCCAGCAGCGGCCTGATG ACCAAGAAGAACAGCACCTTCGTGAGGGTGCACGAGAAGGA CAAGACCCACACCTGCCCTCCTTGCCCTGCCCCTGAGCTGCT GGGCGGCCCTAGCGTGTTCCTGTTCCCTCCTAAGCCTAAGGA CACCCTGATGATCAGCAGGACCCCTGAGGTGACCTGCGTGGT GGTGGACGTGAGCCACGAGGACCCTGAGGTGAAGTTCAACT GGTACGTGGACGGCGTGGAGGTGCACAACGCCAAGACCAAG CCTAGGGAGGAGCAGTACAACAGCACCTACAGGGTGGTGAG CGTGCTGACCGTGCTGCACCAGGACTGGCTGAACGGCAAGG AGTACAAGTGCAAGGTGAGCAACAAGGCCCTGCCTGCCCCT ATCGAGAAGACCATCAGCAAGGCCAAGGGCCAGCCTAGGGA GCCTCAGGTGTACACCCTGCCTCCTAGCAGGGACGAGCTGAC CAAGAACCAGGTGAGCCTGACCTGCCTGGTGAAGGGCTTCTA CCCTAGCGACATCGCCGTGGAGTGGGAGAGCAACGGCCAGC CTGAGAACAACTACAAGACCACCCCTCCTGTGCTGGACAGCG ACGGCAGCTTCTTCCTGTACAGCAAGCTGACCGTGGACAAGA GCAGGTGGCAGCAGGGCAACGTGTTCAGCTGCAGCGTGATG CACGAGGCCCTGCACAACCACTACACCCAGAAGAGCCTGAG CCTGAGCCCTGGCAAGTGATTCGAAAATAAAATATCTTTATT TTCATTACATCTGTGTGTTGGTTTTTTGTGTGGCATGCTGGGG AGAGATCAACCCCACTCCCTCTCTGCGCGCTCGCTCGCTCAC TGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCGGGCTTTG CCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGCAAGCTG TAGCCAACCACTAGAACTATAGCTAGAGTCCTGGGCGAACA AACGATGCTCGCCTTCCAGAAAACCGAGGATGCGAACCACTT CATCCGGGGTCAGCACCACCGGCAAGCGCCGCGACGGCCGA GGTCTTCCGATCTCCTGAAGCCAGGGCAGATCCGTGCACAGC ACCTTGCCGTAGAAGAACAGCAAGGCCGCCAATGCCTGACG ATGCGTGGAGACCGAAACCTTGCGCTCGTTCGCCAGCCAGGA CAGAAATGCCTCGACTTCGCTGCTGCCCAAGGTTGCCGGGTG ACGCACACCGTGGAAACGGATGAAGGCACGAACCCAGTTGA CATAAGCCTGTTCGGTTCGTAAACTGTAATGCAAGTAGCGTA TGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCG GTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACT GTTTTTTTGTACAGTCTATGCCTCGGGCATCCAAGCAGCAAG CGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCA ACGATGTTACGCAGCAGCAACGATGTTACGCAGCAGGGCAG TCGCCCTAAAACAAAGTTAGGTGGCTCAAGTATGGGCATCAT TCGCACATGTAGGCTCGGCCCTGACCAAGTCAAATCCATGCG GGCTGCTCTTGATCTTTTCGGTCGTGAGTTCGGAGACGTAGC CACCTACTCCCAACATCAGCCGGACTCCGATTACCTCGGGAA CTTGCTCCGTAGTAAGACATTCATCGCGCTTGCTGCCTTCGAC CAAGAAGCGGTTGTTGGCGCTCTCGCGGCTTACGTTCTGCCC AGGTTTGAGCAGCCGCGTAGTGAGATCTATATCTATGATCTC GCAGTCTCCGGCGAGCACCGGAGGCAGGGCATTGCCACCGC

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SEQ ID NO:	Annotation	Sequence
		GCTCATCAATCTCCTCAAGCATGAGGCCAACGCGCTTGGTGC TTATGTGATCTACGTGCAAGCAGATTACGGTGACGATCCCGC AGTGGCTCTCTATACAAAGTTGGGCATACGGGAAGAAGTGAT GCACTTTGATATCGACCCAAGTACCGCCACCTAACAATTCGT TCAAGCCGAGATCGGCTTCCCGGCCGCGGAGTTGTTCGGTAA ATTGTCACAACGCCGCGAATATAGTCTTTACCATGCCCTTGG CCACGCCCCTCTTTAATACGACGGGCAATTTGCACTTCAGAA AATGAAGAGTTTGCTTTAGCCATAACAAAAGTCCAGTATGCT TTTTCACAGCATAACTGGACTGATTTCAGTTTACAACTATTCT GTCTAGTTTAAGACTTTATTGTCATAGTTTAGATCTATTTTGT TCAGTTTAAGACTTTATTGTCCGCCCACACCCGCTTACGCAG GGCATCCATTTATTACTCAACCGTAACCGATTTTGCCAGGTTA CGCGGCTGGTCTGCGGTGTGAAATACCGCACAGATGCGTAAG GAGAAAATACCGCATCAGGCGCTCTTCCGCTTCCTCGCTCAC TGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATC AGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGG GGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAA AGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCC ATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCT CAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATAC CAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTC CGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTC GGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCT CAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCA CGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAA CTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCC ACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGT ATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACT ACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGC TGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGAT CCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTT GCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAA GATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAAC GAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAA AGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTT AAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGT TACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGT CTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGA TAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTG CAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTAT CAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGT GGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTT GCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGC GCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCT CGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATC AAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGT TAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGC CGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTC

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SEQ ID NO:	Annotation	Sequence
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18	AMI120 plasmid	CATTCGCCATTCAGGCTGCAAATAAGCGTTGATATTCAGTCA ATTACAAACATTAATAACGAAGAGATGACAGAAAAATTTTC ATTCTGTGACAGAGAAAAAGTAGCCGAAGATGACGGTTTGTC ACATGGAGTTGGCAGGATGTTTGATTAAAAACATAACAGGA AGAAAAATGCCCCGCTGTGGGCGGACAAAATAGTTGGGAAC TGGGAGGGGTGGAAATGGAGTTTTTAAGGATTATTTAGGGAA GAGTGACAAAATAGATGGGAACTGGGTGTAGCGTCGTAAGC TAATACGAAAATTAAAAATGACAAAATAGTTTGGAACTAGA TTTCACTTATCTGGTTCGGATCTCCTAGGCTCAAGCAGTGATC AGATCCAGACATGATAAGATACATTGATGAGTTTGGACAAAC CACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAAT TTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAAT AAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAG GTTCAGGGGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAA CCTCTACAAATGTGGTATGGCTGATTATGATCCTCTAGTACTT CTCGACAAGCTCGGATCCTGGCGCGCTCGCTCGCTCACTGAG GCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGC CCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGC CAACTCCATCACTAGGGGTTCCTAGGAAGCTGATCTGAATTC

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		GGTACCCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTG ACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTA TGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACA TCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAA TGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGAC CTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTC ATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAAT GGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTC CACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAAT CAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTG ACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATAT AAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACG CCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCG ATCCAGCCTCCGGACTCTAGAGTTAACTGGTAAGTTTAGTCT TTTTGTCTTTTATTTCAGGTCCCGGATCCGGTGGTGGTGCAAA TCAAAGAACTGCTCCTCAGTGGATGTTGCCTTTACTTCTAGGC CTGCCGCCACCATGGTGAGCTACTGGGACACCGGCGTGCTGC TGTGCGCCCTGCTGAGCTGCCTGCTGCTGACCGGCAGCAGCA GCGGCAGCGACACCGGCAGGCCCTTCGTGGAGATGTACTCCG AGATCCCCGAGATCATCCACATGACCGAGGGCAGGGAGCTG GTGATCCCCTGCAGGGTGACCTCCCCCAACATCACCGTGACC CTGAAGAAGTTCCCCCTGGACACCCTGATCCCCGACGGCAAG AGGATCATCTGGGACTCCAGGAAGGGCTTCATCATCTCCAAC GCCACCTACAAGGAGATCGGCCTGCTGACCTGCGAGGCCACC GTGAACGGCCACCTGTACAAGACCAACTACCTGACCCACAG GCAGACCAACACCATCATCGACGTGGTGCTGTCCCCCTCCCA CGGCATCGAGCTGTCCGTGGGCGAGAAGCTGGTGCTGAACTG CACCGCCAGGACCGAGCTGAACGTGGGCATCGACTTCAACTG GGAGTACCCCTCCTCCAAGCACCAGCACAAGAAGCTGGTGA ACAGGGACCTGAAGACCCAGTCCGGCTCCGAGATGAAGAAG TTCCTGTCCACCCTGACCATCGACGGCGTGACCAGGTCCGAC CAGGGCCTGTACACCTGCGCCGCCTCCTCCGGCCTGATGACC AAGAAGAACTCCACCTTCGTGAGGGTGCACGAGAAGGACAA GACCCACACCTGCCCCCCCTGCCCCGCCCCCGAGCTGCTGGG CGGCCCCTCCGTGTTCCTGTTCCCCCCCAAGCCCAAGGACAC CCTGATGATCTCCAGGACCCCCGAGGTGACCTGCGTGGTGGT GGACGTGTCCCACGAGGACCCCGAGGTGAAGTTCAACTGGT ACGTGGACGGCGTGGAGGTGCACAACGCCAAGACCAAGCCC AGGGAGGAGCAGTACAACTCCACCTACAGGGTGGTGTCCGT GCTGACCGTGCTGCACCAGGACTGGCTGAACGGCAAGGAGT ACAAGTGCAAGGTGTCCAACAAGGCCCTGCCCGCCCCCATCG AGAAGACCATCTCCAAGGCCAAGGGCCAGCCCAGGGAGCCC CAGGTGTACACCCTGCCCCCCTCCAGGGACGAGCTGACCAAG AACCAGGTGTCCCTGACCTGCCTGGTGAAGGGCTTCTACCCC TCCGACATCGCCGTGGAGTGGGAGTCCAACGGCCAGCCCGA GAACAACTACAAGACCACCCCCCCCGTGCTGGACTCCGACGG CTCCTTCTTCCTGTACTCCAAGCTGACCGTGGACAAGTCCAG

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		GTGGCAGCAGGGCAACGTGTTCTCCTGCTCCGTGATGCACGA GGCCCTGCACAACCACTACACCCAGAAGTCCCTGTCCCTGTC CCCCGGCAAGTGATTCGAAAATAAAATATCTTTATTTTCATT ACATCTGTGTGTTGGTTTTTTGTGTGGCATGCTGGGGAGAGA TCAACCCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGC CGGGCGACCAAAGGTCGCCCGACGCCCGGGCTTTGCCCGGG CGGCCTCAGTGAGCGAGCGAGCGCGCAGCAAGCTGTAGCCA ACCACTAGAACTATAGCTAGAGTCCTGGGCGAACAAACGAT GCTCGCCTTCCAGAAAACCGAGGATGCGAACCACTTCATCCG GGGTCAGCACCACCGGCAAGCGCCGCGACGGCCGAGGTCTT CCGATCTCCTGAAGCCAGGGCAGATCCGTGCACAGCACCTTG CCGTAGAAGAACAGCAAGGCCGCCAATGCCTGACGATGCGT GGAGACCGAAACCTTGCGCTCGTTCGCCAGCCAGGACAGAA ATGCCTCGACTTCGCTGCTGCCCAAGGTTGCCGGGTGACGCA CACCGTGGAAACGGATGAAGGCACGAACCCAGTTGACATAA GCCTGTTCGGTTCGTAAACTGTAATGCAAGTAGCGTATGCGC TCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGTGGT AACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTT TTGTACAGTCTATGCCTCGGGCATCCAAGCAGCAAGCGCGTT ACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATG TTACGCAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCT AAAACAAAGTTAGGTGGCTCAAGTATGGGCATCATTCGCACA TGTAGGCTCGGCCCTGACCAAGTCAAATCCATGCGGGCTGCT CTTGATCTTTTCGGTCGTGAGTTCGGAGACGTAGCCACCTACT CCCAACATCAGCCGGACTCCGATTACCTCGGGAACTTGCTCC GTAGTAAGACATTCATCGCGCTTGCTGCCTTCGACCAAGAAG CGGTTGTTGGCGCTCTCGCGGCTTACGTTCTGCCCAGGTTTGA GCAGCCGCGTAGTGAGATCTATATCTATGATCTCGCAGTCTC CGGCGAGCACCGGAGGCAGGGCATTGCCACCGCGCTCATCA ATCTCCTCAAGCATGAGGCCAACGCGCTTGGTGCTTATGTGA TCTACGTGCAAGCAGATTACGGTGACGATCCCGCAGTGGCTC TCTATACAAAGTTGGGCATACGGGAAGAAGTGATGCACTTTG ATATCGACCCAAGTACCGCCACCTAACAATTCGTTCAAGCCG AGATCGGCTTCCCGGCCGCGGAGTTGTTCGGTAAATTGTCAC AACGCCGCGAATATAGTCTTTACCATGCCCTTGGCCACGCCC CTCTTTAATACGACGGGCAATTTGCACTTCAGAAAATGAAGA GTTTGCTTTAGCCATAACAAAAGTCCAGTATGCTTTTTCACAG CATAACTGGACTGATTTCAGTTTACAACTATTCTGTCTAGTTT AAGACTTTATTGTCATAGTTTAGATCTATTTTGTTCAGTTTAA GACTTTATTGTCCGCCCACACCCGCTTACGCAGGGCATCCAT TTATTACTCAACCGTAACCGATTTTGCCAGGTTACGCGGCTG GTCTGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAA TACCGCATCAGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGC TGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACT CAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAAC GCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCA GGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGC TCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTC

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SEQ ID NO:	Annotation	Sequence
		GAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGC GAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCA CGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGC TACAGGGCGCGTC
19	AMI130 plasmid	CATTCGCCATTCAGGCTGCAAATAAGCGTTGATATTCAGTCA ATTACAAACATTAATAACGAAGAGATGACAGAAAAATTTTC ATTCTGTGACAGAGAAAAAGTAGCCGAAGATGACGGTTTGTC ACATGGAGTTGGCAGGATGTTTGATTAAAAACATAACAGGA AGAAAAATGCCCCGCTGTGGGCGGACAAAATAGTTGGGAAC TGGGAGGGGTGGAAATGGAGTTTTTAAGGATTATTTAGGGAA GAGTGACAAAATAGATGGGAACTGGGTGTAGCGTCGTAAGC TAATACGAAAATTAAAAATGACAAAATAGTTTGGAACTAGA TTTCACTTATCTGGTTCGGATCTCCTAGGCTCAAGCAGTGATC AGATCCAGACATGATAAGATACATTGATGAGTTTGGACAAAC CACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAAT TTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAAT AAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAG GTTCAGGGGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAA CCTCTACAAATGTGGTATGGCTGATTATGATCCTCTAGTACTT CTCGACAAGCTCGGATCCTGGCGCGCTCGCTCGCTCACTGAG GCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGC CCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGC CAACTCCATCACTAGGGGTTCCTAGGAAGCTGATCTGAATTC GGTACCCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTG ACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTA TGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACA TCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAA TGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGAC CTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTC ATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAAT GGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTC CACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAAT CAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTG ACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATAT AAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACG CCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCG ATCCAGCCTCCGGACTCTAGAGTTAACTGGTAAGTTTAGTCT TTTTGTCTTTTATTTCAGGTCCCGGATCCGGTGGTGGTGCAAA TCAAAGAACTGCTCCTCAGTGGATGTTGCCTTTACTTCTAGGC CTGCCGCCACCATGGTGAGCTACTGGGACACCGGCGTGCTGC TGTGCGCCCTGCTGAGCTGCCTGCTGCTGACCGGCAGCAGCA GCGGCAGCGACACCGGCAGGCCGTTCGTGGAGATGTACAGC GAGATCCCGGAGATCATCCACATGACCGAGGGCAGGGAGCT GGTGATCCCGTGCAGGGTGACCAGCCCGAACATCACCGTGAC CCTGAAGAAGTTCCCGCTGGACACCCTGATCCCGGACGGCAA GAGGATCATCTGGGACAGCAGGAAGGGCTTCATCATCAGCA ACGCCACCTACAAGGAGATCGGCCTGCTGACCTGCGAGGCC

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		ACCGTGAACGGCCACCTGTACAAGACCAACTACCTGACCCAC AGGCAGACCAACACCATCATCGACGTGGTGCTGAGCCCGAG CCACGGCATCGAGCTGAGCGTGGGCGAGAAGCTGGTGCTGA ACTGCACCGCCAGGACCGAGCTGAACGTGGGCATCGACTTCA ACTGGGAGTACCCGAGCAGCAAGCACCAGCACAAGAAGCTG GTGAACAGGGACCTGAAGACCCAGAGCGGCAGCGAGATGAA GAAGTTCCTGAGCACCCTGACCATCGACGGCGTGACCAGGA GCGACCAGGGCCTGTACACCTGCGCCGCCAGCAGCGGCCTG ATGACCAAGAAGAACAGCACCTTCGTGAGGGTGCACGAGAA GGACAAGACCCACACCTGCCCGCCGTGCCCGGCCCCGGAGCT GCTGGGCGGCCCGAGCGTGTTCCTGTTCCCGCCGAAGCCGAA GGACACCCTGATGATCAGCAGGACCCCGGAGGTGACCTGCG TGGTGGTGGACGTGAGCCACGAGGACCCGGAGGTGAAGTTC AACTGGTACGTGGACGGCGTGGAGGTGCACAACGCCAAGAC CAAGCCGAGGGAGGAGCAGTACAACAGCACCTACAGGGTGG TGAGCGTGCTGACCGTGCTGCACCAGGACTGGCTGAACGGCA AGGAGTACAAGTGCAAGGTGAGCAACAAGGCCCTGCCGGCC CCGATCGAGAAGACCATCAGCAAGGCCAAGGGCCAGCCGAG GGAGCCGCAGGTGTACACCCTGCCGCCGAGCAGGGACGAGC TGACCAAGAACCAGGTGAGCCTGACCTGCCTGGTGAAGGGC TTCTACCCGAGCGACATCGCCGTGGAGTGGGAGAGCAACGG CCAGCCGGAGAACAACTACAAGACCACCCCGCCGGTGCTGG ACAGCGACGGCAGCTTCTTCCTGTACAGCAAGCTGACCGTGG ACAAGAGCAGGTGGCAGCAGGGCAACGTGTTCAGCTGCAGC GTGATGCACGAGGCCCTGCACAACCACTACACCCAGAAGAG CCTGAGCCTGAGCCCCGGCAAGTGATTCGAAAATAAAATATC TTTATTTTCATTACATCTGTGTGTTGGTTTTTTGTGTGGCATGC TGGGGAGAGATCAACCCCACTCCCTCTCTGCGCGCTCGCTCG CTCACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCGGG CTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGCA AGCTGTAGCCAACCACTAGAACTATAGCTAGAGTCCTGGGCG AACAAACGATGCTCGCCTTCCAGAAAACCGAGGATGCGAAC CACTTCATCCGGGGTCAGCACCACCGGCAAGCGCCGCGACG GCCGAGGTCTTCCGATCTCCTGAAGCCAGGGCAGATCCGTGC ACAGCACCTTGCCGTAGAAGAACAGCAAGGCCGCCAATGCC TGACGATGCGTGGAGACCGAAACCTTGCGCTCGTTCGCCAGC CAGGACAGAAATGCCTCGACTTCGCTGCTGCCCAAGGTTGCC GGGTGACGCACACCGTGGAAACGGATGAAGGCACGAACCCA GTTGACATAAGCCTGTTCGGTTCGTAAACTGTAATGCAAGTA GCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACG CAGCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTA TGACTGTTTTTTTGTACAGTCTATGCCTCGGGCATCCAAGCAG CAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC AGCAACGATGTTACGCAGCAGCAACGATGTTACGCAGCAGG GCAGTCGCCCTAAAACAAAGTTAGGTGGCTCAAGTATGGGC ATCATTCGCACATGTAGGCTCGGCCCTGACCAAGTCAAATCC ATGCGGGCTGCTCTTGATCTTTTCGGTCGTGAGTTCGGAGAC GTAGCCACCTACTCCCAACATCAGCCGGACTCCGATTACCTC

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SEQ ID NO:	Annotation	Sequence
		GGGAACTTGCTCCGTAGTAAGACATTCATCGCGCTTGCTGCC TTCGACCAAGAAGCGGTTGTTGGCGCTCTCGCGGCTTACGTT CTGCCCAGGTTTGAGCAGCCGCGTAGTGAGATCTATATCTAT GATCTCGCAGTCTCCGGCGAGCACCGGAGGCAGGGCATTGCC ACCGCGCTCATCAATCTCCTCAAGCATGAGGCCAACGCGCTT GGTGCTTATGTGATCTACGTGCAAGCAGATTACGGTGACGAT CCCGCAGTGGCTCTCTATACAAAGTTGGGCATACGGGAAGAA GTGATGCACTTTGATATCGACCCAAGTACCGCCACCTAACAA TTCGTTCAAGCCGAGATCGGCTTCCCGGCCGCGGAGTTGTTC GGTAAATTGTCACAACGCCGCGAATATAGTCTTTACCATGCC CTTGGCCACGCCCCTCTTTAATACGACGGGCAATTTGCACTTC AGAAAATGAAGAGTTTGCTTTAGCCATAACAAAAGTCCAGTA TGCTTTTTCACAGCATAACTGGACTGATTTCAGTTTACAACTA TTCTGTCTAGTTTAAGACTTTATTGTCATAGTTTAGATCTATT TTGTTCAGTTTAAGACTTTATTGTCCGCCCACACCCGCTTACG CAGGGCATCCATTTATTACTCAACCGTAACCGATTTTGCCAG GTTACGCGGCTGGTCTGCGGTGTGAAATACCGCACAGATGCG TAAGGAGAAAATACCGCATCAGGCGCTCTTCCGCTTCCTCGC TCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGG TATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAAT CAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAG CAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTT TTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCG ACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAA GATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTC CTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCT CCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAG GTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTG TGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATC CGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTT ATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAG CGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGC CTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCG CTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCT CTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTT TTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTC AAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGT GGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTAT CAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAA GTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTG ACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGA TCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGT GTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAG TGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGA TTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCA GAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAA TTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAG TTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTC

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SEQ ID NO:	Annotation	Sequence
		ACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAA CGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAA GCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAG TTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCAT AATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGA CTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGC GGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATA CCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAA AACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGT TGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGAT CTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAA AACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCG ACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATT ATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACA TATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGC GCACATTTCCCCGAAAAGTGCCACCTGAAATTGTAAACGTTA ATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTC ATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAA ATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGT TTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGT CAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTAC GTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCC GTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTT AGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGA AGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCA AGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCG CTTAATGCGCCGCTACAGGGCGCGTC

SEQ ID NO:	Annotation	Sequence
20	Control Plasmid	CATTCGCCATTCAGGCTGCAAATAAGCGTTGATATTCAGTCA ATTACAAACATTAATAACGAAGAGATGACAGAAAAATTTTC ATTCTGTGACAGAGAAAAAGTAGCCGAAGATGACGGTTTGTC ACATGGAGTTGGCAGGATGTTTGATTAAAAACATAACAGGA AGAAAAATGCCCCGCTGTGGGCGGACAAAATAGTTGGGAAC TGGGAGGGGTGGAAATGGAGTTTTTAAGGATTATTTAGGGAA GAGTGACAAAATAGATGGGAACTGGGTGTAGCGTCGTAAGC TAATACGAAAATTAAAAATGACAAAATAGTTTGGAACTAGA TTTCACTTATCTGGTTCGGATCTCCTAGGCTCAAGCAGTGATC AGATCCAGACATGATAAGATACATTGATGAGTTTGGACAAAC CACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAAT TTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAAT AAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAG GTTCAGGGGGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAA CCTCTACAAATGTGGTATGGCTGATTATGATCCTCTAGTACTT CTCGACAAGCTCGGATCCTGGCGCGCTCGCTCGCTCACTGAG GCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGC CCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGC CAACTCCATCACTAGGGGTTCCTAGGAAGCTGATCTGAATTC GGTACCCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTG ACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTA TGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACA TCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAA TGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGAC CTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTC ATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAAT GGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTC CACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAAT CAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTG ACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATAT AAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACG CCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCG ATCCAGCCTCCGGACTCTAGAGTTAACTGGTAAGTTTAGTCT TTTTGTCTTTTATTTCAGGTCCCGGATCCGGTGGTGGTGCAAA TCAAAGAACTGCTCCTCAGTGGATGTTGCCTTTACTTCTAGGC CTGCGCTCACCATGGTCAGCTACTGGGACACCGGGGTCCTGC TGTGCGCGCTGCTCAGCTGTCTGCTTCTCACAGGATCTAGTTC AGGTTCAGATACAGGTAGACCTTTCGTAGAGATGTACAGTGA AATCCCCGAAATTATACACATGACTGAAGGAAGGGAGCTCG TCATTCCCTGCCGGGTTACGTCACCTAACATCACTGTTACTTT AAAAAAGTTTCCACTTGACACTTTGATCCCTGATGGAAAACG CATAATCTGGGACAGTAGAAAGGGCTTCATCATATCAAATGC AACGTACAAAGAAATAGGGCTTCTGACCTGTGAAGCAACAG TCAATGGGCATTTGTATAAGACAAACTATCTCACACATCGAC AAACCAATACAATCATAGATGTCGTTCTGAGTCCGTCTCATG GAATTGAACTATCTGTTGGAGAAAAGCTTGTCTTAAATTGTA CAGCAAGAACTGAACTAAATGTGGGGATTGACTTCAACTGG

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SEQ ID NO:	Annotation	Sequence
		GAATACCCTTCTTCGAAGCATCAGCATAAGAAACTTGTAAAC CGAGACCTAAAAACCCAGTCTGGGAGTGAGATGAAGAAATT TTTGAGCACCTTAACTATAGATGGTGTAACCCGGAGTGACCA AGGATTGTACACCTGTGCAGCATCCAGTGGGCTGATGACCAA GAAGAACAGCACATTTGTCAGGGTCCATGAAAAAGACAAAA CTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGG GACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCC TCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGG ACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTAC GTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCG GGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCC TCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACA AGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGA AAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAG GTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAAC CAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGC GACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAA CAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTC CTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTG GCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGC TCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCC GGGTAAATGATTCGAAAATAAAATATCTTTATTTTCATTACA TCTGTGTGTTGGTTTTTTGTGTGGCATGCTGGGGAGAGATCA ACCCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCG GGCGACCAAAGGTCGCCCGACGCCCGGGCTTTGCCCGGGCG GCCTCAGTGAGCGAGCGAGCGCGCAGCAAGCTGTAGCCAAC CACTAGAACTATAGCTAGAGTCCTGGGCGAACAAACGATGCT CGCCTTCCAGAAAACCGAGGATGCGAACCACTTCATCCGGGG TCAGCACCACCGGCAAGCGCCGCGACGGCCGAGGTCTTCCG ATCTCCTGAAGCCAGGGCAGATCCGTGCACAGCACCTTGCCG TAGAAGAACAGCAAGGCCGCCAATGCCTGACGATGCGTGGA GACCGAAACCTTGCGCTCGTTCGCCAGCCAGGACAGAAATGC CTCGACTTCGCTGCTGCCCAAGGTTGCCGGGTGACGCACACC GTGGAAACGGATGAAGGCACGAACCCAGTTGACATAAGCCT GTTCGGTTCGTAAACTGTAATGCAAGTAGCGTATGCGCTCAC GCAACTGGTCCAGAACCTTGACCGAACGCAGCGGTGGTAAC GGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTG TACAGTCTATGCCTCGGGCATCCAAGCAGCAAGCGCGTTACG CCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTA CGCAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAA AACAAAGTTAGGTGGCTCAAGTATGGGCATCATTCGCACATG TAGGCTCGGCCCTGACCAAGTCAAATCCATGCGGGCTGCTCT TGATCTTTTCGGTCGTGAGTTCGGAGACGTAGCCACCTACTC CCAACATCAGCCGGACTCCGATTACCTCGGGAACTTGCTCCG TAGTAAGACATTCATCGCGCTTGCTGCCTTCGACCAAGAAGC GGTTGTTGGCGCTCTCGCGGCTTACGTTCTGCCCAGGTTTGAG CAGCCGCGTAGTGAGATCTATATCTATGATCTCGCAGTCTCC GGCGAGCACCGGAGGCAGGGCATTGCCACCGCGCTCATCAA

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		TCTCCTCAAGCATGAGGCCAACGCGCTTGGTGCTTATGTGAT CTACGTGCAAGCAGATTACGGTGACGATCCCGCAGTGGCTCT CTATACAAAGTTGGGCATACGGGAAGAAGTGATGCACTTTGA TATCGACCCAAGTACCGCCACCTAACAATTCGTTCAAGCCGA GATCGGCTTCCCGGCCGCGGAGTTGTTCGGTAAATTGTCACA ACGCCGCGAATATAGTCTTTACCATGCCCTTGGCCACGCCCC TCTTTAATACGACGGGCAATTTGCACTTCAGAAAATGAAGAG TTTGCTTTAGCCATAACAAAAGTCCAGTATGCTTTTTCACAGC ATAACTGGACTGATTTCAGTTTACAACTATTCTGTCTAGTTTA AGACTTTATTGTCATAGTTTAGATCTATTTTGTTCAGTTTAAG ACTTTATTGTCCGCCCACACCCGCTTACGCAGGGCATCCATTT ATTACTCAACCGTAACCGATTTTGCCAGGTTACGCGGCTGGT CTGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATA CCGCATCAGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTG CGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCA AAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGC AGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGG AACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTC CGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAG AGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTT TCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTG CCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCG TGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGT GTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCC CGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTT GAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCA GCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGG TGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACAC TAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGT TACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACA AACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCA GATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGA TCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCAC GTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCA CCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCT AAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCT TAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTC ATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGAT ACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACC GCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAA CCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAA CTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGC TAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGT TGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGT ATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTT ACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTC GGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTA TCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCA

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SEQ ID NO:	Annotation	Sequence
		TGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAA CCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCT CTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCA GAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGC GAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGA TGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTAC TTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAA ATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGA ATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATC AGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTT AGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGA AAAGTGCCACCTGAAATTGTAAACGTTAATATTTTGTTAAAA TTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAAT AGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAG ACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGT CCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAA AACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACC CTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAA TCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGG GAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGC GAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCA CGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGC TACAGGGCGCGTC
21	AMI059 coding sequence	ATGGTATCCTATTGGGATACGGGTGTTCTCTTGTGTGCACTCC TTTCCTGTCTCCTGCTCACTGGATCTTCTTCTGGGTCTGATAC TGGTAGACCTTTTGTAGAAATGTATTCAGAAATTCCGGAAAT AATTCATATGACAGAAGGACGAGAACTCGTTATACCATGTCG CGTCACGTCCCCTAATATTACTGTTACGCTCAAGAAGTTTCCT CTCGATACACTTATTCCAGATGGGAAACGCATAATTTGGGAT TCACGCAAAGGGTTTATTATTAGTAACGCAACGTATAAAGAA ATTGGGCTGCTCACATGTGAAGCTACGGTAAATGGGCATCTT TATAAAACAAATTATTTGACTCATCGGCAAACTAATACTATT ATCGATGTAGTACTCTCCCCATCCCATGGTATTGAATTGTCAG TTGGGGAGAAGTTGGTATTGAATTGTACTGCACGGACAGAAC TCAACGTTGGTATTGATTTTAATTGGGAATATCCATCATCTAA ACATCAGCATAAGAAGTTGGTAAATCGTGATCTCAAAACTCA AAGTGGGTCCGAAATGAAGAAGTTTCTGTCCACACTTACGAT TGATGGGGTCACTAGAAGTGATCAAGGGCTCTATACGTGTGC AGCATCTAGTGGGTTGATGACAAAGAAGAATTCAACTTTTGT TCGTGTCCATGAAAAGGATAAAACACATACTTGTCCACCGTG TCCTGCGCCAGAACTTCTCGGTGGTCCATCCGTCTTTCTCTTT CCACCTAAACCAAAAGATACTTTGATGATTTCACGGACTCCA

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SEQ ID NO:	Annotation	Sequence
		GAAGTAACATGTGTTGTCGTTGATGTATCACACGAAGATCCA GAAGTCAAATTTAATTGGTATGTTGATGGTGTAGAAGTTCAT AATGCGAAGACAAAACCACGAGAAGAACAATACAATAGTAC ATATCGGGTAGTATCCGTCTTGACTGTACTTCACCAAGATTG GCTTAATGGGAAAGAATACAAATGTAAAGTTTCTAATAAAGC TCTTCCTGCGCCGATCGAAAAGACAATTTCCAAAGCAAAAGG TCAACCTCGGGAACCTCAAGTTTATACGCTCCCACCATCACG GGATGAACTCACTAAGAATCAAGTATCCTTGACTTGTCTCGT AAAAGGGTTTTATCCTTCAGATATTGCTGTAGAATGGGAATC CAATGGGCAACCAGAAAATAATTATAAAACAACACCACCTG TTCTTGATTCAGATGGTTCATTCTTTCTCTATTCCAAACTTACT GTCGATAAATCACGCTGGCAACAAGGTAATGTTTTCTCTTGT TCCGTCATGCATGAAGCACTCCATAATCACTATACGCAAAAG TCTCTCTCTCTCTCACCAGGTAAATAA
22	AMI066 coding sequence	ATGGAGTTCGGCCTGAGCTGGCTGTTCCTGGTGGCCATCCTT AAGGGCGTGCAGTGCGATACTGGTAGACCTTTTGTAGAAATG TATTCAGAAATTCCGGAAATAATTCATATGACAGAAGGACGA GAACTCGTTATACCATGTCGCGTCACGTCCCCTAATATTACTG TTACGCTCAAGAAGTTTCCTCTCGATACACTTATTCCAGATGG GAAACGCATAATTTGGGATTCACGCAAAGGGTTTATTATTAG TAACGCAACGTATAAAGAAATTGGGCTGCTCACATGTGAAGC TACGGTAAATGGGCATCTTTATAAAACAAATTATTTGACTCA TCGGCAAACTAATACTATTATCGATGTAGTACTCTCCCCATCC CATGGTATTGAATTGTCAGTTGGGGAGAAGTTGGTATTGAAT TGTACTGCACGGACAGAACTCAACGTTGGTATTGATTTTAAT TGGGAATATCCATCATCTAAACATCAGCATAAGAAGTTGGTA AATCGTGATCTCAAAACTCAAAGTGGGTCCGAAATGAAGAA GTTTCTGTCCACACTTACGATTGATGGGGTCACTAGAAGTGA TCAAGGGCTCTATACGTGTGCAGCATCTAGTGGGTTGATGAC AAAGAAGAATTCAACTTTTGTTCGTGTCCATGAAAAGGATAA AACACATACTTGTCCACCGTGTCCTGCGCCAGAACTTCTCGG TGGTCCATCCGTCTTTCTCTTTCCACCTAAACCAAAAGATACT TTGATGATTTCACGGACTCCAGAAGTAACATGTGTTGTCGTT GATGTATCACACGAAGATCCAGAAGTCAAATTTAATTGGTAT GTTGATGGTGTAGAAGTTCATAATGCGAAGACAAAACCACG AGAAGAACAATACAATAGTACATATCGGGTAGTATCCGTCTT GACTGTACTTCACCAAGATTGGCTTAATGGGAAAGAATACAA ATGTAAAGTTTCTAATAAAGCTCTTCCTGCGCCGATCGAAAA GACAATTTCCAAAGCAAAAGGTCAACCTCGGGAACCTCAAG TTTATACGCTCCCACCATCACGGGATGAACTCACTAAGAATC AAGTATCCTTGACTTGTCTCGTAAAAGGGTTTTATCCTTCAGA TATTGCTGTAGAATGGGAATCCAATGGGCAACCAGAAAATA ATTATAAAACAACACCACCTGTTCTTGATTCAGATGGTTCATT CTTTCTCTATTCCAAACTTACTGTCGATAAATCACGCTGGCAA CAAGGTAATGTTTTCTCTTGTTCCGTCATGCATGAAGCACTCC ATAATCACTATACGCAAAAGTCTCTCTCTCTCACCAGGTA AATAA
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SEQ ID NO:	Annotation	Sequence
	AMI067 coding sequence	ATGGTGAGCTACTGGGACACCGGCGTGCTGCTGTGCGCCCTG CTGAGCTGCCTGCTGCTGACCGGCAGCAGCAGCGGCAGCGA CACCGGCAGACCCTTCGTGGAGATGTACAGCGAGATCCCCGA GATCATCCACATGACCGAGGGCAGAGAGCTGGTGATCCCCTG CAGAGTGACCAGCCCCAACATCACCGTGACCCTGAAGAAGTT CCCCCTGGACACCCTGATCCCCGACGGCAAGAGAATCATCTG GGACAGCAGAAAGGGCTTCATCATCAGCAACGCCACCTACA AGGAGATCGGCCTGCTGACCTGCGAGGCCACCGTGAACGGC CACCTGTACAAGACCAACTACCTGACCCACAGACAGACCAA CACCATCATCGACGTGGTGCTGAGCCCCAGCCACGGCATCGA GCTGAGCGTGGGCGAGAAGCTGGTGCTGAACTGCACCGCCA GAACCGAGCTGAACGTGGGCATCGACTTCAACTGGGAGTAC CCCAGCAGCAAGCACCAGCACAAGAAGCTGGTGAACAGAGA CCTGAAGACCCAGAGCGGCAGCGAGATGAAGAAGTTCCTGA GCACCCTGACCATCGACGGCGTGACCAGAAGCGACCAGGGC CTGTACACCTGCGCCGCCAGCAGCGGCCTGATGACCAAGAA GAACAGCACCTTCGTGAGAGTGCACGAGAAGGACAAGACCC ACACCTGCCCCCCCTGCCCCGCCCCCGAGCTGCTGGGCGGCC CCAGCGTGTTCCTGTTCCCCCCCAAGCCCAAGGACACCCTGA TGATCAGCAGAACCCCCGAGGTGACCTGCGTGGTGGTGGAC GTGAGCCACGAGGACCCCGAGGTGAAGTTCAACTGGTACGT GGACGGCGTGGAGGTGCACAACGCCAAGACCAAGCCCAGAG AGGAGCAGTACAACAGCACCTACAGAGTGGTGAGCGTGCTG ACCGTGCTGCACCAGGACTGGCTGAACGGCAAGGAGTACAA GTGCAAGGTGAGCAACAAGGCCCTGCCCGCCCCCATCGAGA AGACCATCAGCAAGGCCAAGGGCCAGCCCAGAGAGCCCCAG GTGTACACCCTGCCCCCCAGCAGAGACGAGCTGACCAAGAA CCAGGTGAGCCTGACCTGCCTGGTGAAGGGCTTCTACCCCAG CGACATCGCCGTGGAGTGGGAGAGCAACGGCCAGCCCGAGA ACAACTACAAGACCACCCCCCCCGTGCTGGACAGCGACGGC AGCTTCTTCCTGTACAGCAAGCTGACCGTGGACAAGAGCAGA TGGCAGCAGGGCAACGTGTTCAGCTGCAGCGTGATGCACGA GGCCCTGCACAACCACTACACCCAGAAGAGCCTGAGCCTGA GCCCCGGCAAGTGA
24	AMI068 coding sequence	ATGGAGTTCGGCCTGAGCTGGCTGTTCCTGGTGGCCATCCTT AAGGGCGTGCAGTGCGACACCGGCAGACCCTTCGTGGAGAT GTACAGCGAGATCCCCGAGATCATCCACATGACCGAGGGCA GAGAGCTGGTGATCCCCTGCAGAGTGACCAGCCCCAACATCA CCGTGACCCTGAAGAAGTTCCCCCTGGACACCCTGATCCCCG ACGGCAAGAGAATCATCTGGGACAGCAGAAAGGGCTTCATC ATCAGCAACGCCACCTACAAGGAGATCGGCCTGCTGACCTGC GAGGCCACCGTGAACGGCCACCTGTACAAGACCAACTACCT GACCCACAGACAGACCAACACCATCATCGACGTGGTGCTGA GCCCCAGCCACGGCATCGAGCTGAGCGTGGGCGAGAAGCTG GTGCTGAACTGCACCGCCAGAACCGAGCTGAACGTGGGCAT CGACTTCAACTGGGAGTACCCCAGCAGCAAGCACCAGCACA AGAAGCTGGTGAACAGAGACCTGAAGACCCAGAGCGGCAGC GAGATGAAGAAGTTCCTGAGCACCCTGACCATCGACGGCGT

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SEQ ID NO:	Annotation	Sequence
		GACCAGAAGCGACCAGGGCCTGTACACCTGCGCCGCCAGCA GCGGCCTGATGACCAAGAAGAACAGCACCTTCGTGAGAGTG CACGAGAAGGACAAGACCCACACCTGCCCCCCCTGCCCCGCC CCCGAGCTGCTGGGCGGCCCCAGCGTGTTCCTGTTCCCCCCC AAGCCCAAGGACACCCTGATGATCAGCAGAACCCCCGAGGT GACCTGCGTGGTGGTGGACGTGAGCCACGAGGACCCCGAGG TGAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCACAAC GCCAAGACCAAGCCCAGAGAGGAGCAGTACAACAGCACCTA CAGAGTGGTGAGCGTGCTGACCGTGCTGCACCAGGACTGGCT GAACGGCAAGGAGTACAAGTGCAAGGTGAGCAACAAGGCCC TGCCCGCCCCCATCGAGAAGACCATCAGCAAGGCCAAGGGC CAGCCCAGAGAGCCCCAGGTGTACACCCTGCCCCCCAGCAG AGACGAGCTGACCAAGAACCAGGTGAGCCTGACCTGCCTGG TGAAGGGCTTCTACCCCAGCGACATCGCCGTGGAGTGGGAG AGCAACGGCCAGCCCGAGAACAACTACAAGACCACCCCCCC CGTGCTGGACAGCGACGGCAGCTTCTTCCTGTACAGCAAGCT GACCGTGGACAAGAGCAGATGGCAGCAGGGCAACGTGTTCA GCTGCAGCGTGATGCACGAGGCCCTGCACAACCACTACACCC AGAAGAGCCTGAGCCTGAGCCCCGGCAAGTGA
25	AMI119 coding sequence	ATGGTGAGCTACTGGGACACCGGCGTGCTGCTGTGCGCCCTG CTGAGCTGCCTGCTGCTGACCGGCAGCAGCAGCGGCAGCGA CACCGGCAGGCCTTTCGTGGAGATGTACAGCGAGATCCCTGA GATCATCCACATGACCGAGGGCAGGGAGCTGGTGATCCCTTG CAGGGTGACCAGCCCTAACATCACCGTGACCCTGAAGAAGTT CCCTCTGGACACCCTGATCCCTGACGGCAAGAGGATCATCTG GGACAGCAGGAAGGGCTTCATCATCAGCAACGCCACCTACA AGGAGATCGGCCTGCTGACCTGCGAGGCCACCGTGAACGGC CACCTGTACAAGACCAACTACCTGACCCACAGGCAGACCAA CACCATCATCGACGTGGTGCTGAGCCCTAGCCACGGCATCGA GCTGAGCGTGGGCGAGAAGCTGGTGCTGAACTGCACCGCCA GGACCGAGCTGAACGTGGGCATCGACTTCAACTGGGAGTAC CCTAGCAGCAAGCACCAGCACAAGAAGCTGGTGAACAGGGA CCTGAAGACCCAGAGCGGCAGCGAGATGAAGAAGTTCCTGA GCACCCTGACCATCGACGGCGTGACCAGGAGCGACCAGGGC CTGTACACCTGCGCCGCCAGCAGCGGCCTGATGACCAAGAA GAACAGCACCTTCGTGAGGGTGCACGAGAAGGACAAGACCC ACACCTGCCCTCCTTGCCCTGCCCCTGAGCTGCTGGGCGGCC CTAGCGTGTTCCTGTTCCCTCCTAAGCCTAAGGACACCCTGAT GATCAGCAGGACCCCTGAGGTGACCTGCGTGGTGGTGGACGT GAGCCACGAGGACCCTGAGGTGAAGTTCAACTGGTACGTGG ACGGCGTGGAGGTGCACAACGCCAAGACCAAGCCTAGGGAG GAGCAGTACAACAGCACCTACAGGGTGGTGAGCGTGCTGAC CGTGCTGCACCAGGACTGGCTGAACGGCAAGGAGTACAAGT GCAAGGTGAGCAACAAGGCCCTGCCTGCCCCTATCGAGAAG ACCATCAGCAAGGCCAAGGGCCAGCCTAGGGAGCCTCAGGT GTACACCCTGCCTCCTAGCAGGGACGAGCTGACCAAGAACC AGGTGAGCCTGACCTGCCTGGTGAAGGGCTTCTACCCTAGCG ACATCGCCGTGGAGTGGGAGAGCAACGGCCAGCCTGAGAAC

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SEQ ID NO:	Annotation	Sequence
		AACTACAAGACCACCCCTCCTGTGCTGGACAGCGACGGCAGC TTCTTCCTGTACAGCAAGCTGACCGTGGACAAGAGCAGGTGG CAGCAGGGCAACGTGTTCAGCTGCAGCGTGATGCACGAGGC CCTGCACAACCACTACACCCAGAAGAGCCTGAGCCTGAGCCC TGGCAAGTGA
26	AMI120 coding sequence	ATGGTGAGCTACTGGGACACCGGCGTGCTGCTGTGCGCCCTG CTGAGCTGCCTGCTGCTGACCGGCAGCAGCAGCGGCAGCGA CACCGGCAGGCCCTTCGTGGAGATGTACTCCGAGATCCCCGA GATCATCCACATGACCGAGGGCAGGGAGCTGGTGATCCCCTG CAGGGTGACCTCCCCCAACATCACCGTGACCCTGAAGAAGTT CCCCCTGGACACCCTGATCCCCGACGGCAAGAGGATCATCTG GGACTCCAGGAAGGGCTTCATCATCTCCAACGCCACCTACAA GGAGATCGGCCTGCTGACCTGCGAGGCCACCGTGAACGGCC ACCTGTACAAGACCAACTACCTGACCCACAGGCAGACCAAC ACCATCATCGACGTGGTGCTGTCCCCCTCCCACGGCATCGAG CTGTCCGTGGGCGAGAAGCTGGTGCTGAACTGCACCGCCAGG ACCGAGCTGAACGTGGGCATCGACTTCAACTGGGAGTACCCC TCCTCCAAGCACCAGCACAAGAAGCTGGTGAACAGGGACCT GAAGACCCAGTCCGGCTCCGAGATGAAGAAGTTCCTGTCCAC CCTGACCATCGACGGCGTGACCAGGTCCGACCAGGGCCTGTA CACCTGCGCCGCCTCCTCCGGCCTGATGACCAAGAAGAACTC CACCTTCGTGAGGGTGCACGAGAAGGACAAGACCCACACCT GCCCCCCCTGCCCCGCCCCCGAGCTGCTGGGCGGCCCCTCCG TGTTCCTGTTCCCCCCCAAGCCCAAGGACACCCTGATGATCT CCAGGACCCCCGAGGTGACCTGCGTGGTGGTGGACGTGTCCC ACGAGGACCCCGAGGTGAAGTTCAACTGGTACGTGGACGGC GTGGAGGTGCACAACGCCAAGACCAAGCCCAGGGAGGAGCA GTACAACTCCACCTACAGGGTGGTGTCCGTGCTGACCGTGCT GCACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGG TGTCCAACAAGGCCCTGCCCGCCCCCATCGAGAAGACCATCT CCAAGGCCAAGGGCCAGCCCAGGGAGCCCCAGGTGTACACC CTGCCCCCCTCCAGGGACGAGCTGACCAAGAACCAGGTGTCC CTGACCTGCCTGGTGAAGGGCTTCTACCCCTCCGACATCGCC GTGGAGTGGGAGTCCAACGGCCAGCCCGAGAACAACTACAA GACCACCCCCCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTG TACTCCAAGCTGACCGTGGACAAGTCCAGGTGGCAGCAGGG CAACGTGTTCTCCTGCTCCGTGATGCACGAGGCCCTGCACAA CCACTACACCCAGAAGTCCCTGTCCCTGTCCCCCGGCAAGTG A
27	AMI130 coding sequence	ATGGTGAGCTACTGGGACACCGGCGTGCTGCTGTGCGCCCTG CTGAGCTGCCTGCTGCTGACCGGCAGCAGCAGCGGCAGCGA CACCGGCAGGCCGTTCGTGGAGATGTACAGCGAGATCCCGG AGATCATCCACATGACCGAGGGCAGGGAGCTGGTGATCCCG TGCAGGGTGACCAGCCCGAACATCACCGTGACCCTGAAGAA GTTCCCGCTGGACACCCTGATCCCGGACGGCAAGAGGATCAT CTGGGACAGCAGGAAGGGCTTCATCATCAGCAACGCCACCT ACAAGGAGATCGGCCTGCTGACCTGCGAGGCCACCGTGAAC GGCCACCTGTACAAGACCAACTACCTGACCCACAGGCAGAC

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SEQ ID NO:	Annotation	Sequence
		CAACACCATCATCGACGTGGTGCTGAGCCCGAGCCACGGCAT CGAGCTGAGCGTGGGCGAGAAGCTGGTGCTGAACTGCACCG CCAGGACCGAGCTGAACGTGGGCATCGACTTCAACTGGGAG TACCCGAGCAGCAAGCACCAGCACAAGAAGCTGGTGAACAG GGACCTGAAGACCCAGAGCGGCAGCGAGATGAAGAAGTTCC TGAGCACCCTGACCATCGACGGCGTGACCAGGAGCGACCAG GGCCTGTACACCTGCGCCGCCAGCAGCGGCCTGATGACCAAG AAGAACAGCACCTTCGTGAGGGTGCACGAGAAGGACAAGAC CCACACCTGCCCGCCGTGCCCGGCCCCGGAGCTGCTGGGCGG CCCGAGCGTGTTCCTGTTCCCGCCGAAGCCGAAGGACACCCT GATGATCAGCAGGACCCCGGAGGTGACCTGCGTGGTGGTGG ACGTGAGCCACGAGGACCCGGAGGTGAAGTTCAACTGGTAC GTGGACGGCGTGGAGGTGCACAACGCCAAGACCAAGCCGAG GGAGGAGCAGTACAACAGCACCTACAGGGTGGTGAGCGTGC TGACCGTGCTGCACCAGGACTGGCTGAACGGCAAGGAGTAC AAGTGCAAGGTGAGCAACAAGGCCCTGCCGGCCCCGATCGA GAAGACCATCAGCAAGGCCAAGGGCCAGCCGAGGGAGCCGC AGGTGTACACCCTGCCGCCGAGCAGGGACGAGCTGACCAAG AACCAGGTGAGCCTGACCTGCCTGGTGAAGGGCTTCTACCCG AGCGACATCGCCGTGGAGTGGGAGAGCAACGGCCAGCCGGA GAACAACTACAAGACCACCCCGCCGGTGCTGGACAGCGACG GCAGCTTCTTCCTGTACAGCAAGCTGACCGTGGACAAGAGCA GGTGGCAGCAGGGCAACGTGTTCAGCTGCAGCGTGATGCAC GAGGCCCTGCACAACCACTACACCCAGAAGAGCCTGAGCCT GAGCCCCGGCAAGTGA
28	Control coding sequence (reference VEGF-Trap)	ATGGTCAGCTACTGGGACACCGGGGTCCTGCTGTGCGCGCTG CTCAGCTGTCTGCTTCTCACAGGATCTAGTTCAGGTTCAGATA CAGGTAGACCTTTCGTAGAGATGTACAGTGAAATCCCCGAAA TTATACACATGACTGAAGGAAGGGAGCTCGTCATTCCCTGCC GGGTTACGTCACCTAACATCACTGTTACTTTAAAAAAGTTTC CACTTGACACTTTGATCCCTGATGGAAAACGCATAATCTGGG ACAGTAGAAAGGGCTTCATCATATCAAATGCAACGTACAAA GAAATAGGGCTTCTGACCTGTGAAGCAACAGTCAATGGGCAT TTGTATAAGACAAACTATCTCACACATCGACAAACCAATACA ATCATAGATGTCGTTCTGAGTCCGTCTCATGGAATTGAACTA TCTGTTGGAGAAAAGCTTGTCTTAAATTGTACAGCAAGAACT GAACTAAATGTGGGGATTGACTTCAACTGGGAATACCCTTCT TCGAAGCATCAGCATAAGAAACTTGTAAACCGAGACCTAAA AACCCAGTCTGGGAGTGAGATGAAGAAATTTTTGAGCACCTT AACTATAGATGGTGTAACCCGGAGTGACCAAGGATTGTACAC CTGTGCAGCATCCAGTGGGCTGATGACCAAGAAGAACAGCA CATTTGTCAGGGTCCATGAAAAAGACAAAACTCACACATGCC CACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCT TCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCC GGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCAC GAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGT GGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGT ACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGC

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SEQ ID NO:	Annotation	Sequence
		ACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTC TCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCC AAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCT GCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCT GACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGT GGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGA CCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTA CAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGA ACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACC ACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGA
30	Amino acid sequence of aflibercept (encoded by AMI120)	DTGRPFVEMYSEIPEIIHMTEGRELVIPCRVTSPNITVTLKKFPLD TLIPDGKRIIWDSRKGFIISNATYKEIGLLTCEATVNGHLYKTNY LTHRQTNTIIDVVLSPSHGIELSVGEKLVLNCTARTELNVGIDFN WEYPSSKHQHKKLVNRDLKTQSGSEMKKFLSTLTIDGVTRSDQ GLYTCAASSGLMTKKNSTFVRVHEKDKTHTCPPCPAPELLGGPS VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD KSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK*
31	Nucleic acid sequence encoding aflibercept (AMI120)	GACACCGGCAGGCCCTTCGTGGAGATGTACTCCGAGATCCCC GAGATCATCCACATGACCGAGGGCAGGGAGCTGGTGATCCC CTGCAGGGTGACCTCCCCCAACATCACCGTGACCCTGAAGAA GTTCCCCCTGGACACCCTGATCCCCGACGGCAAGAGGATCAT CTGGGACTCCAGGAAGGGCTTCATCATCTCCAACGCCACCTA CAAGGAGATCGGCCTGCTGACCTGCGAGGCC ACCGTGAACGGCCACCTGTACAAGACCAACTACCTGACCCAC AGGCAGACCAACACCATCATCGACGTGGTGCTGTCCCCCTCC CACGGCATCGAGCTGTCCGTGGGCGAGAAGCTGGTGCTGAA CTGCACCGCCAGGACCGAGCTGAACGTGGGCATCGACTTCAA CTGGGAGTACCCCTCCTCCAAGCACCAGCACAAGAAGCTGGT GAACAGGGACCTGAAGACCCAGTCCGGCTCCGAGATGAAGA AGTTCCTGTCCACCCTGACCATCGACGGCGTGACCAGGTCCG ACCAGGGCCTGTACACCTGCGCCGCCTCCTCCGGCCTGATGA CCAAGAAGAACTCCACCTTCGTGAGGGTGCACGAGAAGGAC AAGACCCACACCTGCCCCCCCTGCCCCGCCCCCGAGCTGCTG GGCGGCCCCTCCGTGTTCCTGTTCCCCCCCAAGCCCAAGGAC ACCCTGATGATCTCCAGGACCCCCGAGGTGACCTGCGTGGTG GTGGACGTGTCCCACGAGGACCCCGAGGTGAAGTTCAACTG GTACGTGGACGGCGTGGAGGTGCACAACGCCAAGACCAAGC CCAGGGAGGAGCAGTACAACTCCACCTACAGGGTGGTGTCC GTGCTGACCGTGCTGCACCAGGACTGGCTGAACGGCAAGGA GTACAAGTGCAAGGTGTCCAACAAGGCCCTGCCCGCCCCCAT CGAGAAGACCATCTCCAAGGCCAAGGGCCAGCCCAGGGAGC CCCAGGTGTACACCCTGCCCCCCTCCAGGGACGAGCTGACCA AGAACCAGGTGTCCCTGACCTGCCTGGTGAAGGGCTTCTACC

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SEQ ID NO:	Annotation	Sequence
		CCTCCGACATCGCCGTGGAGTGGGAGTCCAACGGCCAGCCCG AGAACAACTACAAGACCACCCCCCCCGTGCTGGACTCCGACG GCTCCTTCTTCCTGTACTCCAAGCTGACCGTGGACAAGTCCA GGTGGCAGCAGGGCAACGTGTTCTCCTGCTCCGTGATGCACG AGGCCCTGCACAACCACTACACCCAGAAGTCCCTGTCCCTGT CCCCCGGCAAGTGA
70	Reference nucleic acid sequence encoding VEGF-Trap	atgtacagaa tgcagctgct gctgctgatc gccctgagcc tggccctggt gaccaacagc agcgacaccg gcagaccctt cgtggagatg tacagcgaga tccccgagat catccacatg accgagggca gagagctggt gatcccctgc agagtgacca gccccaacat caccgtgacc ctgaagaagt tcccctgga caccctgatc cccgacggca agagaatcat ctgggacagc agaaagggct tcatcatcag caacgccacc tacaaggaga tcggcctgct gacctgcgag gccaccgtga acggccacct gtacaagacc aactacctga cccacagaca gaccaacacc atcatcgacg tggtgctgag ccccagccac ggcatcgagc tgagcgtggg cgagaagctg gtgctgaact gcaccgccag aaccgagctg aacgtgggca tcgacttcaa ctgggagtac cccagcagca agcaccagca caagaagctg gtgaacagag acctgaagac ccagagcggc agcgagatga agaagttcct gagcaccctg accatcgacg gcgtgaccag aagcgaccag ggcctgtaca cctgcgccgc cagcagcggc ctgatgacca agaagaacag caccttcgtg agagtgcacg agaaggacaa gacccacacc tgcccccct gccccgcccc cgagctgctg ggcggcccca gcgtgttcct gttcccccc aagcccaagg acaccctgat gatcagcaga acccccgagg tgacctgcgt ggtggtggac gtgagccacg aggaccccga ggtgaagttc aactggtacg tggacggcgt ggaggtgcac aacgccaaga ccaagcccag agaggagcag tacaacagca cctacagagt ggtgagcgtg ctgaccgtgc tgcaccagga ctggctgaac ggcaaggagt acaagtgcaa ggtgagcaac aaggccctgc ccgccccat cgagaagacc atcagcaagg ccaagggcca gcccagagag ccccaggtgt acaccctgcc ccccagcaga gacgagctga ccaagaacca ggtgagcctg acctgcctgg tgaagggctt ctaccccagc gacatcgccg tggagtggga gagcaacggc cagcccgaga acaactacaa gaccaccccc cccgtgctgg acagcgacgg cagcttcttc ctgtacagca agctgaccgt ggacaagagc agatggcagc agggcaacgt gttcagctgc agcgtgatgc acgaggccct gcacaaccac tacacccaga agagcctgag cctgagcccc ggc

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CLAIMS

WHAT IS CLAIMED IS:

1. An isolated, non-naturally occurring nucleic acid comprising a sequence encoding a biologic comprising an anti-angiogenic agent, said sequence comprising a modification in a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region, said modification comprising a replacement of at least four non-AGG arginine codons with AGG.

2. The isolated, non-naturally occurring nucleic acid of claim 1, wherein the sequence that encodes the anti-angiogenic agent further comprises a second modification.

3. The isolated, non-naturally occurring nucleic acid of claim 2, wherein the second modification is in at least one codon of the coding region of the sequence, and wherein the second modification is selected from the group consisting of:

(a) replacement of at least one non-CCC proline codon with CCC;

(b) replacement of at least one non-TCC serine codon with TCC;

(c) replacement of at least one non-CCG proline codon with CCG; and

(d) any combination of (a)-(c).

4. The isolated, non-naturally occurring nucleic acid of claim 3, wherein the second modification comprises (a).

5. The isolated, non-naturally occurring nucleic acid of claim 4, wherein the at least one non-CCC proline codon is CCT.

6. The isolated, non-naturally occurring nucleic acid of claim 3, wherein the second modification comprises (b).

7. The isolated, non-naturally occurring nucleic acid of claim 6, wherein the at least one non-TCC serine codon is AGC.

8. The isolated, non-naturally occurring nucleic acid of claim 3, wherein the second modification comprises (c).

9. The isolated, non-naturally occurring nucleic acid of claim 8, wherein the at least one non-CCG proline codon is CCC.

10. The isolated, non-naturally occurring nucleic acid of claim 3, wherein the second modification comprises (d).

11. The isolated, non-naturally occurring nucleic acid of claim 10, wherein the at least one non-CCC proline codon of (a) is CCT; the at least one non-TCC serine codon of (b) is AGC; and the at least one non-CCG proline codon of (c) is CCC.

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12. The isolated, non-naturally occurring nucleic acid of any one of claims 1-11, wherein the anti-angiogenic agent is selected from the group consisting of: a VEGF inhibitor, a multi-tyrosine kinase inhibitor, a receptor tyrosine kinase inhibitor, an inhibitor of Akt phosphorylation, a PDGF-1 inhibitor, a PDGF-2 inhibitor, a NP-1 inhibitor, a NP-2 inhibitor, a Del 1 inhibitor, and an integrin inhibitor.

13. The isolated, non-naturally occurring nucleic acid of claim 12, wherein the anti-angiogenic agent comprises the VEGF inhibitor, and wherein the VEGF inhibitor is a non-antibody inhibitor.

14. The isolated, non-naturally occurring nucleic acid of claim 13, wherein the non-antibody inhibitor is a fusion protein that comprises human VEGF receptors 1 and 2.

15. The isolated, non-naturally occurring nucleic acid of claim 14, wherein the fusion protein comprises VEGF-Trap or a modified version thereof.

16. The isolated, non-naturally occurring nucleic acid of any one of claims 1-15, wherein the isolated, non-naturally occurring nucleic acid further comprises a signal peptide.

17. The isolated, non-naturally occurring nucleic acid of claim 16, wherein the signal peptide is selected from the group consisting of: human antibody heavy chain (Vh), human antibody light chain (Vl), and VEGF-Trap.

18. The isolated, non-naturally occurring nucleic acid of claim 17, wherein the signal peptide is from the human antibody heavy chain.

19. The isolated, non-naturally occurring nucleic acid of claim 16, wherein the signal peptide is derived from VEGF-Trap.

20. The isolated, non-naturally occurring nucleic acid of any one of claims 1-19, wherein the isolated, non-naturally occurring nucleic acid further comprises an intronic sequence.

21. The isolated, non-naturally occurring nucleic acid of claim 20, wherein the intronic sequence is selected from the group consisting of: hCMV intron A, adenovirus tripartite leader sequence intron, SV40 intron, hamster EF-1 alpha gene intron 1, intervening sequence intron, human growth hormone intron, and human beta globin intron.

22. The isolated, non-naturally occurring nucleic acid of claim 21, wherein the intronic sequence is the SV40 intron.

23. The isolated, non-naturally occurring nucleic acid of any one of claims 1-22, wherein the isolated, non-naturally occurring nucleic acid further comprises a promoter.

24. The isolated, non-naturally occurring nucleic acid of claim 23, wherein the promoter is selected from the group consisting of: a cytomegalovirus (CMV) promoter, an elongation factor 1 alpha (EF1α) promoter, a simian vacuolating virus (SV40) promoter, a phosphoglycerate kinase (PGK1) promoter, a ubiquitin C (Ubc) promoter, a human beta actin promoter, a CAG promoter,

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a Tetracycline response element (TRE) promoter, a UAS promoter, an Actin 5c (Ac5) promoter, a polyhedron promoter, a Ca2+/calmodulin-dependent protein kinase II (CaMKIIa) promoter, a GAL1 promoter, a GAL 10 promoter, a TEF1 promoter, a glyceraldehyde 3-phosphage dehydrogenase (GDS) promoter, an ADH1 promoter, a CaMV35S promoter, a Ubi promoter, a human polymerase III RNA (H1) promoter, a U6 promoter, a polyadenylated construct thereof, and any combination thereof.

25. The isolated, non-naturally occurring nucleic acid of claim 24, wherein the promoter is the CMV promoter.

26. The isolated, non-naturally occurring nucleic acid of any one of claims 1-25, wherein the sequence is modified to replace non-AGG arginine codon with AGG in at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, or at least 16 codons of the coding region of the sequence.

27. The isolated, non-naturally occurring nucleic acid of claim 26, wherein the sequence is modified to replace every non-AGG arginine codon with AGG of the coding region of the sequence.

28. The isolated, non-naturally occurring nucleic acid of any one of claims 1-27, wherein the sequence is modified to replace non-AGG arginine codon with AGG in at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, or at least 16 codon positions as compared to **SEQ ID NO: 70**.

29. The isolated, non-naturally occurring nucleic acid of any one of claims 1-28, wherein the sequence is modified to replace every non-AGG arginine codon with AGG as compared to **SEQ ID NO: 70**.

30. The isolated, non-naturally occurring nucleic acid of any one of claims 1-29, wherein the non-AGG arginine codon comprises CGT, CGC, CGA, CGG, or AGA.

31. The isolated, non-naturally occurring nucleic acid of claim 30, wherein the non-AGG arginine codon is AGA.

32. The isolated, non-naturally occurring nucleic acid of claim 31, wherein the sequence is modified to replace AGA with AGG in at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, or at least 16 codons of the coding region of the sequence.

33. The isolated, non-naturally occurring nucleic acid of claim 32, wherein the sequence is modified to replace every AGA with AGG of the coding region of the sequence.

34. The isolated, non-naturally occurring nucleic acid of claim 31, wherein the sequence is modified to replace AGA with AGG in at least 5, at least 6, at least 7, at least 8, at least 9, at least

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10, at least 11, at least 12, at least 13, at least 14, at least 15, or at least 16 codon positions as compared to **SEQ ID NO: 70**.

35. The isolated, non-naturally occurring nucleic acid of claim 34, wherein the sequence is modified to replace every AGA with AGG as compared to **SEQ ID NO: 70**.

36. The isolated, non-naturally occurring nucleic acid of any one of claims 3-35, wherein the sequence is modified to replace non-CCC proline codons with CCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or at least 30 codons of the coding region of the sequence.

37. The isolated, non-naturally occurring nucleic acid of claim 36, wherein the sequence is modified to replace every non-CCC proline codons with CCC of the coding region of the sequence.

38. The isolated, non-naturally occurring nucleic acid of any one of claims 3-35, wherein the sequence is modified to replace non-CCC proline codon with CCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or up to 30 codon positions as compared to **SEQ ID NO: 70**.

39. The isolated, non-naturally occurring nucleic acid of claim 38, wherein the sequence is modified to replace every non-CCC proline codon with CCC as compared to **SEQ ID NO: 70**.

40. The isolated, non-naturally occurring nucleic acid of any one of claims 3-35, wherein the non-CCC proline codon comprises CCT or CCA.

41. The isolated, non-naturally occurring nucleic acid of claim 40, wherein the non-CCC proline codon is CCT.

42. The isolated, non-naturally occurring nucleic acid of claim 41, wherein the sequence is modified to replace CCT with CCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or at least 30 codons of the coding region of the sequence.

43. The isolated, non-naturally occurring nucleic acid of claim 42, wherein the sequence is modified to replace every CCT with CCC of the coding region of the sequence.

44. The isolated, non-naturally occurring nucleic acid of claim 41, wherein the sequence is modified to replace CCT with CCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 29, or at least 30 codon positions as compared to **SEQ ID NO: 70**.

45. The isolated, non-naturally occurring nucleic acid of claim 44, wherein the sequence is modified to replace every CCT with CCC as compared to **SEQ ID NO: 70**.

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46. The isolated, non-naturally occurring nucleic acid of any one of claims 3-35, wherein the sequence is modified to replace non-TCC serine codon with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30, at least 33, or at least 36 codons of the coding region of the sequence.

47. The isolated, non-naturally occurring nucleic acid of claim 46, wherein the sequence is modified to replace every non-TCC serine codon with TCC of the coding region of the sequence.

48. The isolated, non-naturally occurring nucleic acid of any one of claims 3-35, wherein the sequence is modified to replace non-TCC serine codon with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30, at least 33, or at least 36 codon positions as compared to **SEQ ID NO: 70**.

49. The isolated, non-naturally occurring nucleic acid of claim 48, wherein the sequence is modified to replace every non-TCC serine codon with TCC as compared to **SEQ ID NO: 70**.

50. The isolated, non-naturally occurring nucleic acid of any one of claims 3-35, wherein the non-TCC serine codon comprises TCT, TCA, TCG, AGT, or AGC.

51. The isolated, non-naturally occurring nucleic acid of claim 50, wherein the non-TCC serine codon is AGC.

52. The isolated, non-naturally occurring nucleic acid of claim 51, wherein the sequence is modified to replace AGC with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30, at least 33, or at least 36 codons of the coding region of the sequence.

53. The isolated, non-naturally occurring nucleic acid of claim 52, wherein the sequence is modified to replace every AGC with TCC of the coding region of the sequence.

54. The isolated, non-naturally occurring nucleic acid of claim 51, wherein the sequence is modified to replace AGC with TCC in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30, at least 33, or at least 36 codon positions as compared to **SEQ ID NO: 70**.

55. The isolated, non-naturally occurring nucleic acid of claim 54, wherein the sequence is modified to replace every AGC with TCC as compared to **SEQ ID NO: 70**.

56. The isolated, non-naturally occurring nucleic acid of any one of claims 3-35, wherein the sequence is modified to replace non-CCG proline codon with CCG in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, or at least 30 codons of the coding region of the sequence.

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57. The isolated, non-naturally occurring nucleic acid of claim 56, wherein the sequence is modified to replace every non-CCG proline codon with CCG of the coding region of the sequence.

58. The isolated, non-naturally occurring nucleic acid of any one of claims 3-35, wherein the sequence is modified to replace non-CCG proline codon with CCG in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, at least 30 codon positions as compared to **SEQ ID NO: 70**.

59. The isolated, non-naturally occurring nucleic acid of claim 58, wherein the sequence is modified to replace every non-CCG proline codon with CCG as compared to **SEQ ID NO: 70**.

60. The isolated, non-naturally occurring nucleic acid of any one of claims 3-35, wherein the non-CCG proline codon comprises CCC or CCA.

61. The isolated, non-naturally occurring nucleic acid of claim 60, wherein the non-CCG proline codon is CCC.

62. The isolated, non-naturally occurring nucleic acid of claim 61, wherein the sequence is modified to replace CCC with CCG in in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, or at least 30 codons of the coding region of the sequence.

63. The isolated, non-naturally occurring nucleic acid of claim 62, wherein the sequence is modified to replace every CCC with CCG of the coding region of the sequence.

64. The isolated, non-naturally occurring nucleic acid of claim 61, wherein the sequence is modified to replace CCC with CCG in at least 3, at least 6, at least 9, at least 12, at least 15, at least 18, at least 21, at least 24, at least 27, or at least 30 codon positions as compared to **SEQ ID NO: 70**.

65. The isolated, non-naturally occurring nucleic acid of claim 64, wherein the sequence is modified to replace every CCC with CCG as compared to **SEQ ID NO: 70**.

66. The isolated, non-naturally occurring nucleic acid of any one of claims 1-65, wherein the nucleic acid comprises a viral vector sequence.

67. The isolated, non-naturally occurring nucleic acid of claim 66, wherein the viral vector sequence is a scAAV vector sequence.

68. The isolated, non-naturally occurring nucleic acid of claim 67, wherein the AAV vector sequence is of serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, or any combination thereof.

69. The isolated, non-naturally occurring nucleic acid of claim 68, wherein the AAV vector sequence is of the AAV2 serotype.

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70. The isolated, non-naturally occurring nucleic acid of any one of claims 66-69, wherein the viral vector sequence comprises sequences of at least two AAV serotypes.

71. The isolated, non-naturally occurring nucleic acid of claim 70, wherein the at least two serotypes are selected from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV8, AAV9, AAV11, and AAV12.

72. The isolated, non-naturally occurring nucleic acid of any one of claims 1-71, wherein the isolated, non-naturally occurring nucleic acid comprises at least about 60% sequence identity or similarity with any one of **SEQ ID NOS: 13-19, 21-27, 31, 62, 64, 66,** or **68**.

73. The isolated, non-naturally occurring nucleic acid of claim 72, wherein the sequence identity is from about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100%.

74. The isolated, non-naturally occurring nucleic acid of claim 72, wherein the isolated, non-naturally occurring nucleic acid comprises about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100% sequence identity to **SEQ ID NO: 31**.

75. The isolated, non-naturally occurring nucleic acid of claim 72, wherein the isolated, non-naturally occurring nucleic acid comprises the nucleic acid sequence of **SEQ ID NO: 31**.

76. The isolated, non-naturally occurring nucleic acid of claim 72, wherein the isolated, non-naturally occurring nucleic acid consists of the nucleic acid sequence of **SEQ ID NO: 31**.

77. The isolated, non-naturally occurring nucleic acid of claim 72, wherein the isolated, non-naturally occurring nucleic acid comprises about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100% sequence identity to **SEQ ID NO: 66**.

78. The isolated, non-naturally occurring nucleic acid of claim 72, wherein the isolated, non-naturally occurring nucleic acid comprises the nucleic acid sequence of **SEQ ID NO: 66**.

79. The isolated, non-naturally occurring nucleic acid of claim 72, wherein the isolated, non-naturally occurring nucleic acid consists of the nucleic acid sequence of **SEQ ID NO: 66**.

80. The isolated, non-naturally occurring nucleic acid of any one of claims 66-79, wherein the viral vector sequence is single-stranded.

81. The isolated, non-naturally occurring nucleic acid of any one of claims 66-79, wherein the viral vector sequence is double-stranded.

82. The isolated, non-naturally occurring nucleic acid of any one of claims 1-79, wherein the isolated, non-naturally occurring nucleic acid is single-stranded.

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83. The isolated, non-naturally occurring nucleic acid of any one of claims 1-79, wherein the isolated, non-naturally occurring nucleic acid is double-stranded.

84. The isolated, non-naturally occurring nucleic acid of any one of claims 1-83, wherein the isolated, non-naturally occurring nucleic acid, upon contacting with a plurality of cells, increases expression of the biologic post transfection or post transduction in the plurality of cells as compared to an otherwise comparable isolated, non-naturally occurring nucleic acid that lacks the otherwise comparable sequence lacking the modification in a comparable plurality of cells.

85. The isolated, non-naturally occurring nucleic acid of claim 84, wherein the increased expression comprises at least a 5-fold, at least a 10-fold, at least a 20-fold, at least a 50-fold, at least a 100-fold, at least a 200-fold, or at least a 500-fold increase as determined by enzyme-linked immunoassay (ELISA) assay.

86. An isolated, non-naturally occurring nucleic acid that comprises at least about 60% sequence identity or similarity with any one of the nucleic acid sequences of **SEQ ID NOS: 13-19, 21-27, 31, 62, 64, 66,** or **68**.

87. The isolated, non-naturally occurring nucleic acid of claim 86, wherein the isolated, non-naturally occurring nucleic acid comprises the nucleic acid sequence of **SEQ ID NO: 31**.

88. The isolated, non-naturally occurring nucleic acid of claim 86, wherein the isolated, non-naturally occurring nucleic acid comprises the nucleic acid sequence of **SEQ ID NO: 66**.

89. A biologic encoded by the isolated non-naturally occurring nucleic acid of any one of claims 86-88.

90. The biologic of claim 89, wherein the biologic comprises at least about 60% sequence identity with **SEQ ID NO: 12**.

91. The biologic of claim 90, wherein the sequence identity is from about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100%.

92. An engineered cell comprising the isolated, non-naturally occurring nucleic acid of any one of claims 1 to 88.

93. A plurality of adeno-associated viral (AAV) particles comprising the isolated, non-naturally occurring nucleic acid of any one of claims 1-88.

94. A composition comprising the plurality of AAV particles of claim 93 in unit dosage form.

95. The composition of claim 106, wherein the composition is cryopreserved.

96. A container comprising: (a) the isolated, non-naturally occurring nucleic acid of any one of claims 1-88; (b) the biologic of any one of any one of claims 89-91; (c) the engineered cell of claim 92, or (d) the plurality of AAV particles of claim 93.

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97. A method of modifying cells, the method comprising contacting:

(a) a plurality of cells with the isolated non-naturally occurring nucleic acid of any one of claims 1-88;

(b) a plurality of cells with the plurality of adeno-associated viral (AAV) particles of claim 93; or

(c) both (a) and (b).

98. A pharmaceutical composition comprising: (a) the isolated, non-naturally occurring nucleic acid of any one of claims 1-88; (b) the biologic of any one of claims 89-91; (c) or the plurality of AAV particles of claim 93.

99. The pharmaceutical composition of claim 98, wherein the pharmaceutical composition is for treating an ocular disease or condition.

100. The pharmaceutical composition of claim 98 or 99, wherein the ocular disease or condition is selected from the group consisting of: Achromatopsia, Age-related macular degeneration (AMD), Diabetic retinopathy (DR), Glaucoma, Bardet-Biedl Syndrome, Best Disease, Choroideremia, Leber Congenital Amaurosis, Macular degeneration, Polypoidal choroidal vasculopathy (PCV), Retinitis pigmentosa, Refsum disease, Stargardt disease, Usher syndrome, X-linked retinoschisis (XLRS), Rod-cone dystrophy, Cone-rod dystrophy, Oguchi disease, Malattia Leventinese (Familial Dominant Drusen), and Blue-cone monochromacy.

101. The pharmaceutical composition of claim 100, wherein the ocular disease or condition is AMD.

102. A method of treating a disease or condition in a subject in need thereof, the method comprising administering an effective amount of the pharmaceutical composition of any one of claims 98-101 to the subject, thereby treating the disease.

103. A method for treating a disease or condition in a subject in need thereof, the method comprising administering a therapeutically effective amount of a pharmaceutical composition that comprises an isolated, non-naturally occurring nucleic acid that comprises a sequence that encodes a biologic that comprises an anti-angiogenic agent, wherein the sequence is modified to replace non-AGG arginine codons with AGG in at least four codons of a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region, thereby treating the disease or condition in the subject in need thereof.

104. A method for treating a disease or condition in a subject in need thereof, the method comprising: administering a therapeutically effective amount of a pharmaceutical composition that comprises an isolated, non-naturally occurring nucleic acid that comprises a sequence that encodes a biologic that comprises an anti-angiogenic agent, wherein the sequence is modified to

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replace non-AGG arginine codons with AGG in at least four codons of a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region, and wherein the modification is effective in increasing a level of the biologic in the subject in need thereof as compared to an otherwise comparable subject administered an otherwise comparable isolated, non-naturally occurring nucleic acid lacking the modification.

105. The method of claim 104, wherein the increased level of the biologic in the subject is at least a 5-fold, at least a 10-fold, at least a 20-fold, at least a 50-fold, at least a 100-fold, at least a 200-fold, or at least a 500-fold increased, as determined by a diagnostic assay.

106. The method of any one of claims 102-105, wherein the sequence that encodes the anti-angiogenic agent further comprises a second modification.

107. The method of claim 106, wherein the second modification is in at least one codon of the coding region of the sequence, and wherein the second modification is selected from the group consisting of:

(a) replacement of at least one non-CCC proline codon with CCC;

(b) replacement of at least one non-TCC serine codon with TCC;

(c) replacement of at least one non-CCG proline codon with CCG; and

(d) any combination of (a)-(c).

108. The method of claim 107, wherein the second modification comprises (a).

109. The method of claim 107, wherein the second modification comprises (b).

110. The method of claim 107, wherein the second modification comprises (c).

111. The method of claim 107, wherein the second modification comprises (d).

112. A method for treating a disease or condition in a subject in need thereof, the method comprising administering a therapeutically effective amount of a pharmaceutical composition that comprises an isolated, non-naturally occurring nucleic acid that comprises a sequence that encodes a biologic that comprises an anti-angiogenic agent, wherein the sequence is modified to replace AGA with AGG in at least four codons of a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region, thereby treating the disease or condition in the subject in need thereof.

113. A method for treating a disease or condition in a subject in need thereof, the method comprising: administering a therapeutically effective amount of a pharmaceutical composition that comprises an isolated, non-naturally occurring nucleic acid that comprises a sequence that encodes a biologic that comprises an anti-angiogenic agent, wherein the sequence is modified to replace AGA with AGG in at least four codons of a coding region of the sequence as compared to an otherwise comparable sequence lacking the modification in the coding region, and wherein

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the modification is effective in increasing a level of the biologic in the subject in need thereof as compared to an otherwise comparable subject administered an otherwise comparable isolated, non-naturally occurring nucleic acid lacking the modification.

114. The method of claim 113, wherein the increased level of the biologic in the subject is at least a 5-fold, at least a 10-fold, at least a 20-fold, at least a 50-fold, at least a 100-fold, at least a 200-fold, or at least a 500-fold increased, as determined by a diagnostic assay.

115. The method of any one of claims 112-114, wherein the sequence that encodes the anti-angiogenic agent further comprises a second modification.

116. The method of claim 115, wherein the second modification is in at least one codon of the coding region of the sequence, and wherein the second modification is selected from the group consisting of:

(a) CCT to CCC;

(b) AGC to TCC;

(c) CCC to CCG; and

(d) any combination of (a)-(c).

117. The method of claim 116, wherein the second modification comprises (a).

118. The method of claim 116, wherein the second modification comprises (b).

119. The method of claim 116, wherein the second modification comprises (c).

120. The method of claim 116, wherein the second modification comprises (d).

121. The method of any one of claims 102-120, wherein the anti-angiogenic agent comprises: a VEGF inhibitor, a multi-tyrosine kinase inhibitor, a receptor tyrosine kinase inhibitor, or an inhibitor of Akt phosphorylation.

122. The method of claim 121, wherein the anti-angiogenic agent comprises the VEGF inhibitor.

123. The method of claim 122, wherein the VEGF inhibitor is a non-antibody inhibitor.

124. The method of claim 123, wherein the non-antibody inhibitor comprises a fusion protein that comprises human VEGF receptors 1 and 2, and wherein the fusion protein comprises VEGF-Trap or a modified version thereof.

125. The method of any one of claims 102-124, wherein the isolated, non-naturally occurring nucleic acid comprises at least about 60% sequence identity or similarity with any one **SEQ ID NOS: 13-19, 21-27, 31, 62, 64, 66,** or **68**.

126. The method of claim 125, wherein the sequence identity is from about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100%.

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127. The method of claim 125 wherein the isolated, non-naturally occurring nucleic acid comprises about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100% sequence identity to **SEQ ID NO: 31**.

128. The method of claim 125, wherein the isolated, non-naturally occurring nucleic acid comprises the nucleic acid sequence of **SEQ ID NO: 31**.

129. The method of claim 125, wherein the isolated, non-naturally occurring nucleic acid consists of the nucleic acid sequence of **SEQ ID NO: 31**.

130. The method of claim 125, wherein the isolated, non-naturally occurring nucleic acid comprises about 70%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, and up to about 100% sequence identity to **SEQ ID NO: 66**.

131. The method of claim 125, wherein the isolated, non-naturally occurring nucleic acid comprises the nucleic acid sequence of **SEQ ID NO: 66**.

132. The method of claim 125, wherein the isolated, non-naturally occurring nucleic acid consists of the nucleic acid sequence of **SEQ ID NO: 66**.

133. The method of any one of claims 102-132, wherein the nucleic acid comprises a viral vector sequence.

134. The method of claim 133, wherein the viral vector sequence is a scAAV vector sequence.

135. The method of claim 134, wherein the AAV vector sequence is of serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, or any combination thereof.

136. The method of claim 135, wherein the AAV vector sequence is of the AAV2 serotype.

137. The method of any one of claims 102-136, wherein the administering is via intravitreal injection, subretinal injection, microinjection, or supraocular injection.

138. The method of claim 137, wherein the administering is via intravitreal injection.

139. The method of any one of claims 102-138, wherein the ocular disease or condition is selected from the group consisting of: Achromatopsia, Age-related macular degeneration (AMD), Diabetic retinopathy (DR), Glaucoma, Bardet-Biedl Syndrome, Best Disease, Choroideremia, Leber Congenital Amaurosis, Macular degeneration, Polypoidal choroidal vasculopathy (PCV), Retinitis pigmentosa, Refsum disease, Stargardt disease, Usher syndrome, X-linked retinoschisis (XLRS), Rod-cone dystrophy, Cone-rod dystrophy, Oguchi disease, Malattia Leventinese (Familial Dominant Drusen), and Blue-cone monochromacy.

140. The method of claim 139, wherein the ocular disease or condition is AMD.

141. The method of claim 140, wherein the AMD is wet AMD.

142. The method of claim 140, wherein the AMD is dry AMD.

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143. The method of any one of claims 102-142, wherein the administering is sufficient to reduce at least a symptom of the disease or condition, treat the disease or condition, and/or eliminate the disease or condition.

144. The method of any one of claims 102-143, wherein the administering comprises delivering a dosage of the isolated, non-naturally occurring nucleic acid of about 1.0×10^9 vg, about 1.0×10^{10}, about 1.0×10^{11} vg, about 3.0×10^{11} vg, about 6×10^{11} vg, about 8.0×10^{11} vg, about 1.0×10^{12} vg, about 1.0×10^{13} vg, about 1.0×10^{14} vg, or about 1.0×10^{15} vg.

145. The method of any one of claims 102-144, wherein the administering is repeated.

146. The method of claim 145, wherein the administering is performed: twice daily, every other day, twice a week, bimonthly, trimonthly, once a month, every other month, semiannually, annually, or biannually.

147. The method of any one of claims 102-146, wherein prior to the administering the subject undergoes genetic testing.

148. The method of claim 147, wherein the genetic testing detects a mutation in a gene sequence as compared to an otherwise comparable wild type sequence.

149. The method of any one of claims 102-148, wherein the method further comprises administering a secondary therapy.

150. The method of claim 149, wherein the secondary therapy comprises at least one of: photodynamic therapy (PDT), an anti-inflammatory agent, an anti-microbial agent, and laser photocoagulation therapy (LPT).

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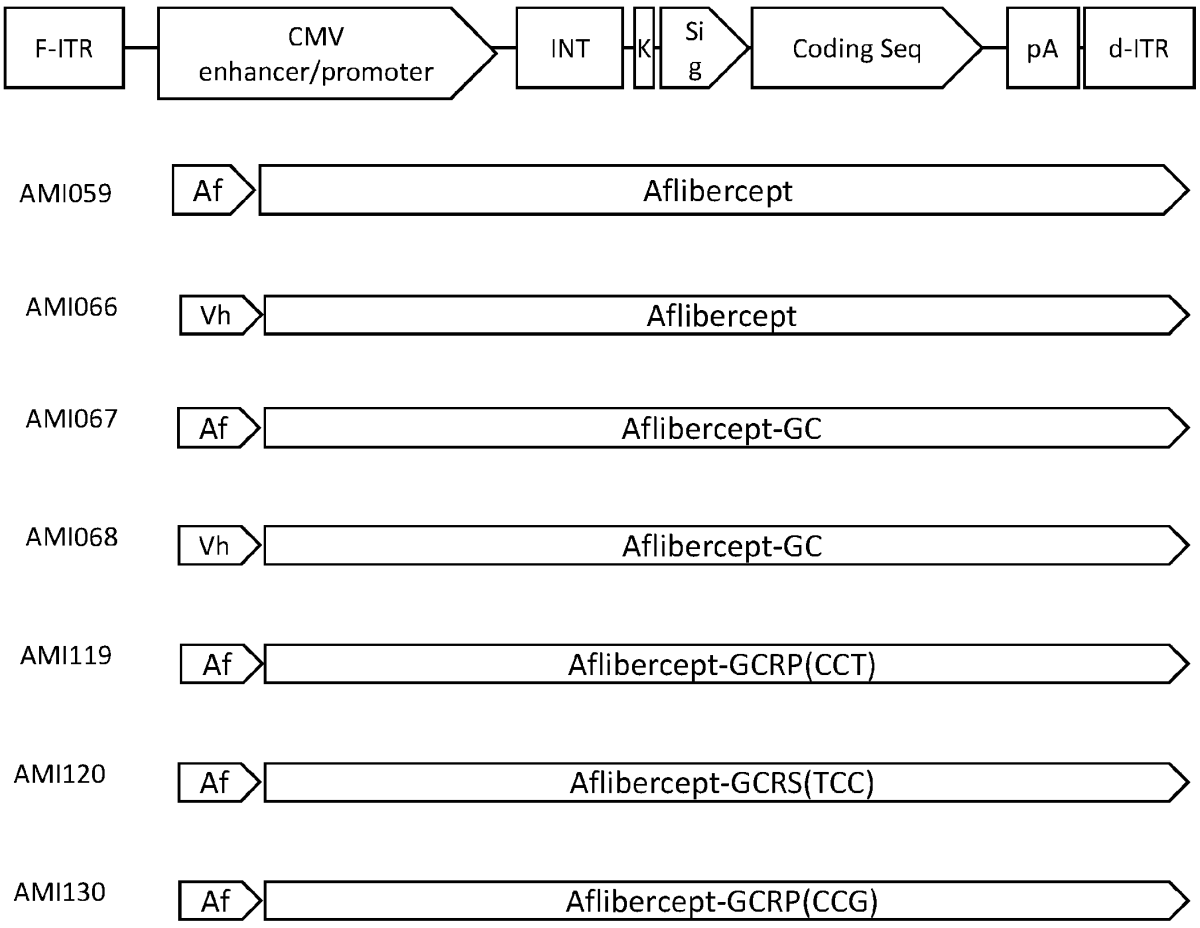

F-ITR
CMV enhancer/promoter
INT
K
Si g
Coding Seq
pA
d-ITR
AMI059
Af
Aflibercept
AMI066
Vh
Aflibercept
AMI067
Af
Aflibercept-GC
AMI068
Vh
Aflibercept-GC
AMI119
Af
Aflibercept-GCRP(CCT)
AMI120
Af
Aflibercept-GCRS(TCC)
AMI130
Af
Aflibercept-GCRP(CCG)

Fig. 1

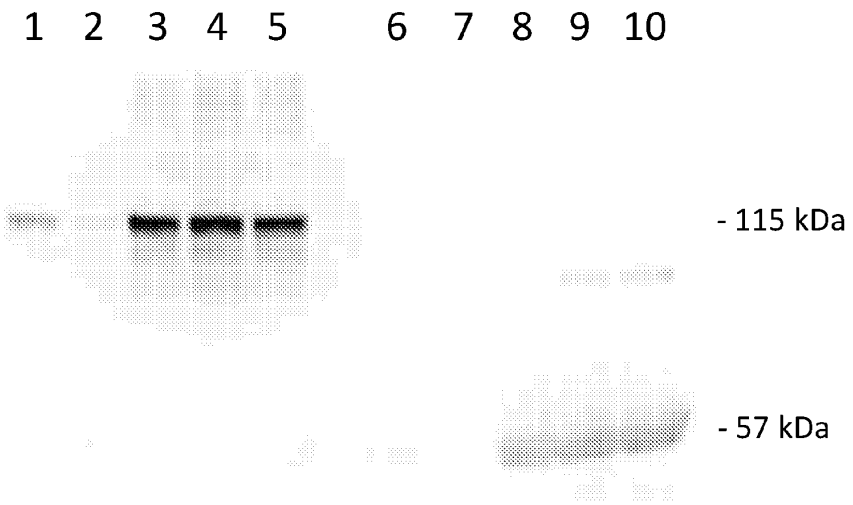


Fig. 2

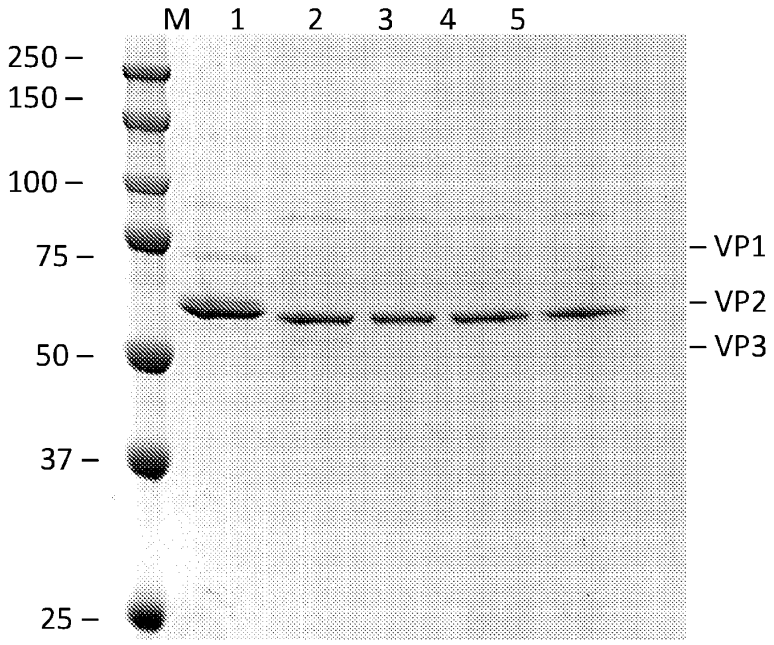

M 1 2 3 4 5
250 –
150 –
100 –
75 –
50 –
37 –
25 –
– VP1
– VP2
– VP3

Fig. 3A

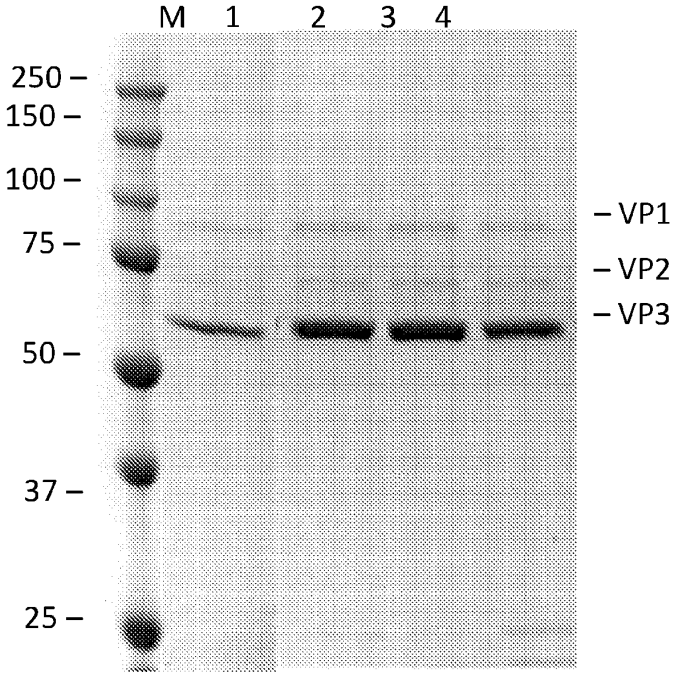

M 1 2 3 4
250 –
150 –
100 –
75 –
50 –
37 –
25 –
– VP1
– VP2
– VP3

Fig. 3B

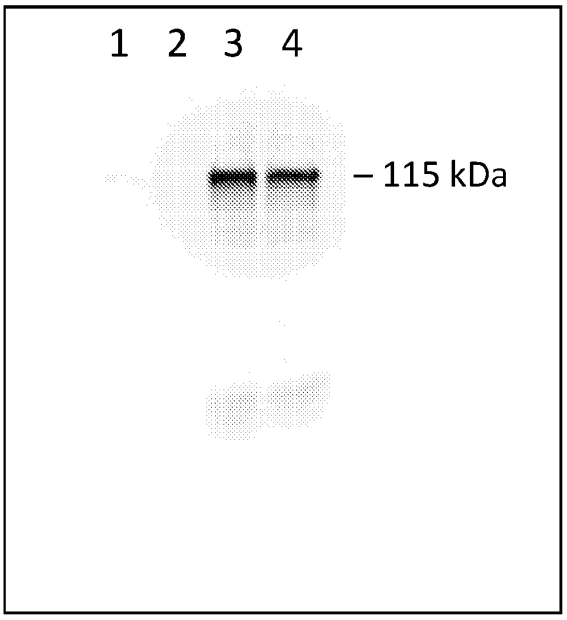


Fig. 4A

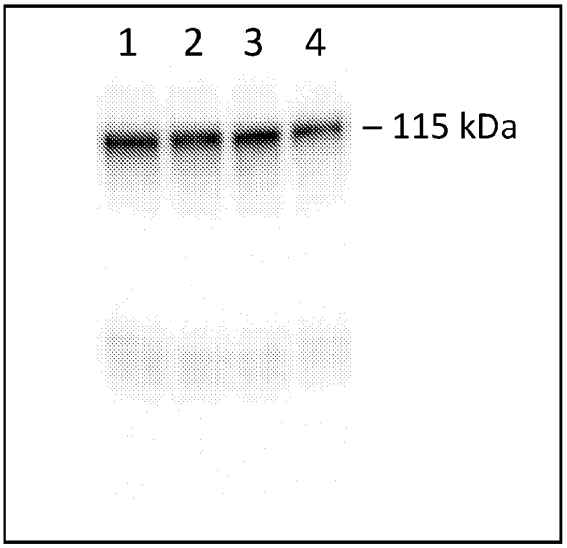


Fig. 4B

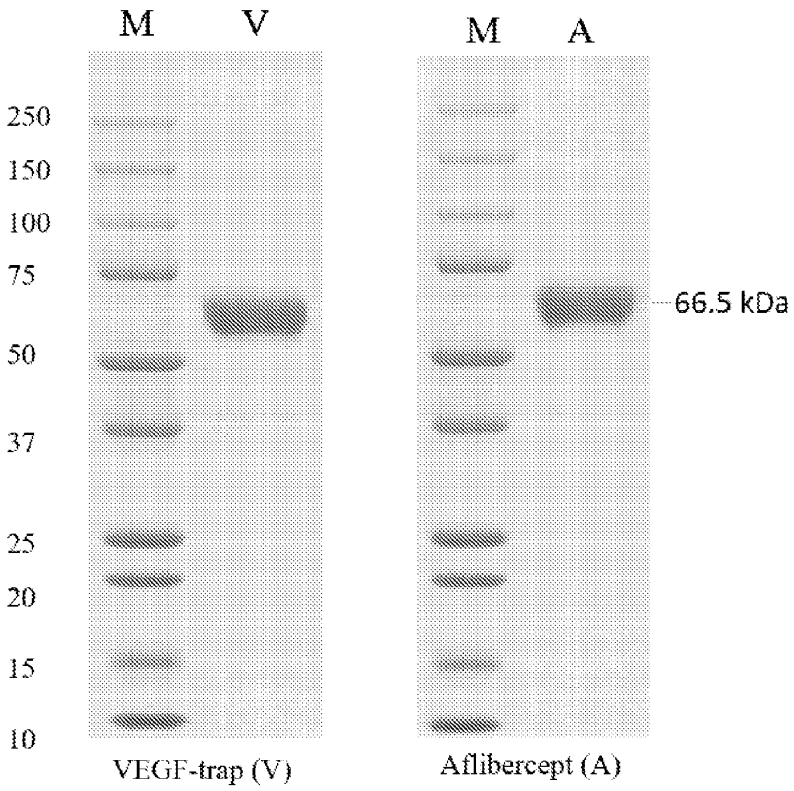

M
V
M
A
250
150
100
75
50
37
25
20
15
10
66.5 kDa
VEGF-trap (V)
Aflibercept (A)

Fig. 5A

Fig. 5B

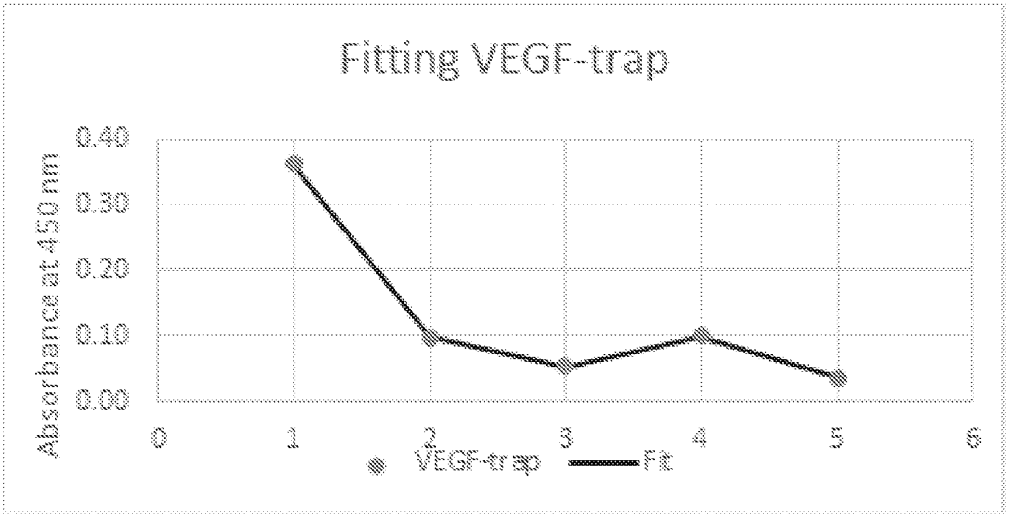
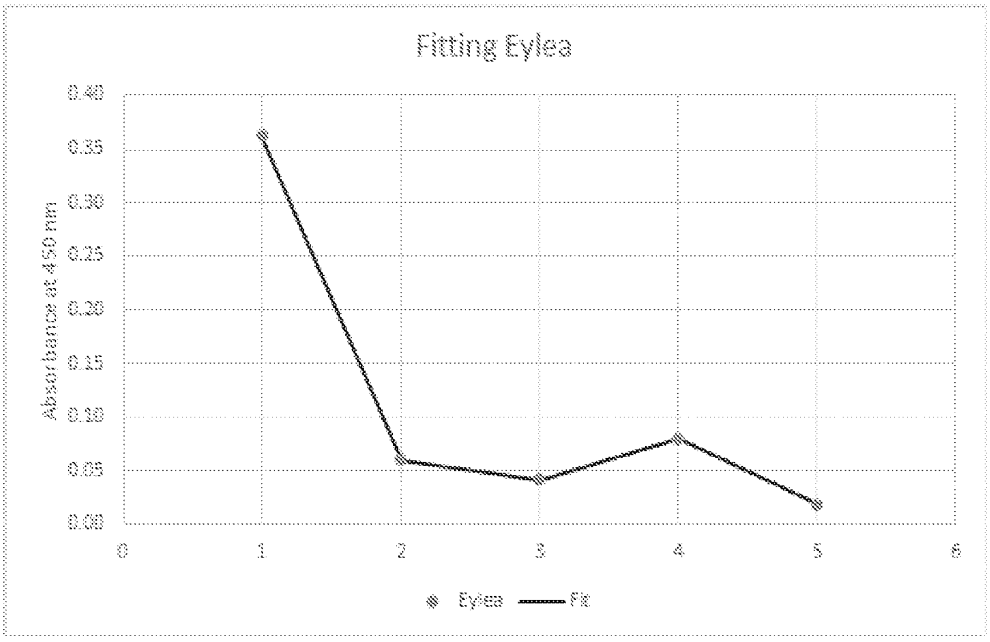

Fitting VEGF-trap
Absorbance at 450 nm
0.40
0.30
0.20
0.10
0.00
0
1
2
3
4
5
6
VEGF-trap
Fit

Fig. 5C


Fitting Eylea
Absorbance at 450 nm
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
1
2
3
4
5
6
Eylea
Fit

AMI068-pFB-scCMV-SV40-intron-kozak-Vh-Aflibercept-GC

```
   1    E  F  G  L  S  W  L  F  L  V  A  I  L  K  G  V  Q  C  D
   1 ATGGAGTTCGGCCTGAGCTGGCTGTTCCTGGTGGCCATCCTTAAGGGCGTGCAGTGCGAC
  20 T  G  R  P  F  V  E  M  Y  S  E  I  P  E  I  I  H  M  T  E
  61 ACCGGCAGACCCTTCGTGGAGATGTACAGCGAGATCCCCGAGATCATCCACATGACCGAG
  40 G  R  E  L  V  I  P  C  R  V  T  S  P  N  I  T  V  T  L  K
 121 GGCAGAGAGCTGGTGATCCCCTGCAGAGTGACCAGCCCCAACATCACCGTGACCCTGAAG
  60 K  F  P  L  D  T  L  I  P  D  G  K  R  I  I  W  D  S  R  K
 181 AAGTTCCCCCTGGACACCCTGATCCCCGACGGCAAGAGAATCATCTGGGACAGCAGAAAG
  80 G  F  I  I  S  N  A  T  Y  K  E  I  G  L  L  T  C  E  A  T
 241 GGCTTCATCATCAGCAACGCCACCTACAAGGAGATCGGCCTGCTGACCTGCGAGGCCACC
 100 V  N  G  H  L  Y  K  T  N  Y  L  T  H  R  Q  T  N  T  I  I
 301 GTGAACGGCCACCTGTACAAGACCAACTACCTGACCCACAGACAGACCAACACCATCATC
 120 D  V  V  L  S  P  S  H  G  I  E  L  S  V  G  E  K  L  V  L
 361 GACGTGGTGCTGAGCCCCAGCCACGGCATCGAGCTGAGCGTGGGCGAGAAGCTGGTGCTG
 140 N  C  T  A  R  T  E  L  N  V  G  I  D  F  N  W  E  Y  P  S
 421 AACTGCACCGCCAGAACCGAGCTGAACGTGGGCATCGACTTCAACTGGGAGTACCCCAGC
 160 S  K  H  Q  H  K  K  L  V  N  R  D  L  K  T  Q  S  G  S  E
 481 AGCAAGCACCAGCACAAGAAGCTGGTGAACAGAGACCTGAAGACCCAGAGCGGCAGCGAG
 180 M  K  K  F  L  S  T  L  T  I  D  G  V  T  R  S  D  Q  G  L
 541 ATGAAGAAGTTCCTGAGCACCCTGACCATCGACGGCGTGACCAGAAGCGACCAGGGCCTG
 200 Y  T  C  A  A  S  S  G  L  M  T  K  K  N  S  T  F  V  R  V
 601 TACACCTGCGCCGCCAGCAGCGGCCTGATGACCAAGAAGAACAGCACCTTCGTGAGAGTG
 220 H  E  K  D  K  T  H  T  C  P  P  C  P  A  P  E  L  L  G  G
 661 CACGAGAAGGACAAGACCCACACCTGCCCCCCCTGCCCCGCCCCCGAGCTGCTGGGCGGC
 240 P  S  V  F  L  F  P  P  K  P  K  D  T  L  M  I  S  R  T  P
 721 CCCAGCGTGTTCCTGTTCCCCCCCAAGCCCAAGGACACCCTGATGATCAGCAGAACCCCC
 260 E  V  T  C  V  V  V  D  V  S  H  E  D  P  E  V  K  F  N  W
 781 GAGGTGACCTGCGTGGTGGTGGACGTGAGCCACGAGGACCCCGAGGTGAAGTTCAACTGG
 280 Y  V  D  G  V  E  V  H  N  A  K  T  K  P  R  E  E  Q  Y  N
 841 TACGTGGACGGCGTGGAGGTGCACAACGCCAAGACCAAGCCCAGAGAGGAGCAGTACAAC
 300 S  T  Y  R  V  V  S  V  L  T  V  L  H  Q  D  W  L  N  G  K
 901 AGCACCTACAGAGTGGTGAGCGTGCTGACCGTGCTGCACCAGGACTGGCTGAACGGCAAG
 320 E  Y  K  C  K  V  S  N  K  A  L  P  A  P  I  E  K  T  I  S
 961 GAGTACAAGTGCAAGGTGAGCAACAAGGCCCTGCCCGCCCCCATCGAGAAGACCATCAGC
 340 K  A  K  G  Q  P  R  E  P  Q  V  Y  T  L  P  P  S  R  D  E
1021 AAGGCCAAGGGCCAGCCCAGAGAGCCCCAGGTGTACACCCTGCCCCCCAGCAGAGACGAG
 360 L  T  K  N  Q  V  S  L  T  C  L  V  K  G  F  Y  P  S  D  I
1081 CTGACCAAGAACCAGGTGAGCCTGACCTGCCTGGTGAAGGGCTTCTACCCCAGCGACATC
 380 A  V  E  W  E  S  N  G  Q  P  E  N  N  Y  K  T  T  P  P  V
1141 GCCGTGGAGTGGGAGAGCAACGGCCAGCCCGAGAACAACTACAAGACCACCCCCCCCGTG
 400 L  D  S  D  G  S  F  F  L  Y  S  K  L  T  V  D  K  S  R  W
1201 CTGGACAGCGACGGCAGCTTCTTCCTGTACAGCAAGCTGACCGTGGACAAGAGCAGATGG
 420 Q  Q  G  N  V  F  S  C  S  V  M  H  E  A  L  H  N  H  Y  T
1261 CAGCAGGGCAACGTGTTCAGCTGCAGCGTGATGCACGAGGCCCTGCACAACCACTACACC
 440 Q  K  S  L  S  L  S  P  G  K  *
1321 CAGAAGAGCCTGAGCCTGAGCCCCGGCAAGTGA
```

Fig. 6

AMI119-pFB-scCMV-SV40-intron-kozak-Af-Aflibercept-GCRP (CCT)

```
   1 M  V  S  Y  W  D  T  G  V  L  L  C  A  L  L  S  C  L  L  L
   1 ATGGTGAGCTACTGGGACACCGGCGTGCTGCTGTGCGCCCTGCTGAGCTGCCTGCTGCTG
  21 T  G  S  S  S  G  S  D  T  G  R  P  F  V  E  M  Y  S  E  I
  61 ACCGGCAGCAGCAGCGGCAGCGACACCGGCAGGCCTTTCGTGGAGATGTACAGCGAGATC
  41 P  E  I  I  H  M  T  E  G  R  E  L  V  I  P  C  R  V  T  S
 121 CCTGAGATCATCCACATGACCGAGGGCAGGGAGCTGGTGATCCCTTGCAGGGTGACCAGC
  61 P  N  I  T  V  T  L  K  K  F  P  L  D  T  L  I  P  D  G  K
 181 CCTAACATCACCGTGACCCTGAAGAAGTTCCCTCTGGACACCCTGATCCCTGACGGCAAG
  81 R  I  I  W  D  S  R  K  G  F  I  I  S  N  A  T  Y  K  E  I
 241 AGGATCATCTGGGACAGCAGGAAGGGCTTCATCATCAGCAACGCCACCTACAAGGAGATC
 101 G  L  L  T  C  E  A  T  V  N  G  H  L  Y  K  T  N  Y  L  T
 301 GGCCTGCTGACCTGCGAGGCCACCGTGAACGGCCACCTGTACAAGACCAACTACCTGACC
 121 H  R  Q  T  N  T  I  I  D  V  V  L  S  P  S  H  G  I  E  L
 361 CACAGGCAGACCAACACCATCATCGACGTGGTGCTGAGCCCTAGCCACGGCATCGAGCTG
 141 S  V  G  E  K  L  V  L  N  C  T  A  R  T  E  L  N  V  G  I
 421 AGCGTGGGCGAGAAGCTGGTGCTGAACTGCACCGCCAGGACCGAGCTGAACGTGGGCATC
 161 D  F  N  W  E  Y  P  S  S  K  H  Q  H  K  K  L  V  N  R  D
 481 GACTTCAACTGGGAGTACCCTAGCAGCAAGCACCAGCACAAGAAGCTGGTGAACAGGGAC
 181 L  K  T  Q  S  G  S  E  M  K  K  F  L  S  T  L  T  I  D  G
 541 CTGAAGACCCAGAGCGGCAGCGAGATGAAGAAGTTCCTGAGCACCCTGACCATCGACGGC
 201 V  T  R  S  D  Q  G  L  Y  T  C  A  A  S  S  G  L  M  T  K
 601 GTGACCAGGAGCGACCAGGGCCTGTACACCTGCGCCGCCAGCAGCGGCCTGATGACCAAG
 221 K  N  S  T  F  V  R  V  H  E  K  D  K  T  H  T  C  P  P  C
 661 AAGAACAGCACCTTCGTGAGGGTGCACGAGAAGGACAAGACCCACACCTGCCCTCCTTGC
 241 P  A  P  E  L  L  G  G  P  S  V  F  L  F  P  P  K  P  K  D
 721 CCTGCCCCTGAGCTGCTGGGCGGCCCTAGCGTGTTCCTGTTCCCTCCTAAGCCTAAGGAC
 261 T  L  M  I  S  R  T  P  E  V  T  C  V  V  V  D  V  S  H  E
 781 ACCCTGATGATCAGCAGGACCCCTGAGGTGACCTGCGTGGTGGTGGACGTGAGCCACGAG
 281 D  P  E  V  K  F  N  W  Y  V  D  G  V  E  V  H  N  A  K  T
 841 GACCCTGAGGTGAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCACAACGCCAAGACC
 301 K  P  R  E  E  Q  Y  N  S  T  Y  R  V  V  S  V  L  T  V  L
 901 AAGCCTAGGGAGGAGCAGTACAACAGCACCTACAGGGTGGTGAGCGTGCTGACCGTGCTG
 321 H  Q  D  W  L  N  G  K  E  Y  K  C  K  V  S  N  K  A  L  P
 961 CACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTGAGCAACAAGGCCCTGCCT
 341 A  P  I  E  K  T  I  S  K  A  K  G  Q  P  R  E  P  Q  V  Y
1021 GCCCCTATCGAGAAGACCATCAGCAAGGCCAAGGGCCAGCCTAGGGAGCCTCAGGTGTAC
 361 T  L  P  P  S  R  D  E  L  T  K  N  Q  V  S  L  T  C  L  V
1081 ACCCTGCCTCCTAGCAGGGACGAGCTGACCAAGAACCAGGTGAGCCTGACCTGCCTGGTG
 381 K  G  F  Y  P  S  D  I  A  V  E  W  E  S  N  G  Q  P  E  N
1141 AAGGGCTTCTACCCTAGCGACATCGCCGTGGAGTGGGAGAGCAACGGCCAGCCTGAGAAC
 401 N  Y  K  T  T  P  P  V  L  D  S  D  G  S  F  F  L  Y  S  K
1201 AACTACAAGACCACCCCTCCTGTGCTGGACAGCGACGGCAGCTTCTTCCTGTACAGCAAG
 421 L  T  V  D  K  S  R  W  Q  Q  G  N  V  F  S  C  S  V  M  H
1261 CTGACCGTGGACAAGAGCAGGTGGCAGCAGGGCAACGTGTTCAGCTGCAGCGTGATGCAC
 441 E  A  L  H  N  H  Y  T  Q  K  S  L  S  L  S  P  G  K  *
1321 GAGGCCCTGCACAACCACTACACCCAGAAGAGCCTGAGCCTGAGCCCTGGCAAGTGA
```

Fig. 7

AMI120-pFB-scCMV-SV40-intron-kozak-Af-Aflibercept-GCRS (TCC), version 2

```
   1 M  V  S  Y  W  D  T  G  V  L  L  C  A  L  L  S  C  L  L  L
   1 ATGGTGAGCTACTGGGACACCGGCGTGCTGCTGTGCGCCCTGCTGAGCTGCCTGCTGCTG
  21 T  G  S  S  S  G  S  D  T  G  R  P  F  V  E  M  Y  S  E  I
  61 ACCGGCAGCAGCAGCGGCAGCGACACCGGCAGGCCCTTCGTGGAGATGTACTCCGAGATC
  41 P  E  I  I  H  M  T  E  G  R  E  L  V  I  P  C  R  V  T  S
 121 CCCGAGATCATCCACATGACCGAGGGCAGGGAGCTGGTGATCCCCTGCAGGGTGACCTCC
  61 P  N  I  T  V  T  L  K  K  F  P  L  D  T  L  I  P  D  G  K
 181 CCCAACATCACCGTGACCCTGAAGAAGTTCCCCCTGGACACCCTGATCCCCGACGGCAAG
  81 R  I  I  W  D  S  R  K  G  F  I  I  S  N  A  T  Y  K  E  I
 241 AGGATCATCTGGGACTCCAGGAAGGGCTTCATCATCTCCAACGCCACCTACAAGGAGATC
 101 G  L  L  T  C  E  A  T  V  N  G  H  L  Y  K  T  N  Y  L  T
 301 GGCCTGCTGACCTGCGAGGCCACCGTGAACGGCCACCTGTACAAGACCAACTACCTGACC
 121 H  R  Q  T  N  T  I  I  D  V  V  L  S  P  S  H  G  I  E  L
 361 CACAGGCAGACCAACACCATCATCGACGTGGTGCTGTCCCCCTCCCACGGCATCGAGCTG
 141 S  V  G  E  K  L  V  L  N  C  T  A  R  T  E  L  N  V  G  I
 421 TCCGTGGGCGAGAAGCTGGTGCTGAACTGCACCGCCAGGACCGAGCTGAACGTGGGCATC
 161 D  F  N  W  E  Y  P  S  S  K  H  Q  H  K  K  L  V  N  R  D
 481 GACTTCAACTGGGAGTACCCCTCCTCCAAGCACCAGCACAAGAAGCTGGTGAACAGGGAC
 181 L  K  T  Q  S  G  S  E  M  K  K  F  L  S  T  L  T  I  D  G
 541 CTGAAGACCCAGTCCGGCTCCGAGATGAAGAAGTTCCTGTCCACCCTGACCATCGACGGC
 201 V  T  R  S  D  Q  G  L  Y  T  C  A  A  S  S  G  L  M  T  K
 601 GTGACCAGGTCCGACCAGGGCCTGTACACCTGCGCCGCCTCCTCCGGCCTGATGACCAAG
 221 K  N  S  T  F  V  R  V  H  E  K  D  K  T  H  T  C  P  P  C
 661 AAGAACTCCACCTTCGTGAGGGTGCACGAGAAGGACAAGACCCACACCTGCCCCCCCTGC
 241 P  A  P  E  L  L  G  G  P  S  V  F  L  F  P  P  K  P  K  D
 721 CCCGCCCCCGAGCTGCTGGGCGGCCCCTCCGTGTTCCTGTTCCCCCCCAAGCCCAAGGAC
 261 T  L  M  I  S  R  T  P  E  V  T  C  V  V  V  D  V  S  H  E
 781 ACCCTGATGATCTCCAGGACCCCCGAGGTGACCTGCGTGGTGGTGGACGTGTCCCACGAG
 281 D  P  E  V  K  F  N  W  Y  V  D  G  V  E  V  H  N  A  K  T
 841 GACCCCGAGGTGAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCACAACGCCAAGACC
 301 K  P  R  E  E  Q  Y  N  S  T  Y  R  V  V  S  V  L  T  V  L
 901 AAGCCCAGGGAGGAGCAGTACAACTCCACCTACAGGGTGGTGTCCGTGCTGACCGTGCTG
 321 H  Q  D  W  L  N  G  K  E  Y  K  C  K  V  S  N  K  A  L  P
 961 CACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTGTCCAACAAGGCCCTGCCC
 341 A  P  I  E  K  T  I  S  K  A  K  G  Q  P  R  E  P  Q  V  Y
1021 GCCCCCATCGAGAAGACCATCTCCAAGGCCAAGGGCCAGCCCAGGGAGCCCCAGGTGTAC
 361 T  L  P  P  S  R  D  E  L  T  K  N  Q  V  S  L  T  C  L  V
1081 ACCCTGCCCCCCTCCAGGGACGAGCTGACCAAGAACCAGGTGTCCCTGACCTGCCTGGTG
 381 K  G  F  Y  P  S  D  I  A  V  E  W  E  S  N  G  Q  P  E  N
1141 AAGGGCTTCTACCCCTCCGACATCGCCGTGGAGTGGGAGTCCAACGGCCAGCCCGAGAAC
 401 N  Y  K  T  T  P  P  V  L  D  S  D  G  S  F  F  L  Y  S  K
1201 AACTACAAGACCACCCCCCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTGTACTCCAAG
 421 L  T  V  D  K  S  R  W  Q  Q  G  N  V  F  S  C  S  V  M  H
1261 CTGACCGTGGACAAGTCCAGGTGGCAGCAGGGCAACGTGTTCTCCTGCTCCGTGATGCAC
 441 E  A  L  H  N  H  Y  T  Q  K  S  L  S  L  S  P  G  K  *
1321 GAGGCCCTGCACAACCACTACACCCAGAAGTCCCTGTCCCTGTCCCCCGGCAAGTGA
```

Fig. 8

AMI130-pFB-scCMV-SV40-intron-kozak-Af-Aflibercept-GCRP (CCG)

```
   1 M  V  S  Y  W  D  T  G  V  L  L  C  A  L  L  S  C  L  L  L
   1 ATGGTGAGCTACTGGGACACCGGCGTGCTGCTGTGCGCCCTGCTGAGCTGCCTGCTGCTG
  21 T  G  S  S  S  G  S  D  T  G  R  P  F  V  E  M  Y  S  E  I
  61 ACCGGCAGCAGCAGCGGCAGCGACACCGGCAGGCCGTTCGTGGAGATGTACAGCGAGATC
  41 P  E  I  I  H  M  T  E  G  R  E  L  V  I  P  C  R  V  T  S
 121 CCGGAGATCATCCACATGACCGAGGGCAGGGAGCTGGTGATCCCGTGCAGGGTGACCAGC
  61 P  N  I  T  V  T  L  K  K  F  P  L  D  T  L  I  P  D  G  K
 181 CCGAACATCACCGTGACCCTGAAGAAGTTCCCGCTGGACACCCTGATCCCGGACGGCAAG
  81 R  I  I  W  D  S  R  K  G  F  I  I  S  N  A  T  Y  K  E  I
 241 AGGATCATCTGGGACAGCAGGAAGGGCTTCATCATCAGCAACGCCACCTACAAGGAGATC
 101 G  L  L  T  C  E  A  T  V  N  G  H  L  Y  K  T  N  Y  L  T
 301 GGCCTGCTGACCTGCGAGGCCACCGTGAACGGCCACCTGTACAAGACCAACTACCTGACC
 121 H  R  Q  T  N  T  I  I  D  V  V  L  S  P  S  H  G  I  E  L
 361 CACAGGCAGACCAACACCATCATCGACGTGGTGCTGAGCCCGAGCCACGGCATCGAGCTG
 141 S  V  G  E  K  L  V  L  N  C  T  A  R  T  E  L  N  V  G  I
 421 AGCGTGGGCGAGAAGCTGGTGCTGAACTGCACCGCCAGGACCGAGCTGAACGTGGGCATC
 161 D  F  N  W  E  Y  P  S  S  K  H  Q  H  K  K  L  V  N  R  D
 481 GACTTCAACTGGGAGTACCCGAGCAGCAAGCACCAGCACAAGAAGCTGGTGAACAGGGAC
 181 L  K  T  Q  S  G  S  E  M  K  K  F  L  S  T  L  T  I  D  G
 541 CTGAAGACCCAGAGCGGCAGCGAGATGAAGAAGTTCCTGAGCACCCTGACCATCGACGGC
 201 V  T  R  S  D  Q  G  L  Y  T  C  A  A  S  S  G  L  M  T  K
 601 GTGACCAGGAGCGACCAGGGCCTGTACACCTGCGCCGCCAGCAGCGGCCTGATGACCAAG
 221 K  N  S  T  F  V  R  V  H  E  K  D  K  T  H  T  C  P  P  C
 661 AAGAACAGCACCTTCGTGAGGGTGCACGAGAAGGACAAGACCCACACCTGCCCGCCGTGC
 241 P  A  P  E  L  L  G  G  P  S  V  F  L  F  P  P  K  P  K  D
 721 CCGGCCCCGGAGCTGCTGGGCGGCCCGAGCGTGTTCCTGTTCCCGCCGAAGCCGAAGGAC
 261 T  L  M  I  S  R  T  P  E  V  T  C  V  V  V  D  V  S  H  E
 781 ACCCTGATGATCAGCAGGACCCCGGAGGTGACCTGCGTGGTGGTGGACGTGAGCCACGAG
 281 D  P  E  V  K  F  N  W  Y  V  D  G  V  E  V  H  N  A  K  T
 841 GACCCGGAGGTGAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCACAACGCCAAGACC
 301 K  P  R  E  E  Q  Y  N  S  T  Y  R  V  V  S  V  L  T  V  L
 901 AAGCCGAGGGAGGAGCAGTACAACAGCACCTACAGGGTGGTGAGCGTGCTGACCGTGCTG
 321 H  Q  D  W  L  N  G  K  E  Y  K  C  K  V  S  N  K  A  L  P
 961 CACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTGAGCAACAAGGCCCTGCCG
 341 A  P  I  E  K  T  I  S  K  A  K  G  Q  P  R  E  P  Q  V  Y
1021 GCCCCGATCGAGAAGACCATCAGCAAGGCCAAGGGCCAGCCGAGGGAGCCGCAGGTGTAC
 361 T  L  P  P  S  R  D  E  L  T  K  N  Q  V  S  L  T  C  L  V
1081 ACCCTGCCGCCGAGCAGGGACGAGCTGACCAAGAACCAGGTGAGCCTGACCTGCCTGGTG
 381 K  G  F  Y  P  S  D  I  A  V  E  W  E  S  N  G  Q  P  E  N
1141 AAGGGCTTCTACCCGAGCGACATCGCCGTGGAGTGGGAGAGCAACGGCCAGCCGGAGAAC
 401 N  Y  K  T  T  P  P  V  L  D  S  D  G  S  F  F  L  Y  S  K
1201 AACTACAAGACCACCCCGCCGGTGCTGGACAGCGACGGCAGCTTCTTCCTGTACAGCAAG
 421 L  T  V  D  K  S  R  W  Q  Q  G  N  V  F  S  C  S  V  M  H
1261 CTGACCGTGGACAAGAGCAGGTGGCAGCAGGGCAACGTGTTCAGCTGCAGCGTGATGCAC
 441 E  A  L  H  N  H  Y  T  Q  K  S  L  S  L  S  P  G  K  *
1321 GAGGCCCTGCACAACCACTACACCCAGAAGAGCCTGAGCCTGAGCCCCGGCAAGTGA
```

Fig. 9

Fig. 10A

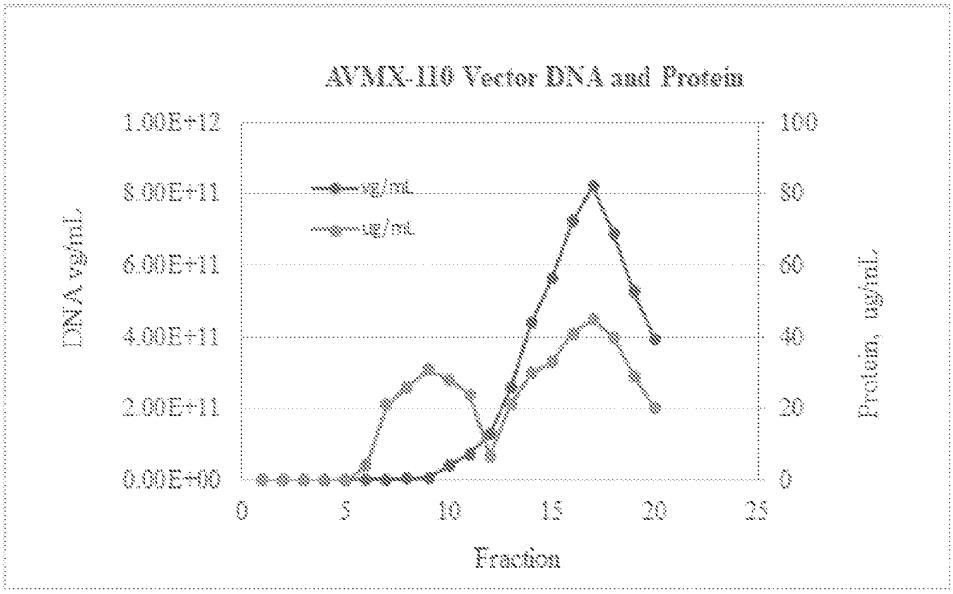
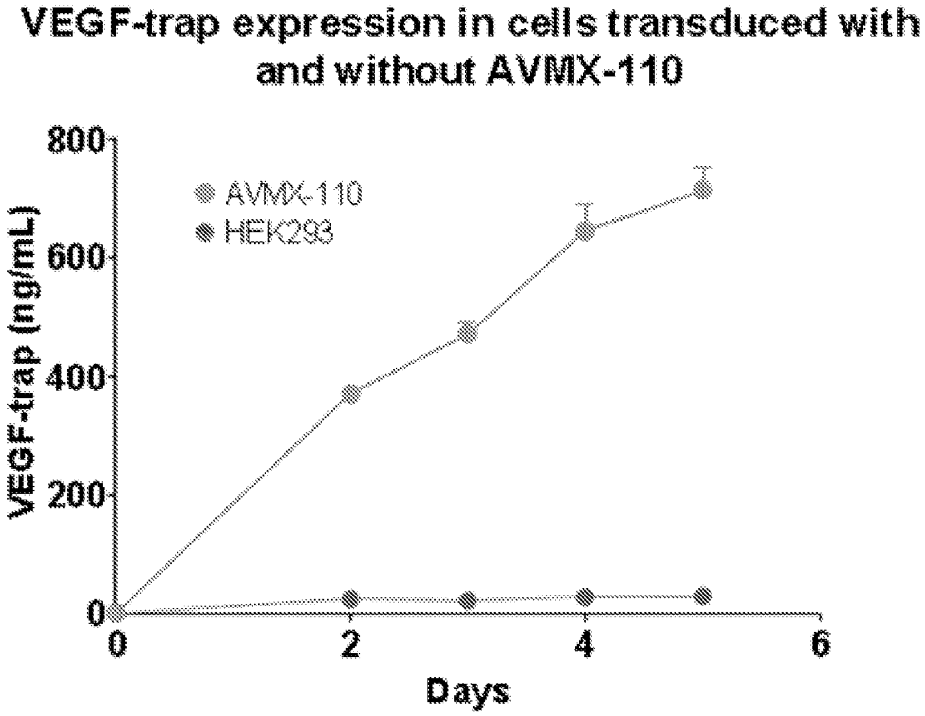
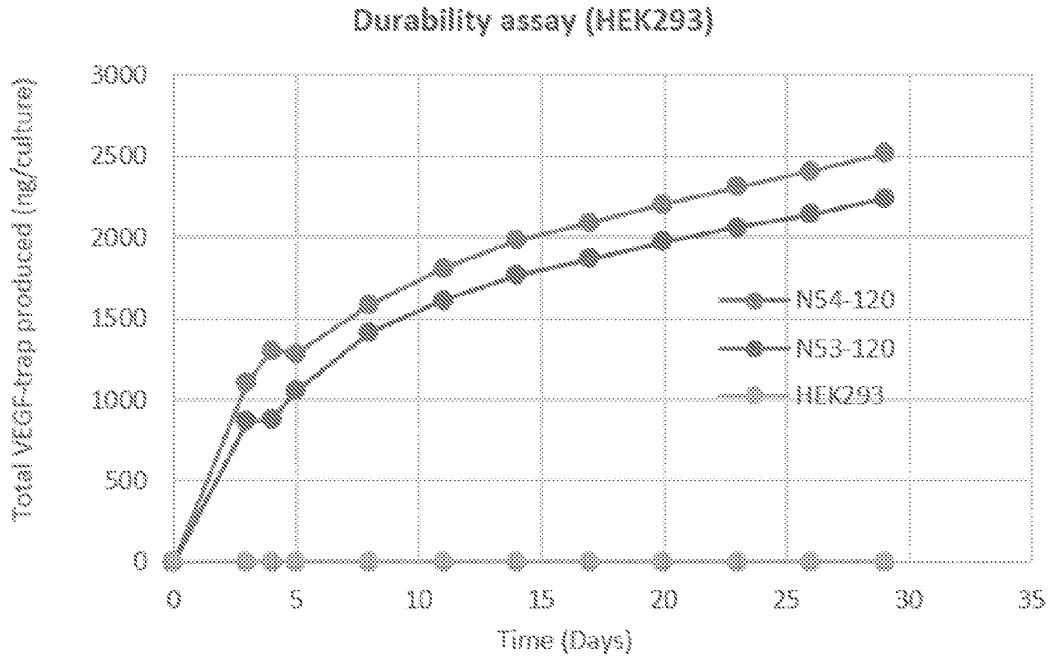


Fig. 10B




Durability assay (HEK293)
Total VEGF-trap produced (ng/culture)
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
35
Time (Days)
N54-120
N53-120
HEK293

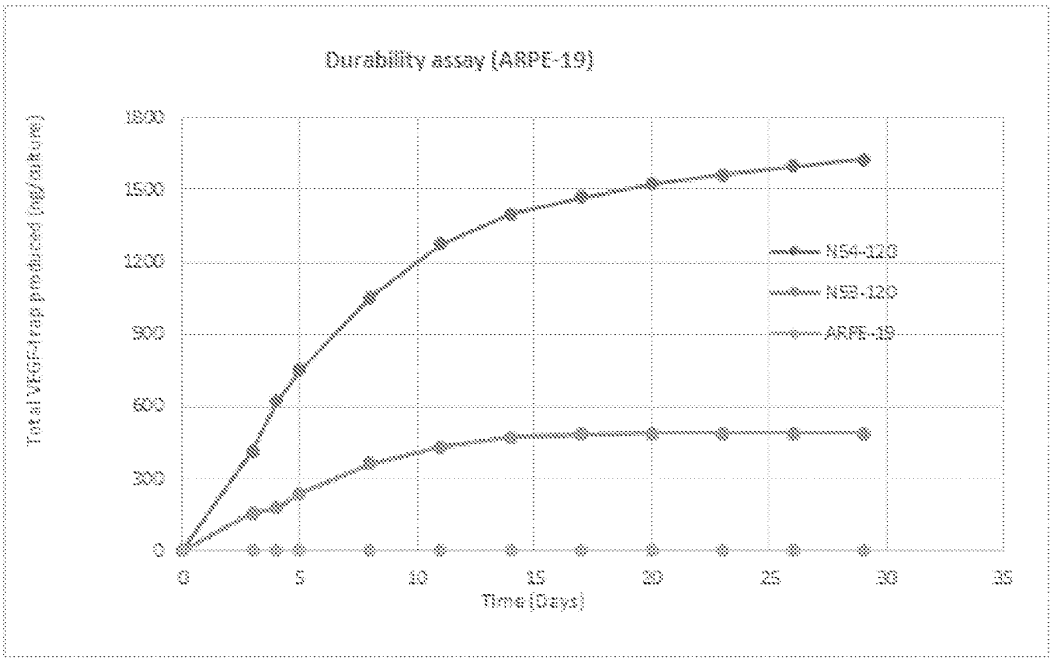

Durability assay (ARPE-19)
Total VEGF-trap produced (ng/culture)
1800
1500
1200
900
600
300
0
0
5
10
15
20
25
30
35
Time (Days)
N54-120
N53-120
ARPE-19

Fig. 11

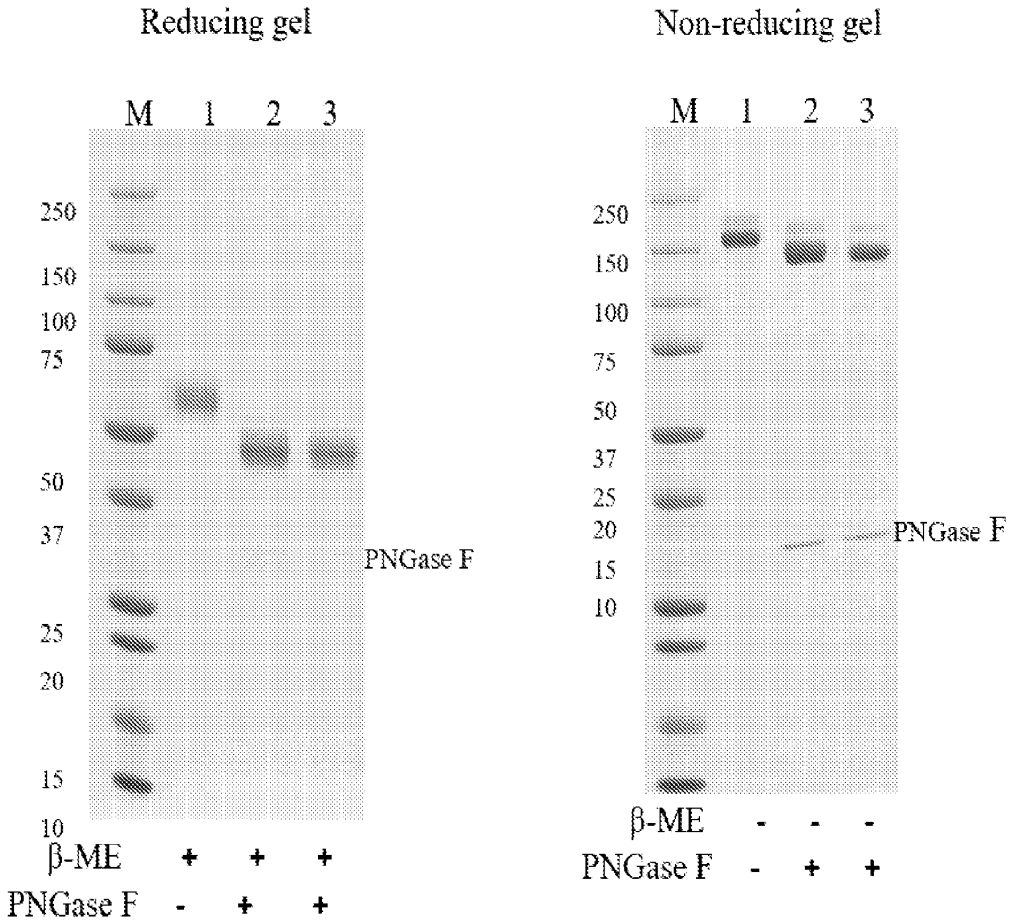

Reducing gel
M 1 2 3
250
150
100
75
50
37
25
20
15
10
PNGase F
β-ME + + +
PNGase F - + +
Non-reducing gel
M 1 2 3
250
150
100
75
50
37
25
20
15
10
PNGase F
β-ME - - -
PNGase F - + +

Fig. 12

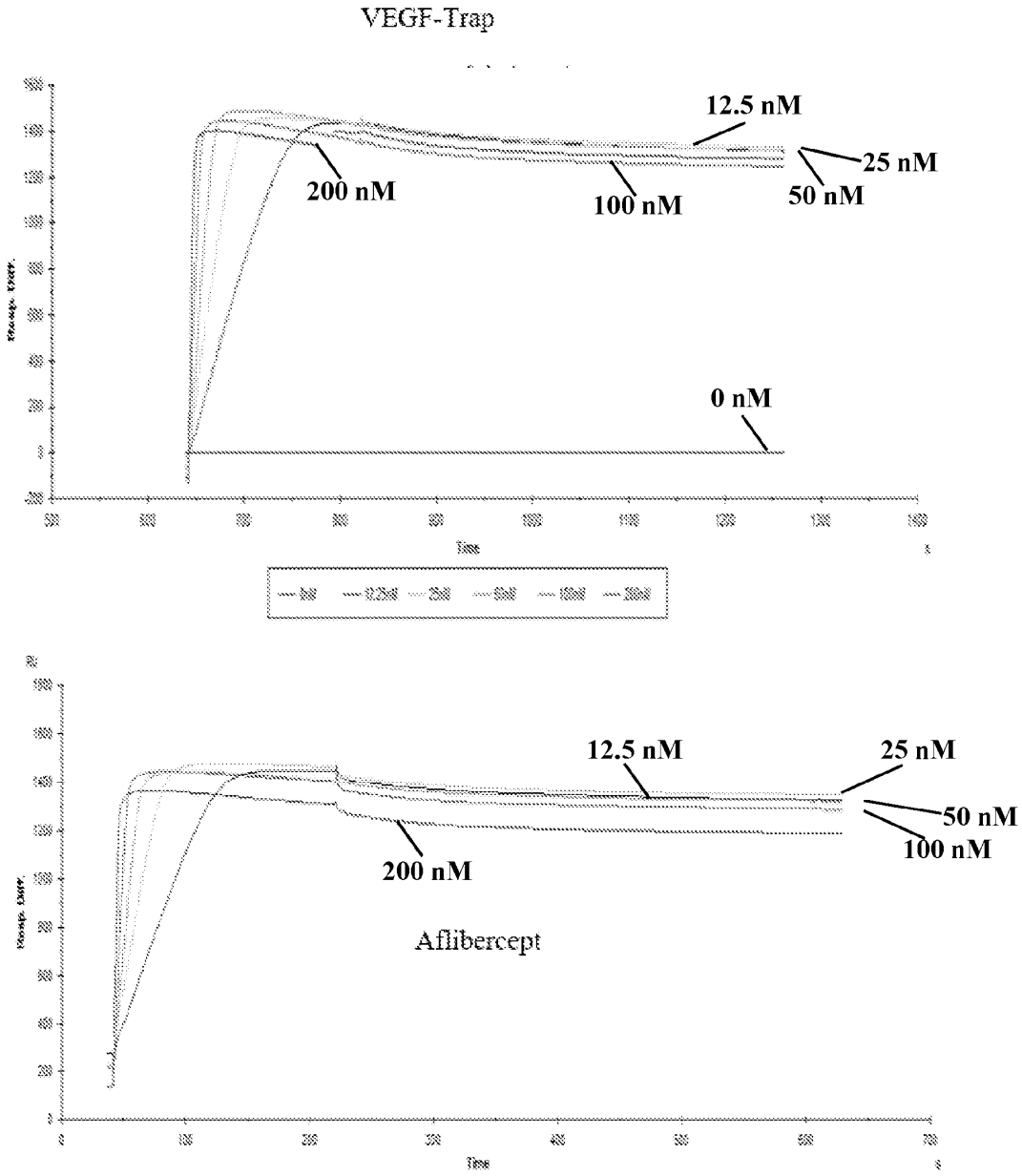

VEGF-Trap
12.5 nM
25 nM
200 nM
100 nM
50 nM
0 nM
Time
12.5 nM
25 nM
50 nM
100 nM
200 nM
Aflibercept
Time

Fig. 13

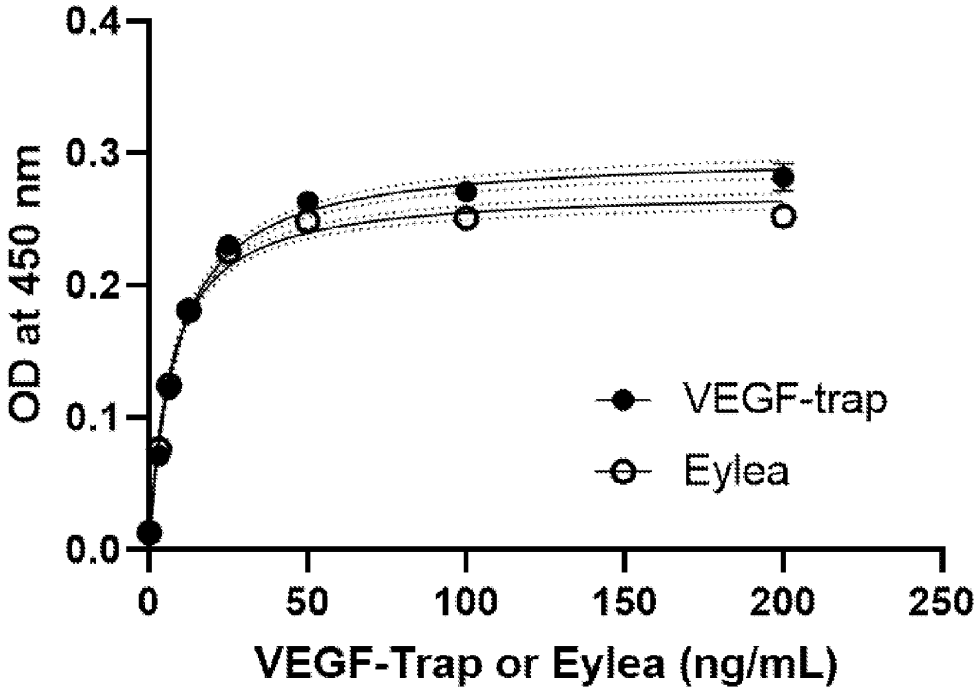

0.4
0.3
0.2
0.1
0.0
OD at 450 nm
0
50
100
150
200
250
VEGF-Trap or Eylea (ng/mL)
VEGF-trap
Eylea

Fig. 14

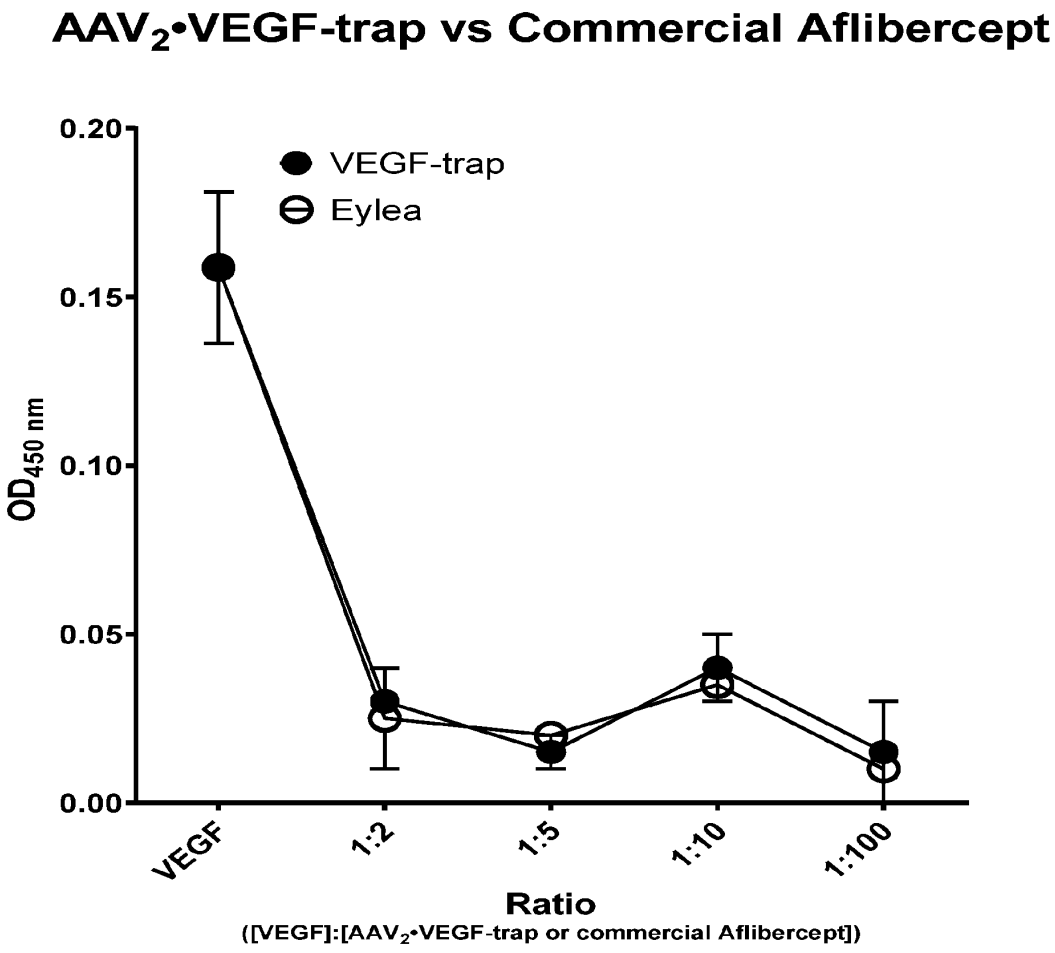

AAV2•VEGF-trap vs Commercial Aflibercept
VEGF-trap
Eylea
0.20
0.15
0.10
0.05
0.00
OD450 nm
VEGF
1:2
1:5
1:10
1:100
Ratio
([VEGF]:[AAV2•VEGF-trap or commercial Aflibercept])

Fig. 15

Fig. 16

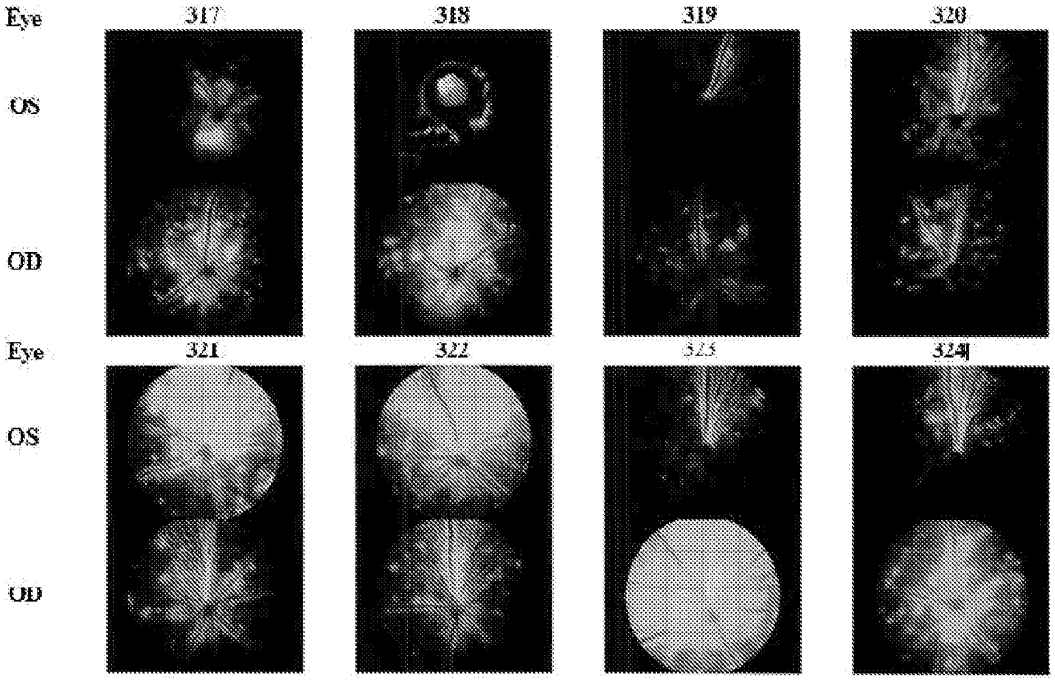


Fig. 17

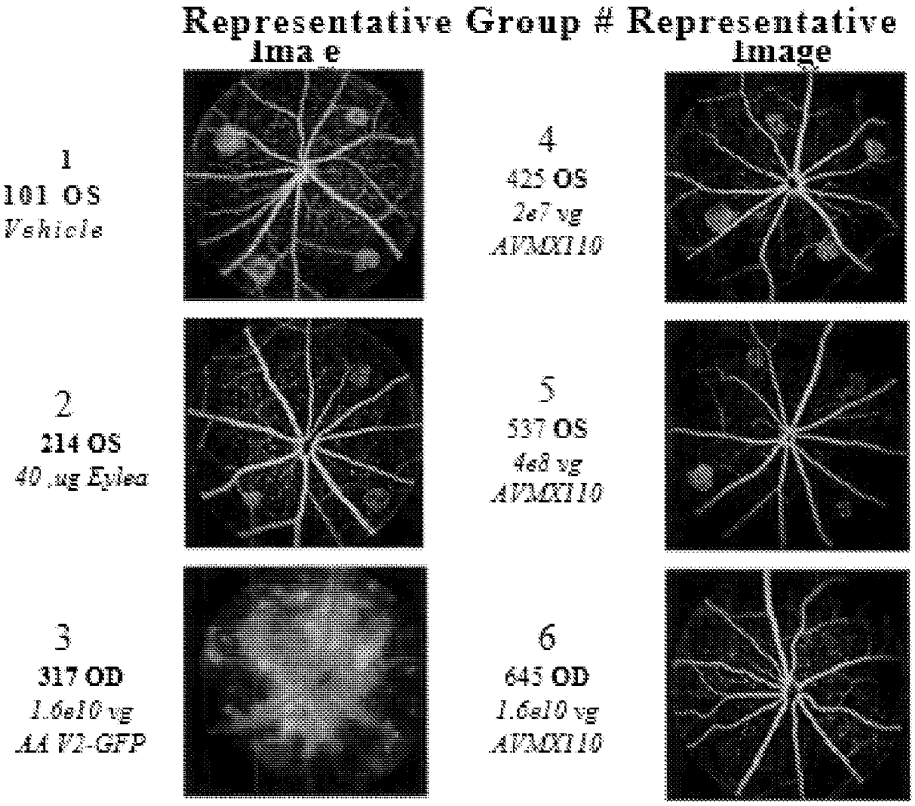

Representative Image
Group #
Representative Image
1
101 OS
Vehicle
4
425 OS
2e7 vg
AVMX110
2
214 OS
40 ug Eylea
5
537 OS
4e8 vg
AVMX110
3
317 OD
1.6e10 vg
AAV2-GFP
6
645 OD
1.6e10 vg
AVMX110

Fig. 18

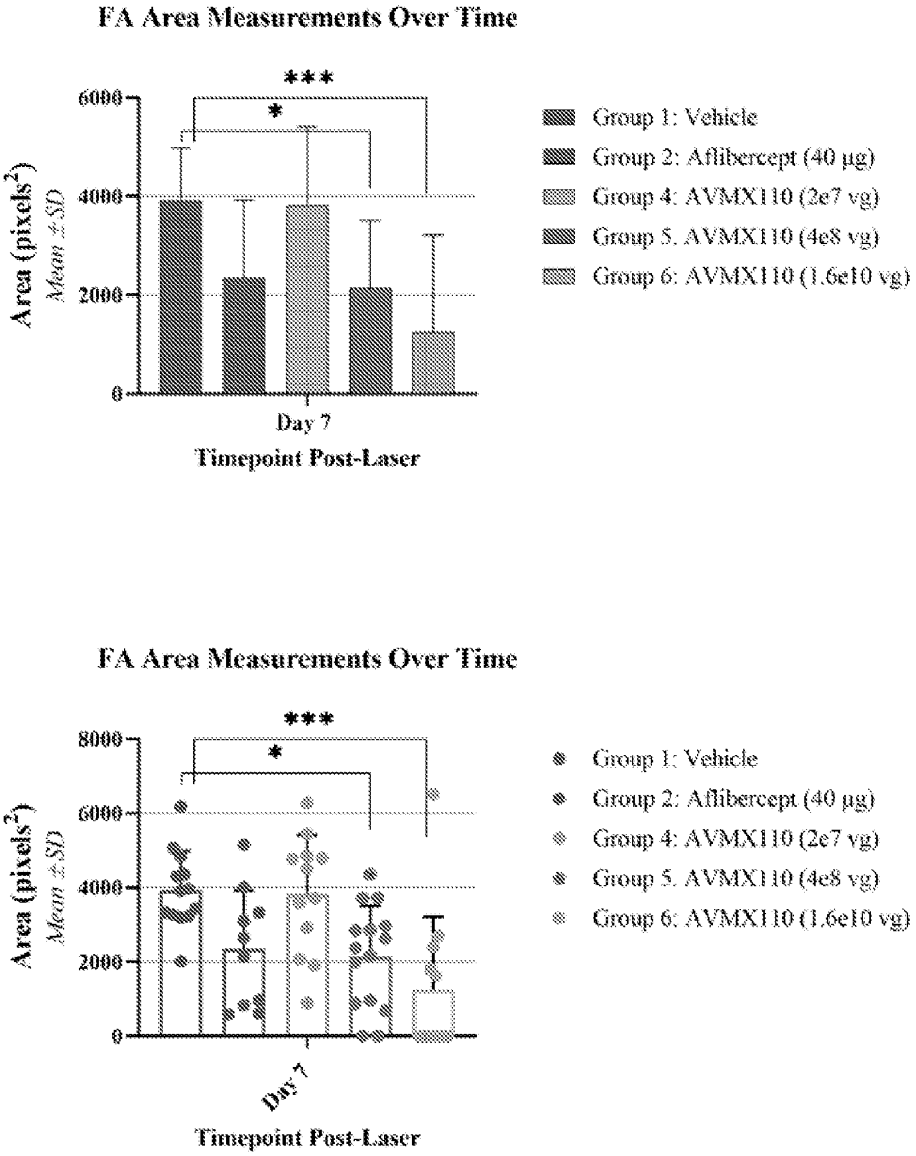

FA Area Measurements Over Time
***
*
6000
4000
2000
0
Area (pixels²)
Mean ±SD
Day 7
Timepoint Post-Laser
Group 1: Vehicle
Group 2: Aflibercept (40 µg)
Group 4: AVMX110 (2e7 vg)
Group 5. AVMX110 (4e8 vg)
Group 6: AVMX110 (1.6e10 vg)
FA Area Measurements Over Time
***
*
8000
6000
4000
2000
0
Area (pixels²)
Mean ±SD
Day 7
Timepoint Post-Laser
Group 1: Vehicle
Group 2: Aflibercept (40 µg)
Group 4: AVMX110 (2e7 vg)
Group 5. AVMX110 (4e8 vg)
Group 6: AVMX110 (1.6e10 vg)

Fig. 19

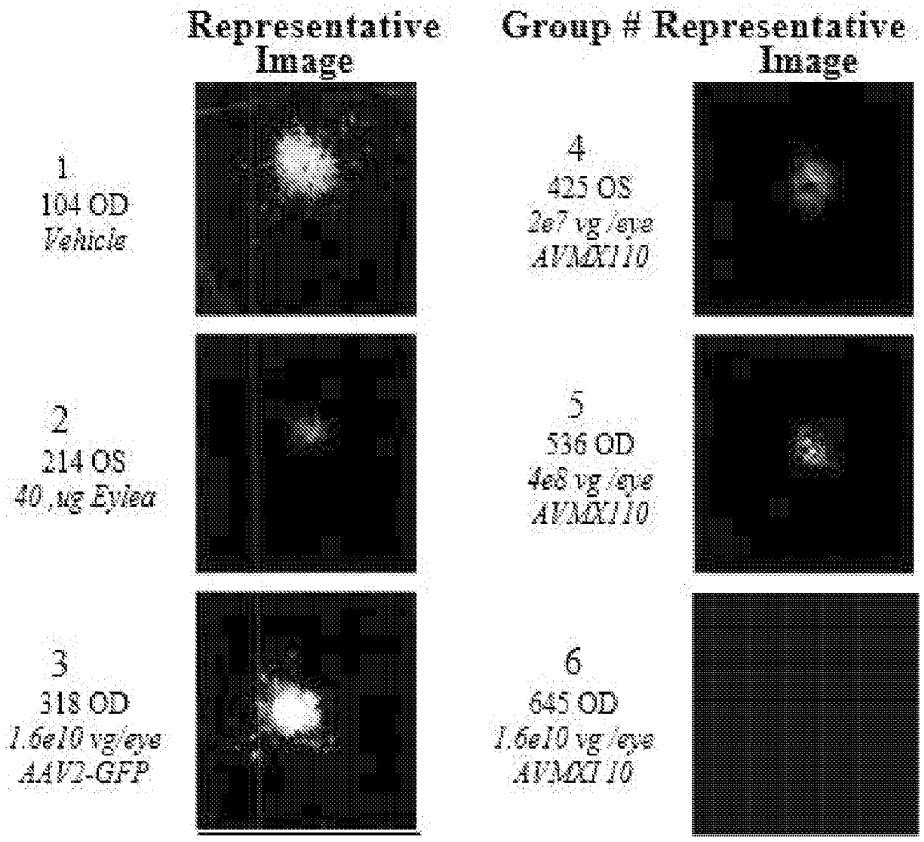
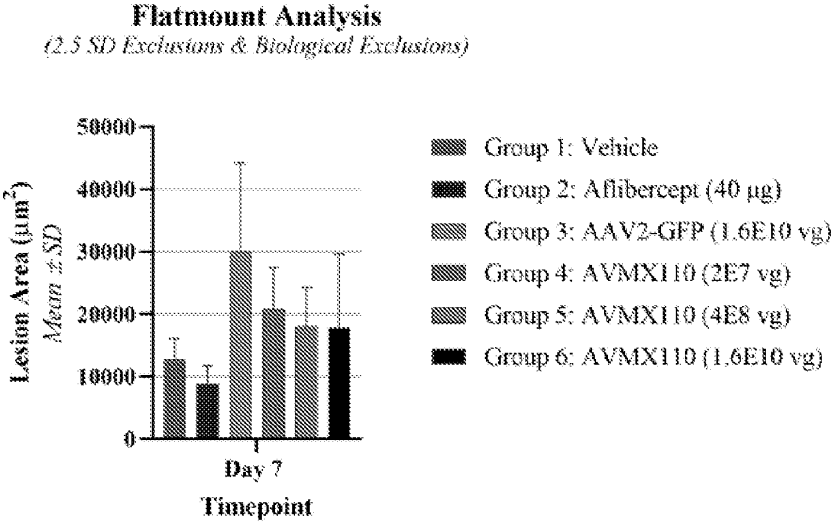

Representative Image
Group # Representative Image
1
104 OD
Vehicle
2
214 OS
40 ,ug Eylea
3
318 OD
1.6e10 vg/eye
AAV2-GFP
4
425 OS
2e7 vg /eye
AVMX110
5
536 OD
4e8 vg /eye
AVMX110
6
645 OD
1.6e10 vg /eye
AVMX110

Fig. 20


Flatmount Analysis
(2.5 SD Exclusions & Biological Exclusions)
Lesion Area (µm²)
Mean ±SD
50000
40000
30000
20000
10000
0
Day 7
Timepoint
Group 1: Vehicle
Group 2: Aflibercept (40 µg)
Group 3: AAV2-GFP (1.6E10 vg)
Group 4: AVMX110 (2E7 vg)
Group 5: AVMX110 (4E8 vg)
Group 6: AVMX110 (1.6E10 vg)

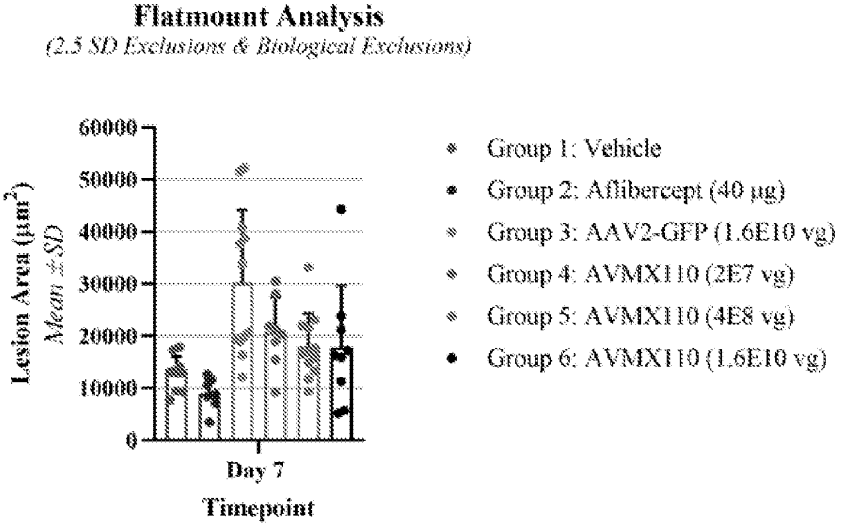

Flatmount Analysis
(2.5 SD Exclusions & Biological Exclusions)
Lesion Area (µm²)
Mean ±SD
60000
50000
40000
30000
20000
10000
0
Day 7
Timepoint
Group 1: Vehicle
Group 2: Aflibercept (40 µg)
Group 3: AAV2-GFP (1.6E10 vg)
Group 4: AVMX110 (2E7 vg)
Group 5: AVMX110 (4E8 vg)
Group 6: AVMX110 (1.6E10 vg)

Fig. 21

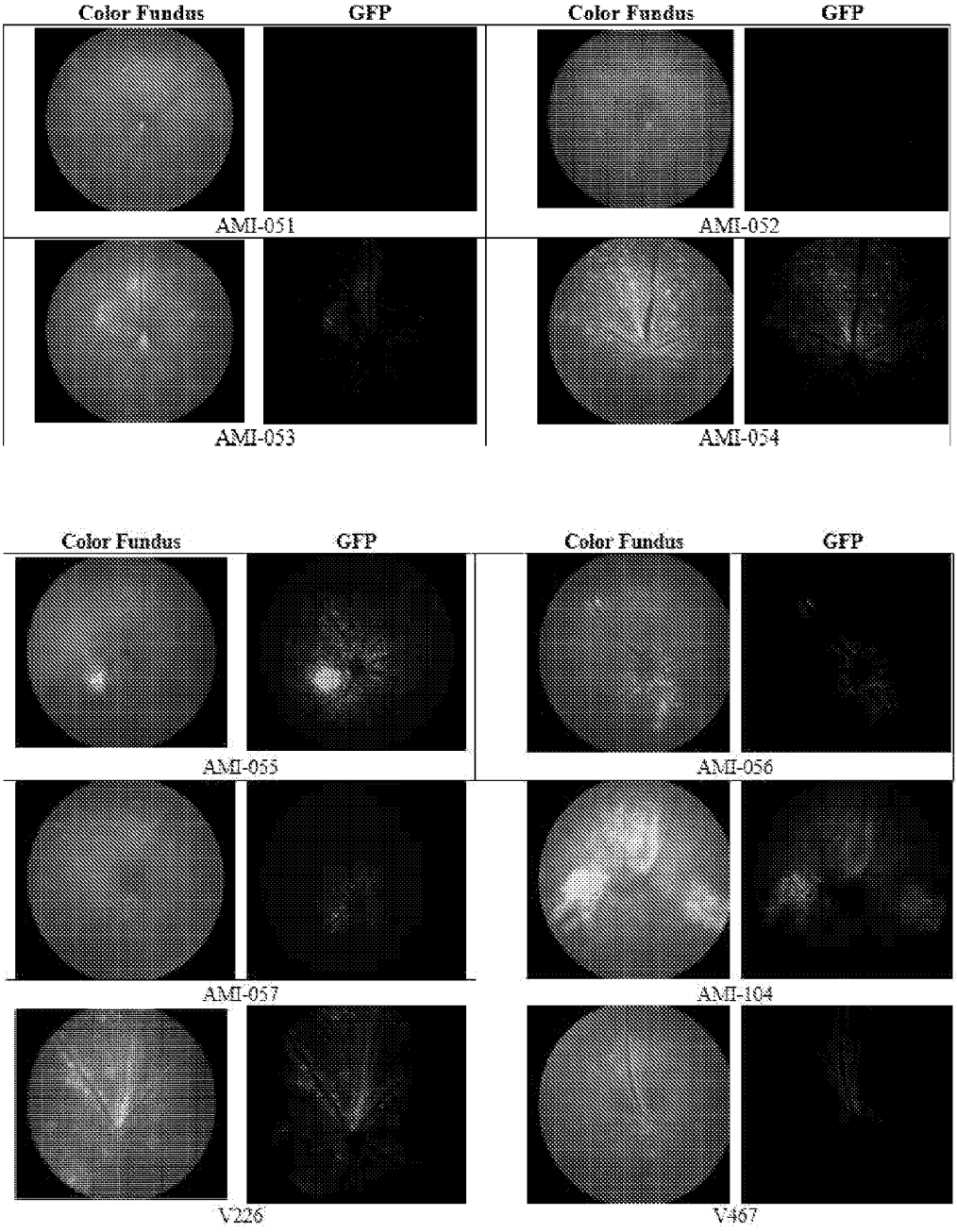

Color Fundus
GFP
Color Fundus
GFP
AMI-051
AMI-052
AMI-053
AMI-054
Color Fundus
GFP
Color Fundus
GFP
AMI-055
AMI-056
AMI-057
AMI-104
V226
V467

Fig. 22

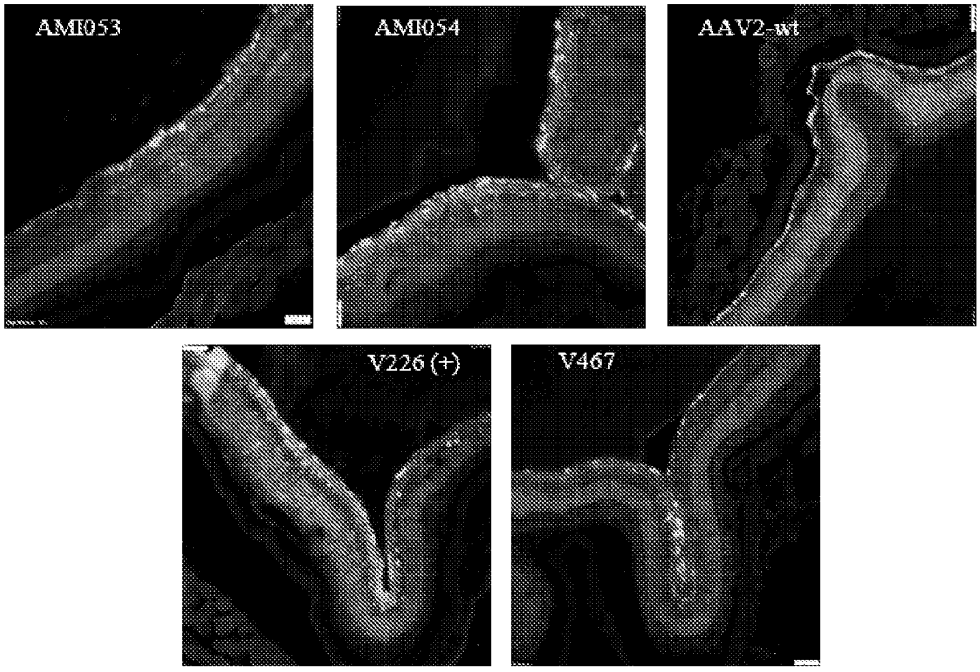


Fig. 23

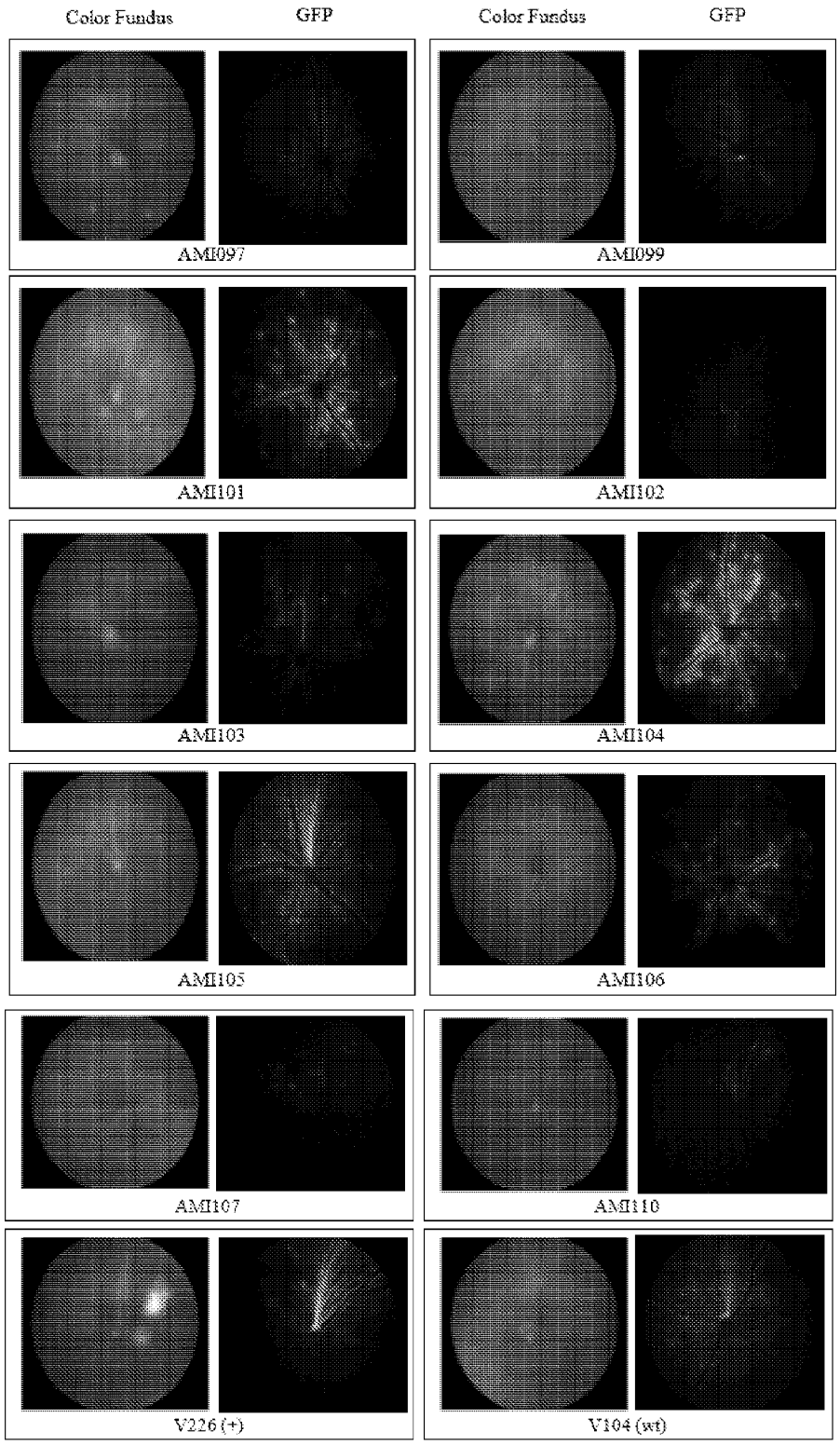

Color Fundus
GFP
Color Fundus
GFP
AMI097
AMI099
AMI101
AMI102
AMI103
AMI104
AMI105
AMI106
AMI107
AMI110
V226 (+)
V104 (wt)

Fig. 24

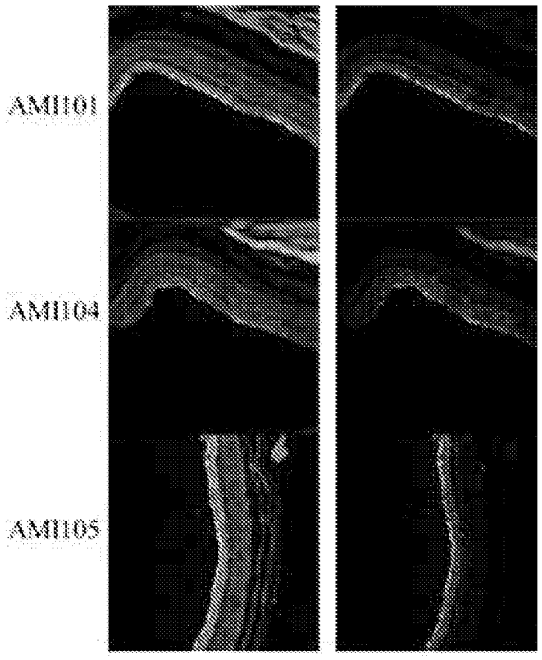


Fig. 25

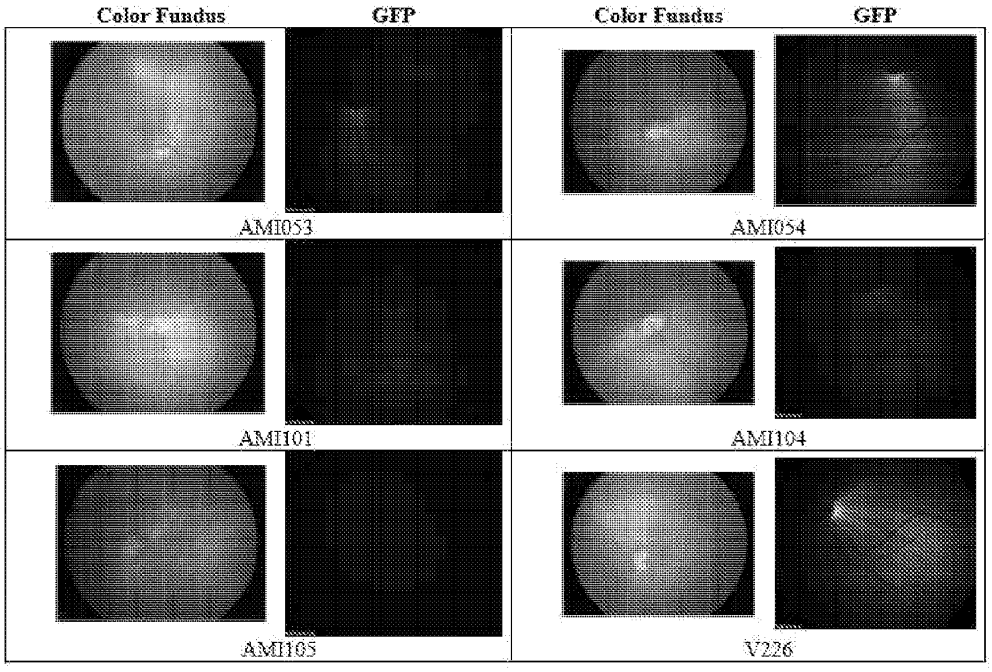

Color Fundus
GFP
Color Fundus
GFP
AMI053
AMI054
AMI101
AMI104
AMI105
V226

Fig. 26

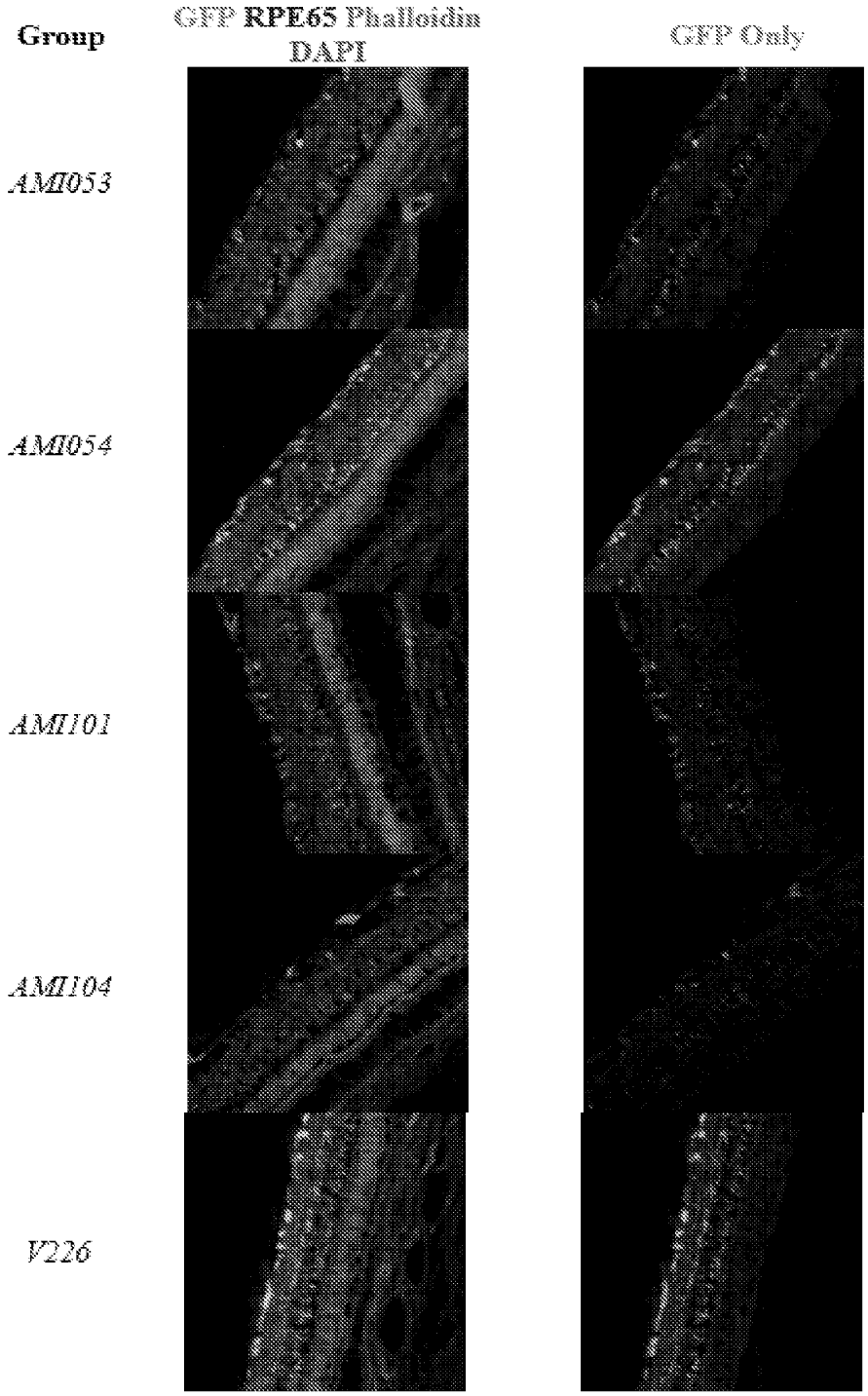

Group
GFP RPE65 Phalloidin DAPI
GFP Only
AMI053
AMI054
AMI101
AMI104
V226

Fig. 27

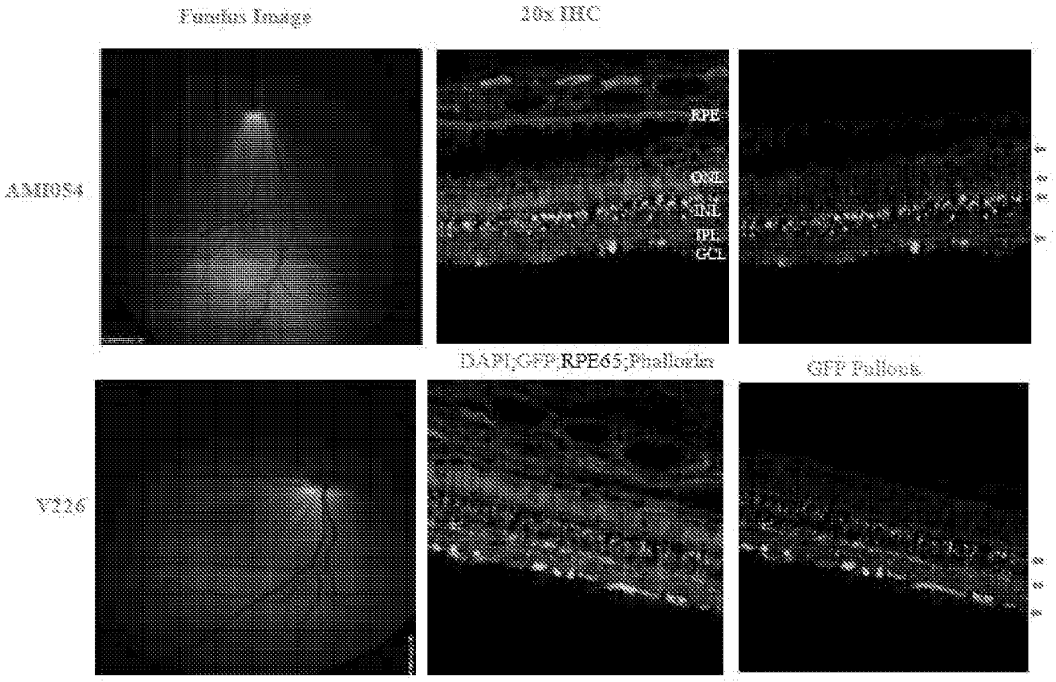

Fundus Image
20x IHC
AAV084
RPE
ONL
INL
IPL
GCL
DAPI;GFP;RPE65;Phalloidin
GFP Pullout
V326

Fig. 28A

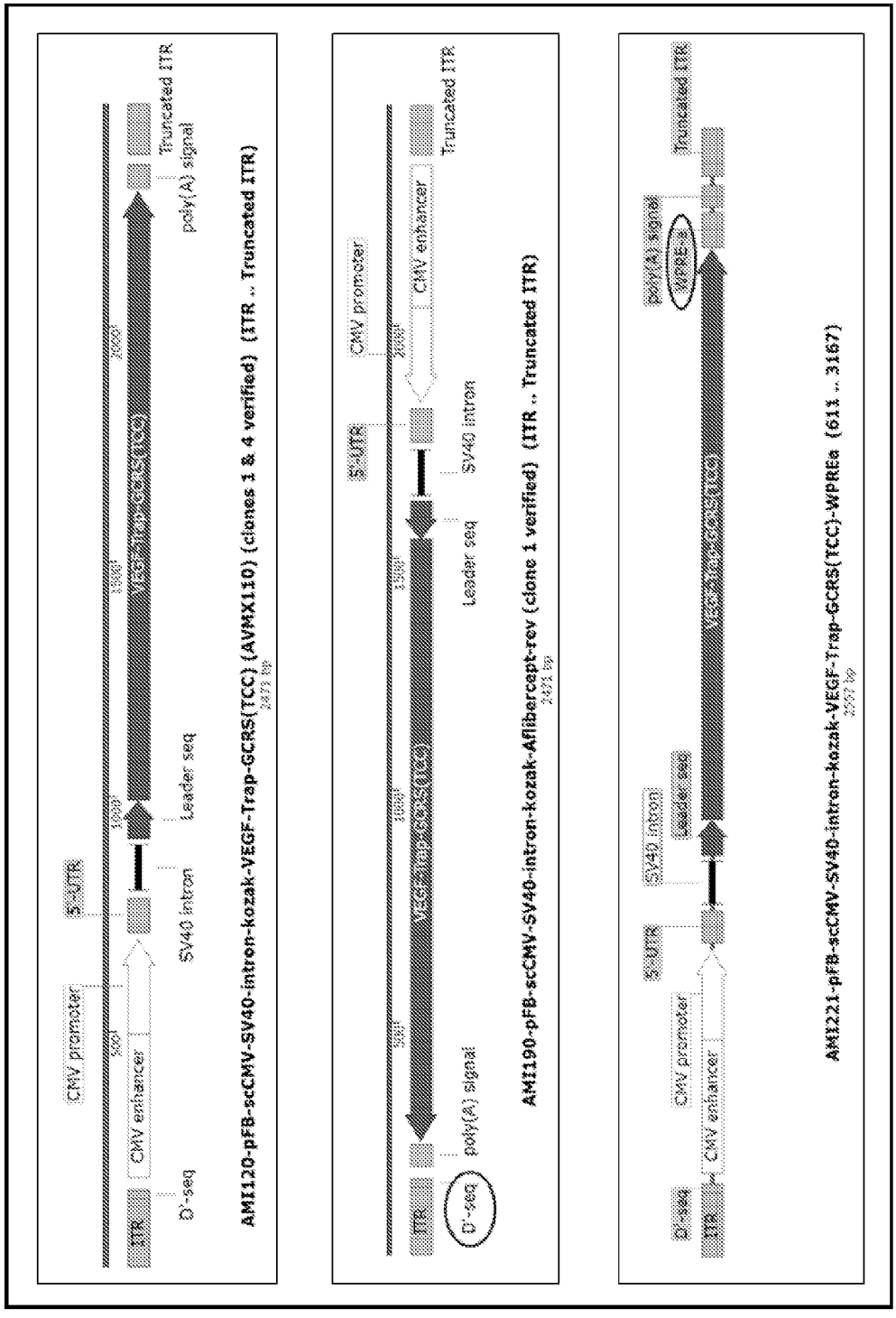

CMV promoter
5'-UTR
Kozak
ATG
CMV enhancer
ITR
5'-seq
SV40 intron
Leader seq
Truncated ITR
poly(A) signal
AMI120-pFB-scCMV-SV40-intron-kozak-VEGF-Trap-GCRS(TCC) (AVMX110) (clones 1 & 4 verified) (ITR .. Truncated ITR)
2471 bp
AMI190-pFB-scCMV-SV40-intron-kozak-Aflibercapt-rev (clone 1 verified) (ITR .. Truncated ITR)
2471 bp
WPREa
AMI221-pFB-scCMV-SV40-intron-kozak-VEGF-Trap-GCRS(TCC)-WPREa (611 .. 3167)
2957 bp

Fig. 28B

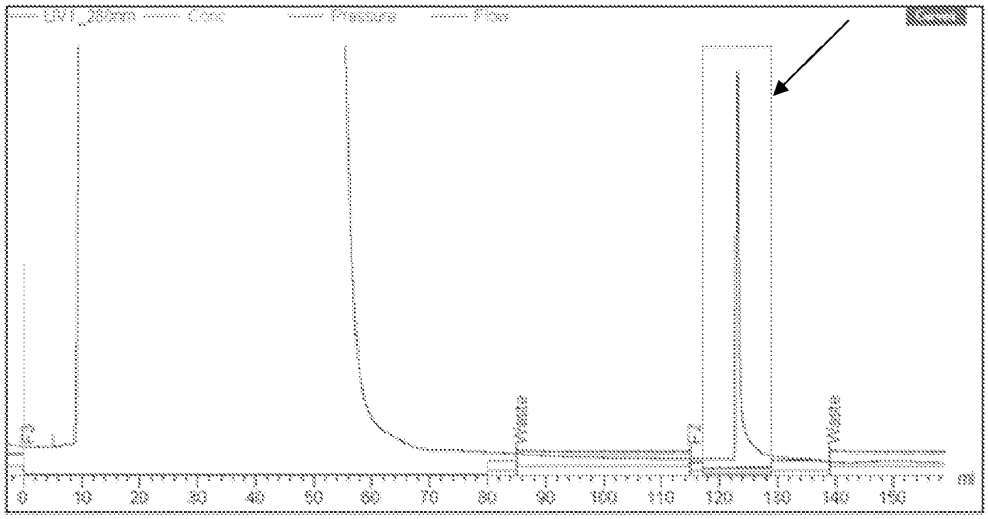
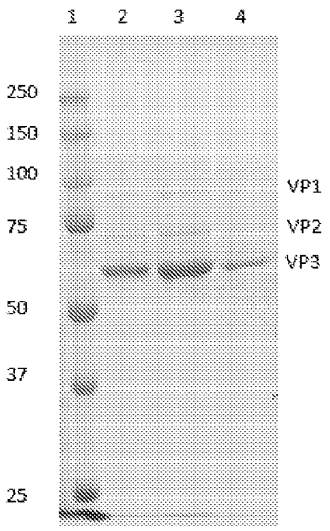


Fig. 28C



1. Marker
2. AAV 6 µg
3. AAV 12 µg
4. AAV control

Fig. 28D

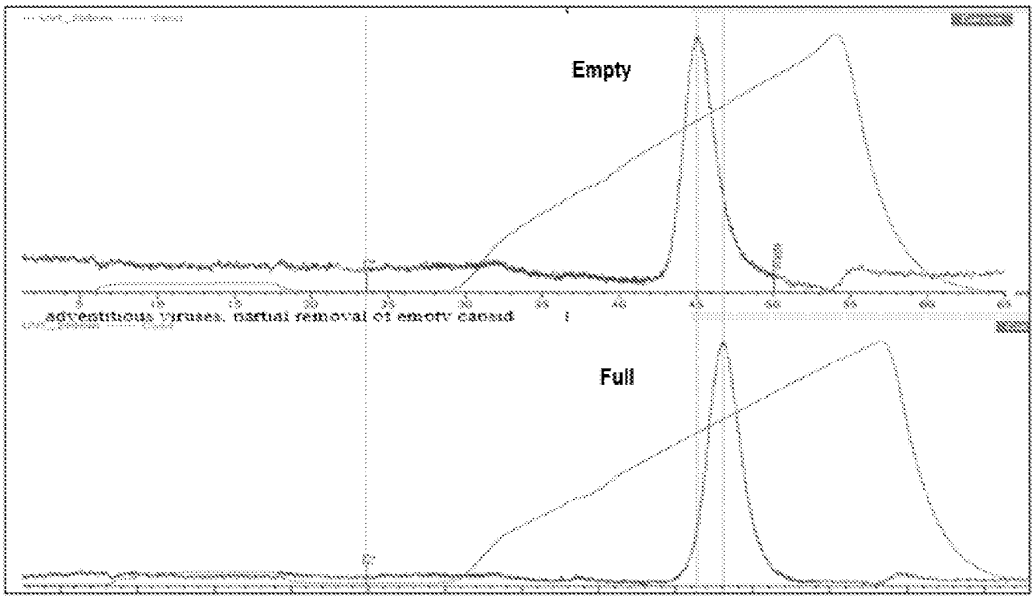


Fig. 28E

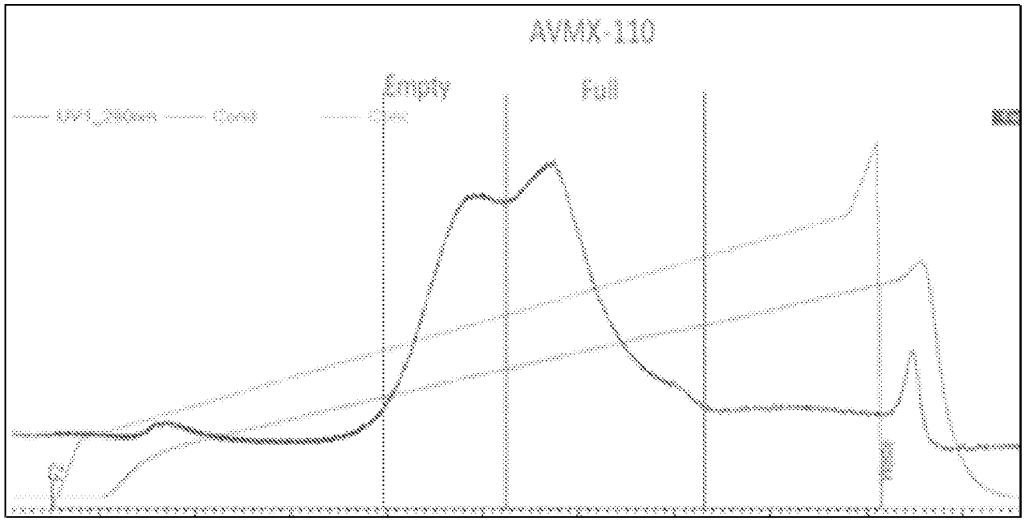


Fig. 28F

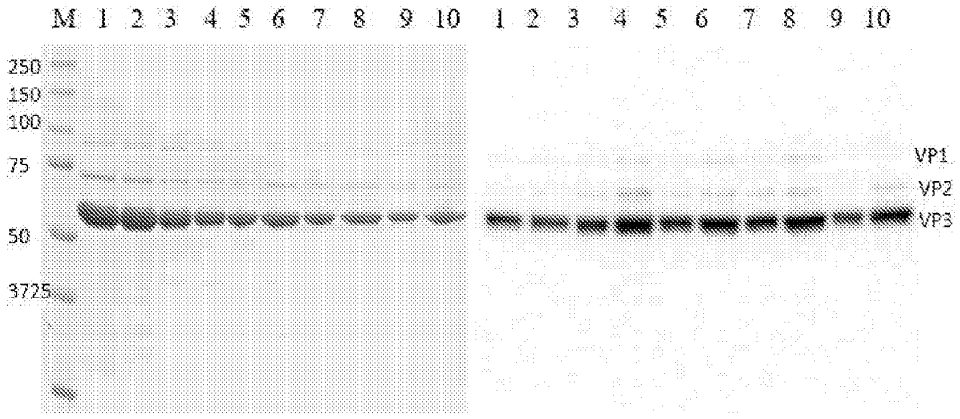


Fig. 28G

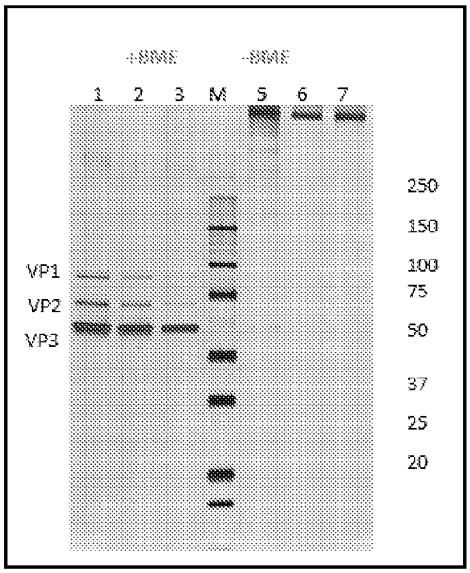
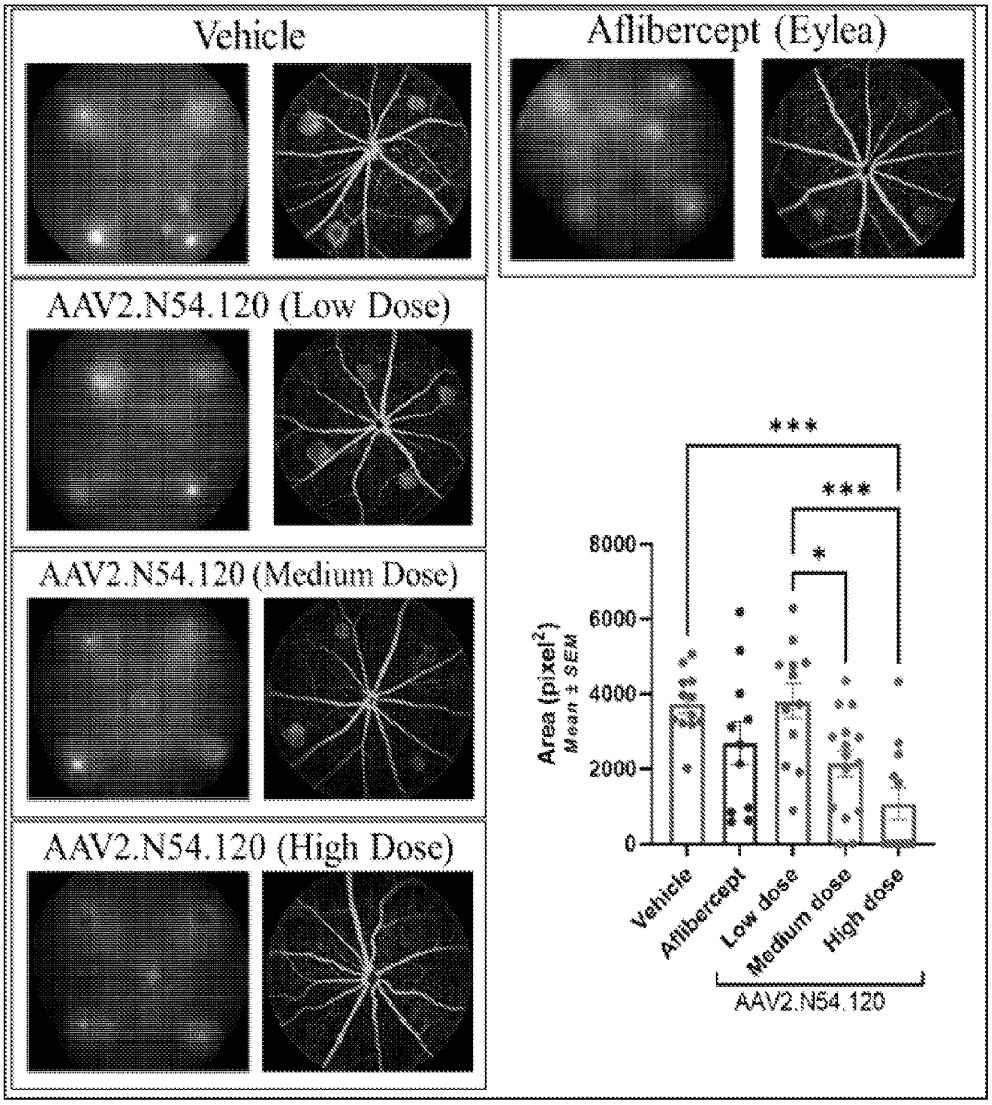


Fig. 29



One-way ANOVA followed by Tukey's multiple comparison (* = 0.0332; *** = 0.0002)

Fig. 30

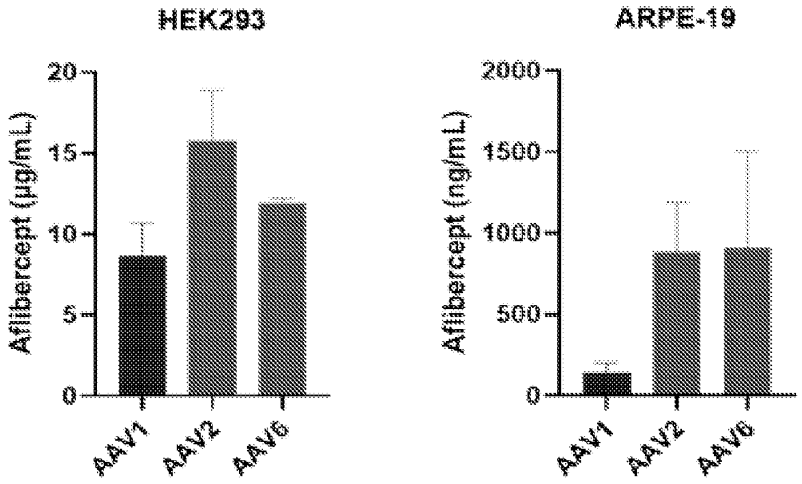


Table 3: Comparing expression level of Aflibercept in HEK293 and ARPE-19 cells

HEK293	Aflibercept (ug/mL)
AAV1	8.7 ± 3.5
AAV2	15.8 ± 5.3
AAV6	11.9 ± 0.5

ARPE-19	Aflibercept (ng/mL)
AAV1	142.1 ± 82.3
AAV2	881 ± 434.7
AAV6	908.4 ± 842.1

Fig. 31

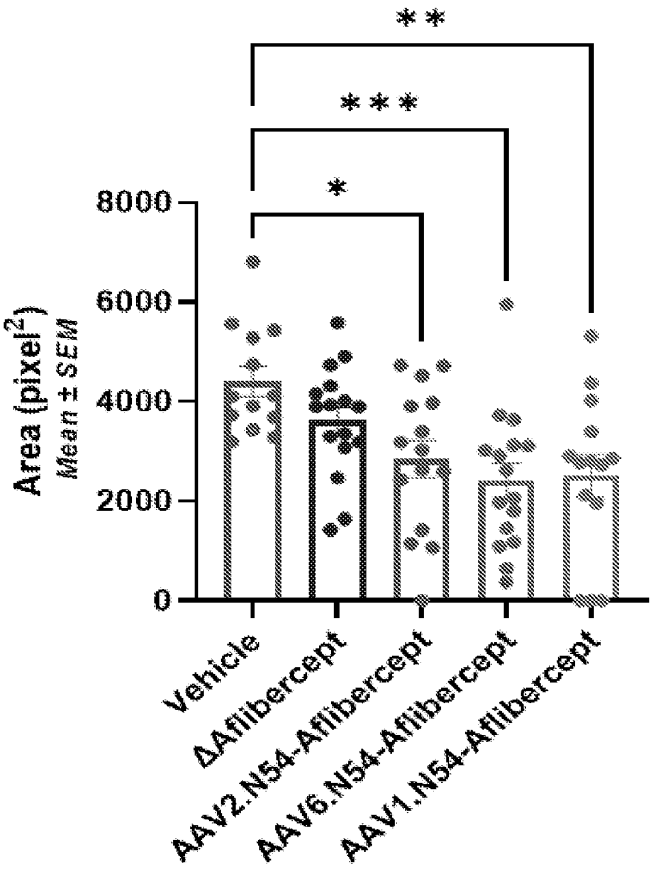

**
***
*
8000
6000
4000
2000
0
Area (pixel^2)
Mean ± SEM
Vehicle
ΔAflibercept
AAV2.N54-Aflibercept
AAV6.N54-Aflibercept
AAV1.N54-Aflibercept

Fig. 32

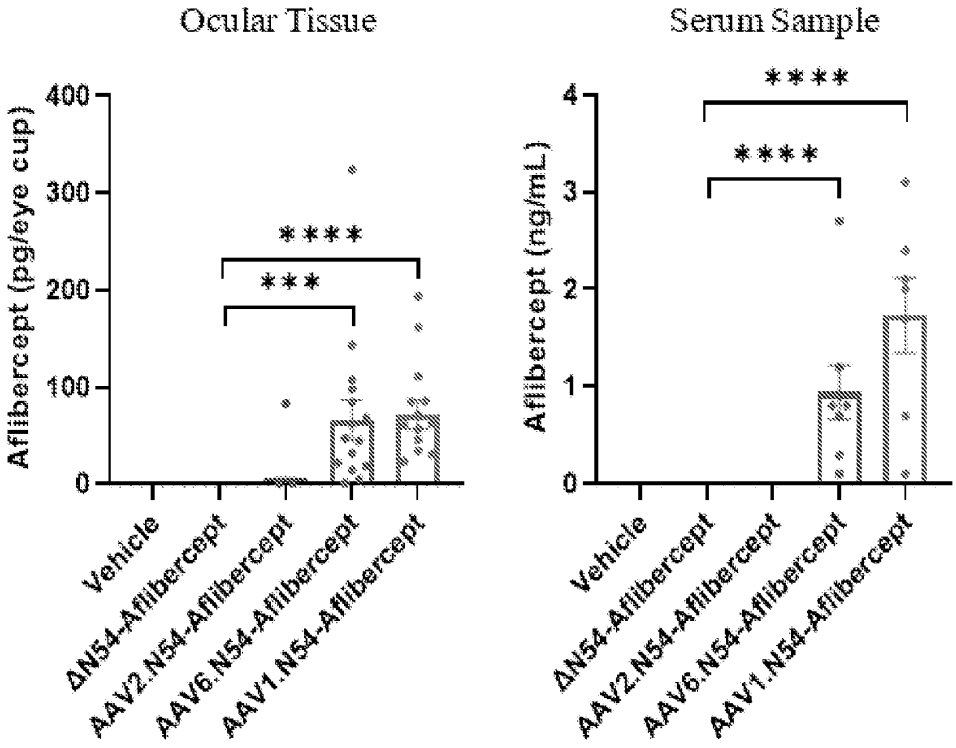

Ocular Tissue
Aflibercept (pg/eye cup)
400
300
200
100
0
****
***
Vehicle
ΔN54-Aflibercept
AAV2.N54-Aflibercept
AAV6.N54-Aflibercept
AAV1.N54-Aflibercept
Serum Sample
Aflibercept (ng/mL)
4
3
2
1
0
****
****
Vehicle
ΔN54-Aflibercept
AAV2.N54-Aflibercept
AAV6.N54-Aflibercept
AAV1.N54-Aflibercept

Fig. 33

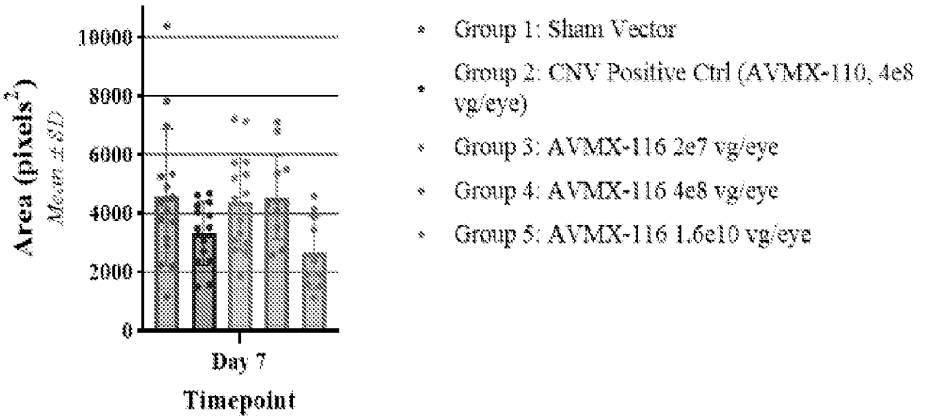

Area (pixels²)
Mean ± SD
10000
8000
6000
4000
2000
0
Day 7
Timepoint
Group 1: Sham Vector
Group 2: CNV Positive Ctrl (AVMX-110, 4e8 vg/eye)
Group 3: AVMX-116 2e7 vg/eye
Group 4: AVMX-116 4e8 vg/eye
Group 5: AVMX-116 1.6e10 vg/eye

Fig. 34

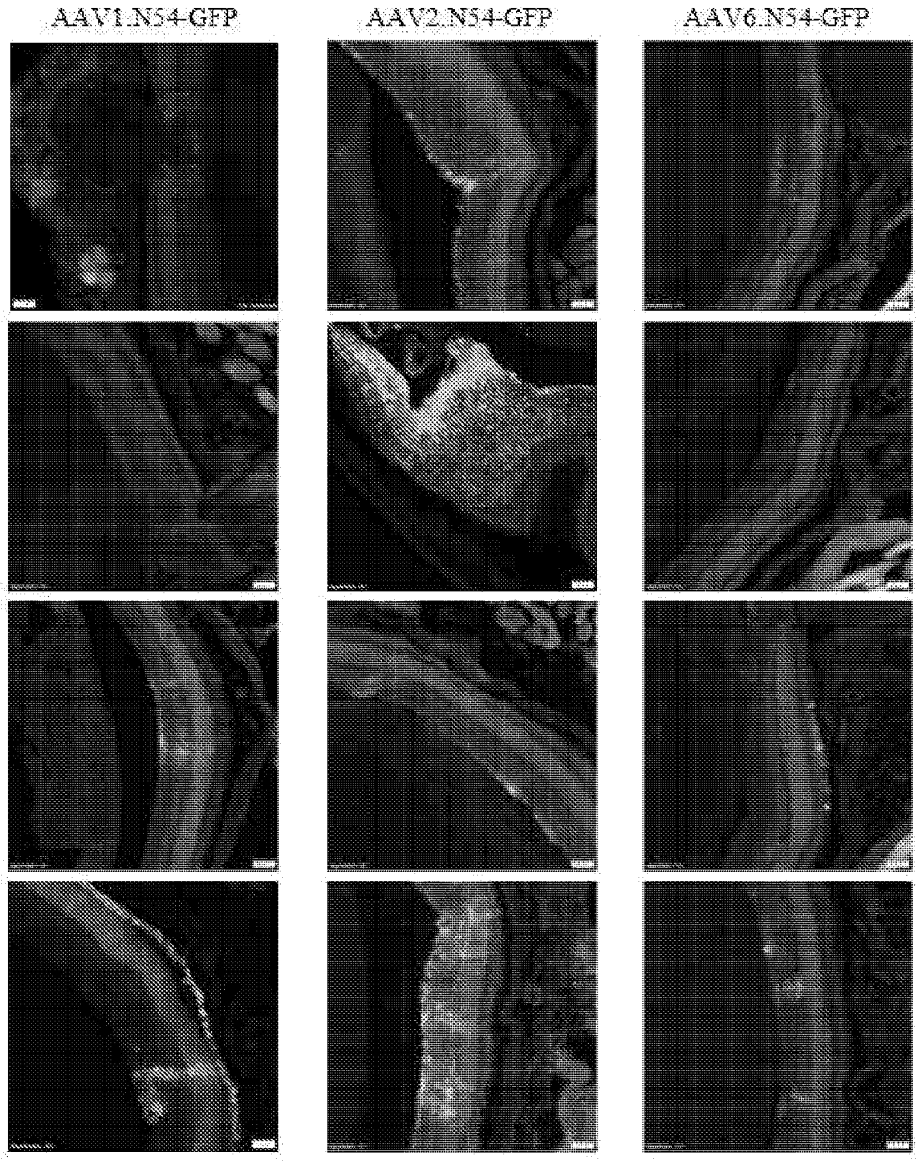


Fig. 35

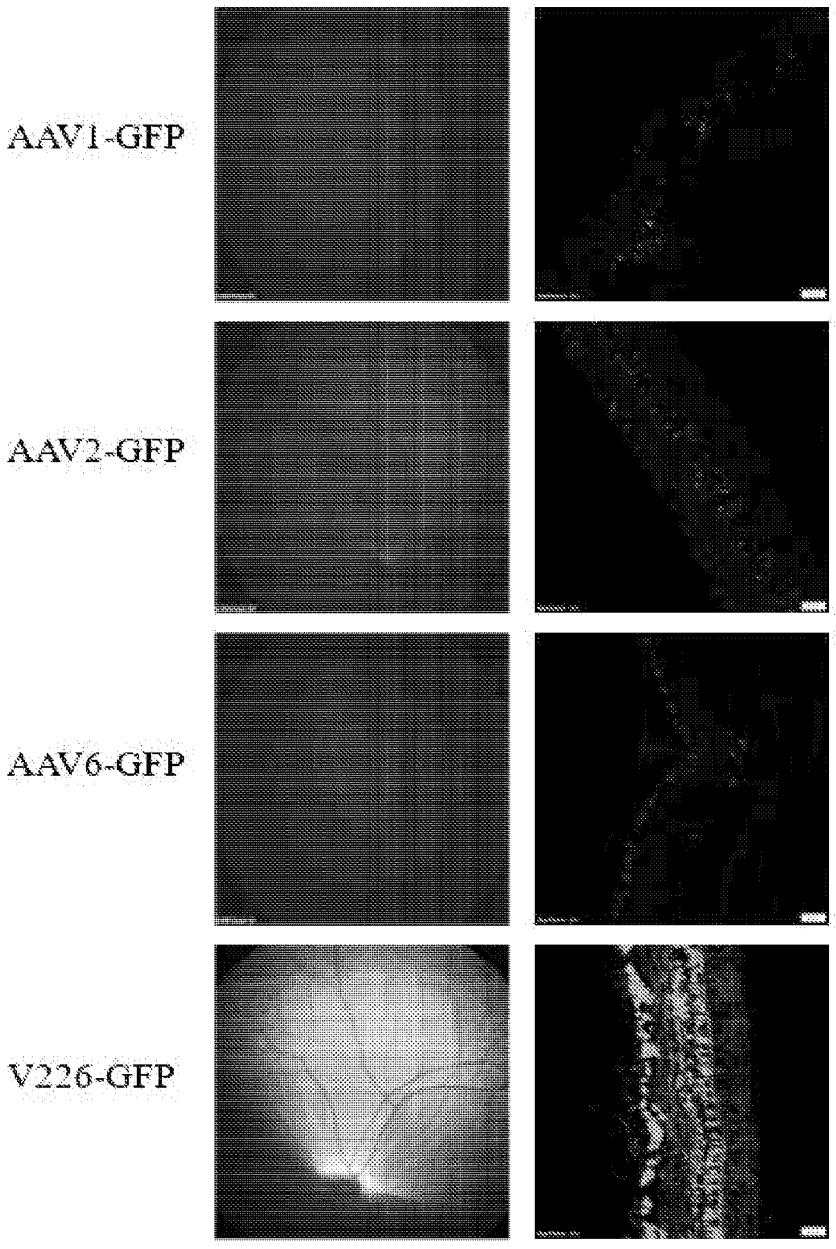


Fig. 36

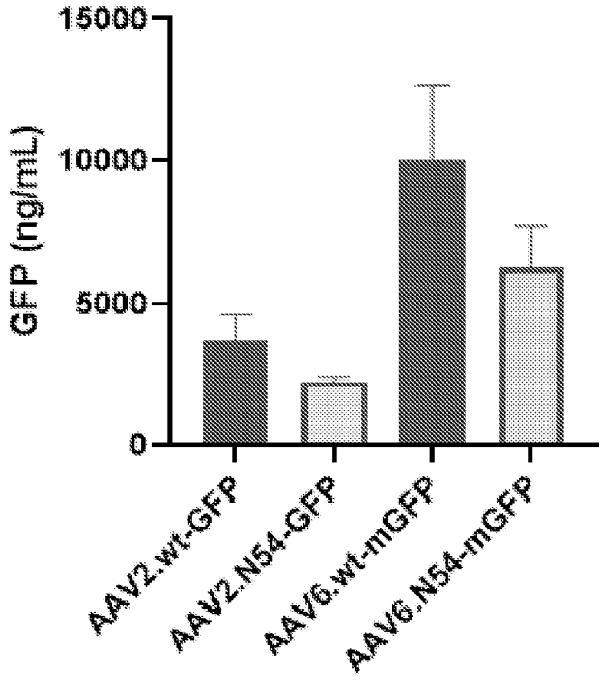

15000
10000
5000
0
GFP (ng/mL)
AAV2.wt-GFP
AAV2.N54-GFP
AAV6.wt-mGFP
AAV6.N54-mGFP

Fig. 37A

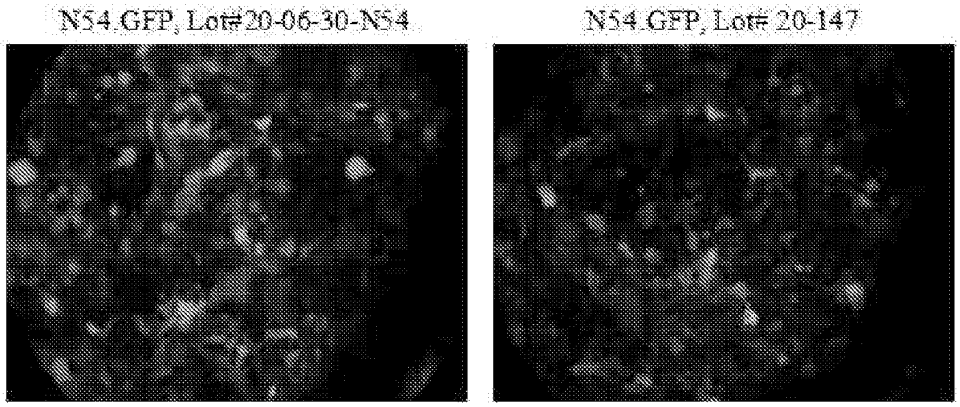


Fig. 37B

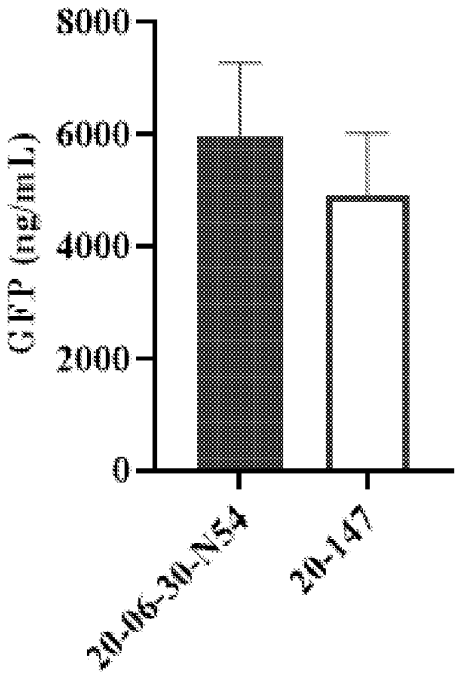


Fig. 38A

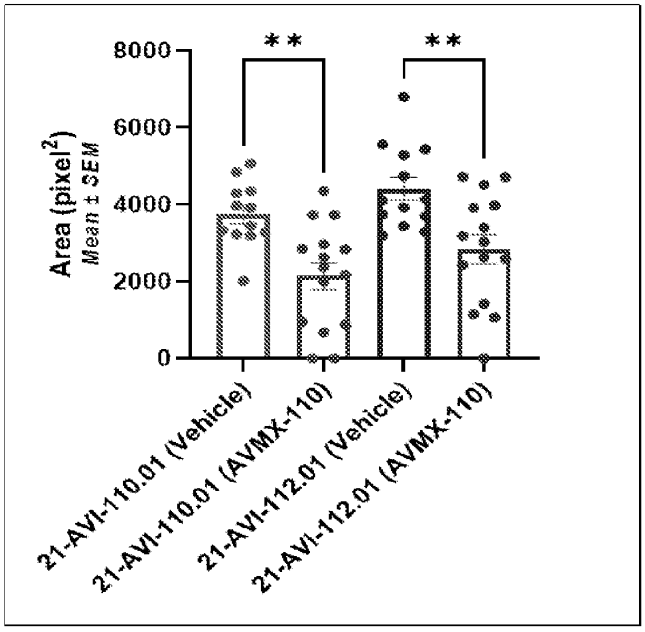


Fig. 38B

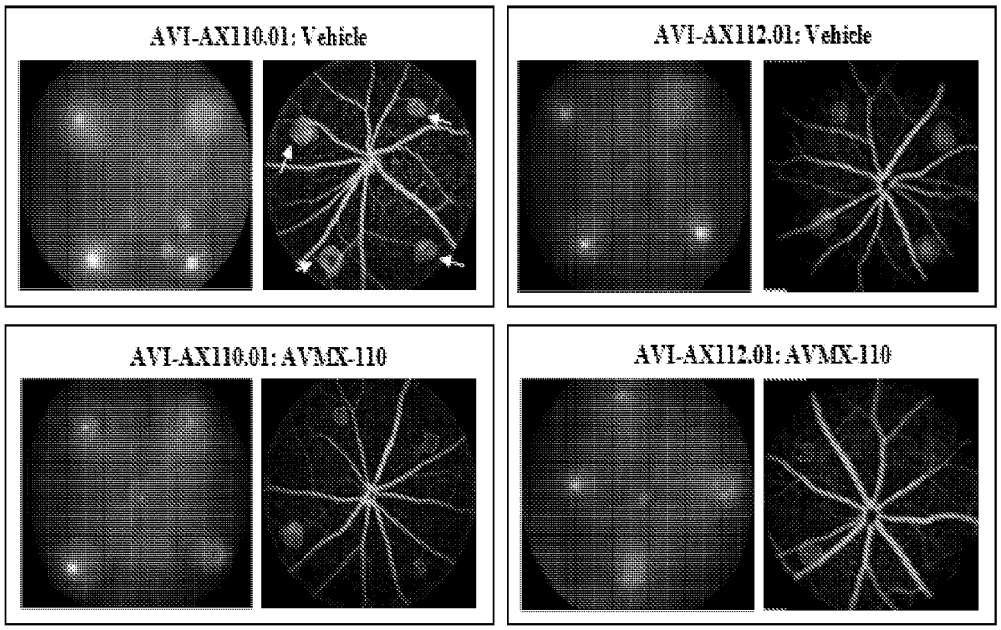


Fig. 39A

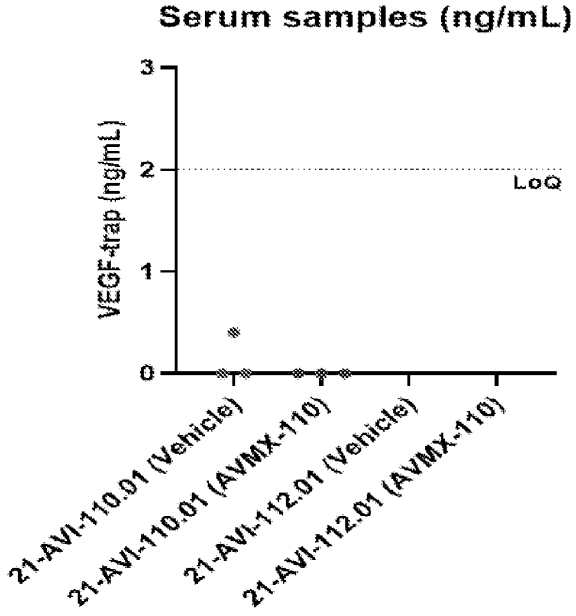


Fig. 39B

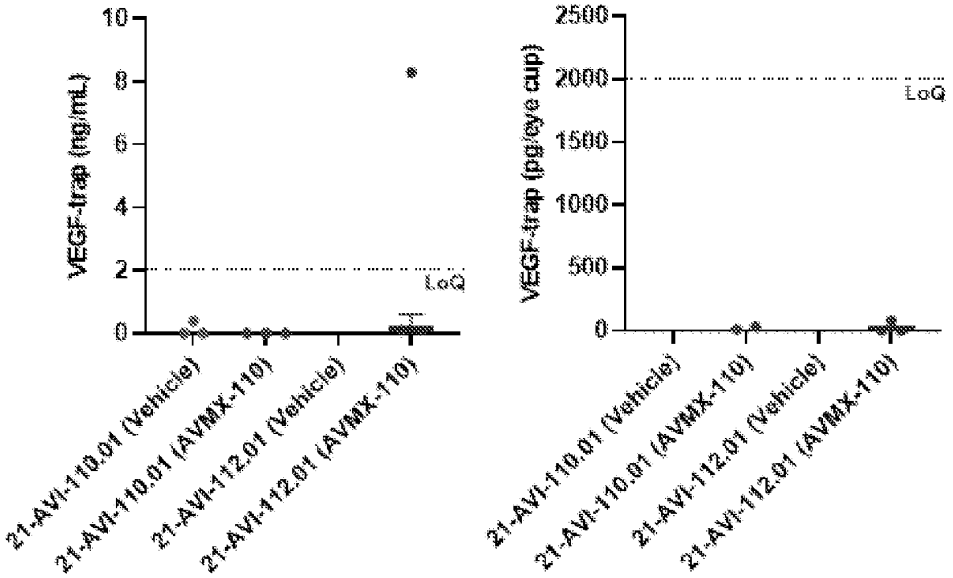


Fig. 40A

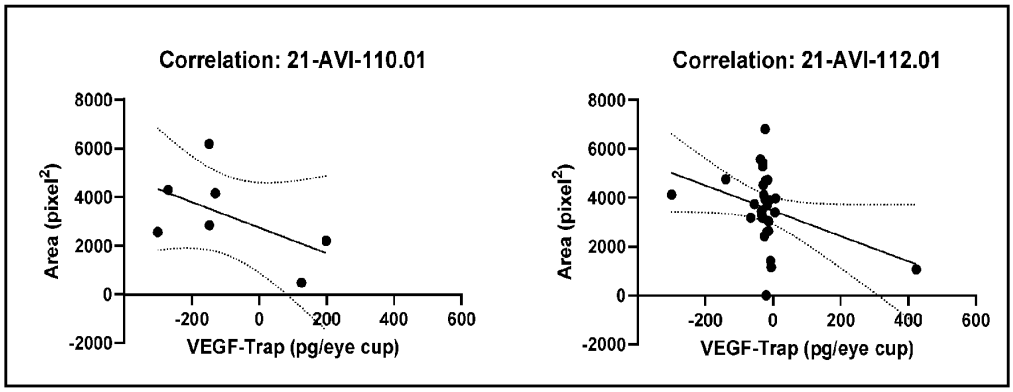


Fig. 40B

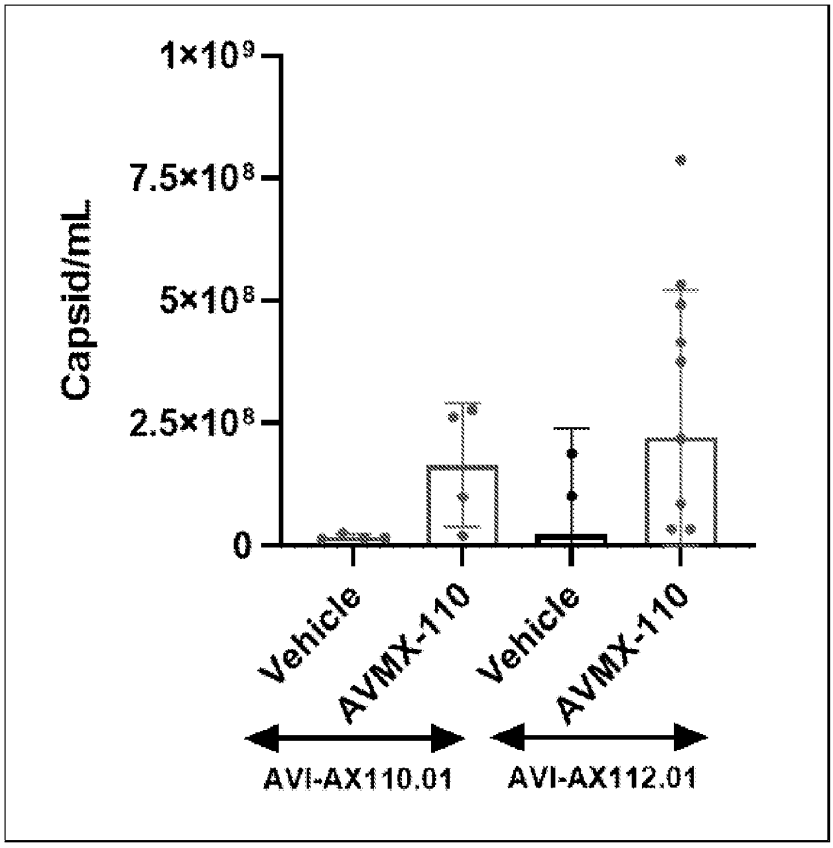


Fig. 41A

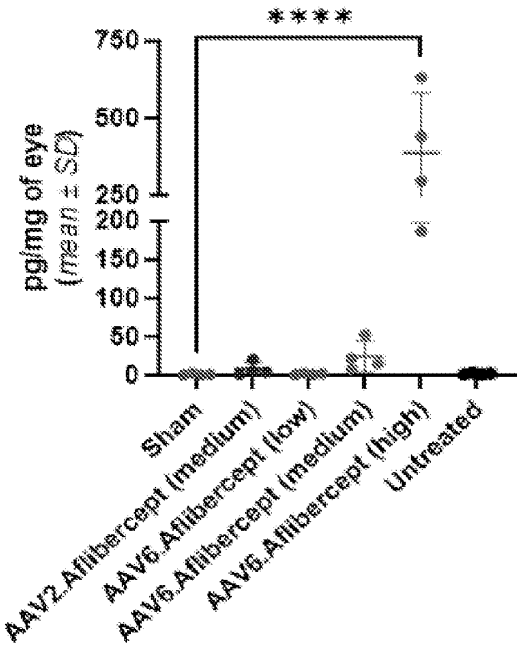


Fig. 41B

