expressing pancreatic cancer patient derived xenograft model (PDX Pan144607). This tumor sample was derived from surgical specimen and propagated in NSG mice (Jackson Laboratories, Bar Harbor, Maine). For efficacy studies, approximately 1-2 mm^3 of tumor fragments were implanted subcutaneously into the lateral flanks of CB17/SCID mice. Animals were randomized by tumor sizes once they reached ~300 mm^3, and single dose of 1.5, 3.0 and 6.0 mg/kg h7E6SVG-N297Q/K222R-PEG6MMAD and the control conjugate were administered through bolus tail vein injection. Tumor volume was measured once a week by a Caliper device and calculated with the following formula: Tumor volume = (length x $width^2$) / 2. Studies were terminated before their tumor volumes reach 2000 mm^3. Figure 13 shows that a single dose of the humanized anti-Trop2 antibody conjugated with PEG6-MMAD resulted in tumor regression in pancreatic Pan144607 PDX model.

Example 17: Anti-Trop-2 7E6 Auristatin Conjugate Induces Tumor Regression in Pancreatic Pan0135 PDX Model

In vivo efficacy study of Trop-2 ADC using humanized anti-Trop-2 antibody (h7E6SVG) conjugated with PEG6MMAD was performed with target-expressing pancreatic cancer patient derived xenograft model (PDX Pan0135) from Jackson Laboratories. For efficacy studies, approximately 1-2 mm^3 of tumor fragments were implanted subcutaneously into the lateral flanks of the animals. Animals were randomized by tumor sizes once they reached ~300 mm^3 and 6mg/kg of h7E6SVG-N297Q/K222R-PEG6MMAD and the control conjugate were administered through bolus tail vein injection with single dose. Tumor volume was measured once a week by a Caliper device and calculated with the following formula: Tumor volume = (length x $width^2$) / 2. Studies were terminated before their tumor volumes reached 2000 mm^3. Figure 14 shows that a single dose of the humanized anti-Trop2 antibody conjugated with PEG6-MMAD resulted in tumor regression in pancreatic Pan0135 PDX model.

Example 18: Antibody Binding Affinity Determination for Humanized Anti-Trop-2 Antibodies

Analysis of Fab/human Trop2-ECD (Extracellular Domain) interactions was performed using a Bio-Rad Proteon XPR36 Surface Plasmon Resonance (SPR)

biosensor (Bio-Rad, Hercules, CA) equipped with a GLC sensor chip. The assay temperature was 25°C, and the assay buffer was 10 mM Sodium Phosphate, 150 mM NaCl, 0.05% Tween-20, pH 7.4. An array of amine-coupled Fabs was prepared using the methodology described in Abdiche et al. (Anal. Biochem., 411: 139-151 (2011)). In each analysis cycle, monovalent human Trop2-ECD antigen was flowed at 30 µL/min over the immobilized Fabs for 3 minutes followed by a buffer wash for 15 minutes to monitor dissociation of the Fab/Trop2 complex. The sensor surface was regenerated between analysis cycles, using three 18-second injections of a 2:1 mixture (by volume) of Pierce IgG Elution buffer:4 M NaCl (Pierce, Rockford, IL). Binding and regeneration cycles were repeated at human Trop2-ECD concentrations of 6, 30 and 150 nM. The resulting data were fit to a 1:1 Langmuir binding model using the Proteon evaluation software. The results appear in the table below.

Table 14

Sequence	k_a (1/Ms)	k_d (1/s)	$t_{1/2}$ (min)	K_D (nM)
DG (h7E6_SVG)	1.7E+05	6.9E-04	17	4.1
DK (h7E6_SVG20)	< 1.7E+05	1.3E-02	0.88	> 75
DA (h7E6_SVG22)	< 1.8E+05	1.3E-02	0.86	> 75
DL (h7E6_SVG28)	< 2.1E+05	1.6E-02	0.72	> 75
DE (h7E6_SVG30)	< 8.5E+05	6.4E-02	0.18	> 75
DS (h7E6_SVG19)	< 6.0E+05	4.5E-02	0.26	> 75

Although the disclosed teachings have been described with reference to various applications, methods, kits, and compositions, it will be appreciated that various changes and modifications can be made without departing from the teachings herein and the claimed invention below. The foregoing examples are provided to better illustrate the disclosed teachings and are not intended to limit the scope of the teachings presented herein. While the present teachings have been described in terms of these exemplary embodiments, the skilled artisan will readily understand that numerous variations and modifications of these exemplary embodiments are possible without

undue experimentation. All such variations and modifications are within the scope of the current teachings.

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 by reference in their entirety. In the event that one or more of the incorporated literature and similar materials differs from or contradicts this application, including but not limited to defined terms, term usage, described techniques, or the like, this application controls.

The foregoing description and Examples detail certain specific embodiments of the invention and describes the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear in text, the invention may be practiced in many ways and the invention should be construed in accordance with the appended claims and any equivalents thereof.

Claims

It is claimed:

1. An isolated antibody, or an antigen binding fragment thereof, which specifically binds to trophoblast cell-surface antigen-2 (Trop-2), wherein the antibody comprises

(a) a heavy chain variable (VH) region complementary determining regions comprising (i) a VH complementary determining region one (CDR1) comprising the sequence SYGVH (SEQ ID NO: 30), GGSISSY (SEQ ID NO: 36), or GGSISSYGVH (SEQ ID NO: 37); (ii) a VH CDR2 comprising the sequence VIWTX$_1$GX$_2$TDYNSALMX$_3$, wherein X$_1$ is G or S; X$_2$ is S or V; X$_3$ is S or G (SEQ ID NO: 49), or WTX$_1$GX$_2$, wherein X$_1$ is G or S, X$_2$ is S or V (SEQ ID NO: 50); and iii) a VH CDR3 comprising the sequence DYDRYTX$_1$DY, wherein X$_1$ is E or M (SEQ ID NO: 82); and/or

(b) a light chain variable region (VL) region complementary determining regions comprising (i) a VL CDR1 comprising the sequence RASKSVSTSX$_1$YSYMH, wherein X$_1$ is G, L, or N (SEQ ID NO: 63); (ii) a VL CDR2 comprising the sequence LASNLES (SEQ ID NO: 55); and (iii) a VL CDR3 comprising the sequence QHSRELPYT (SEQ ID NO: 56);

2. An isolated antibody, or an antigen binding fragment thereof, which specifically binds to Trop-2, wherein the antibody comprises:

a VH region comprising a VH CDR1, VH CDR2, and VH CDR3 of the VH sequence shown in SEQ ID NO: 5 or 85; and/or

a VL region comprising VL CDR1, VL CDR2, and VL CDR3 of the VL sequence shown in SEQ ID NO:3 or 6.

3. The antibody or the antigen binding fragment of claim 2, wherein the VH region comprises (i) a VH CDR1 comprising the sequence SYGVH (SEQ ID NO: 30), GGSISSY (SEQ ID NO: 36), or GGSISSYGVH (SEQ ID NO: 37); (ii) a VH CDR2 comprising the sequence VIWTSGVTDYNSALMG (SEQ ID NO: 38) or WTSGV (SEQ

ID NO: 39); and (iii) a VH CDR3 comprising the sequence DGDYDRYTMDY (SEQ ID NO: 35), DYDRYTMDY (SEQ ID NO: 99), or DYDRYTEDY (SEQ ID NO: 100).

4. The antibody or antigen binding fragment of claim 3, wherein the VL region comprises (i) a VL CDR1 comprising the sequence RASKSVSTSGYSYMH (SEQ ID NO: 54) or RASKSVSTSLYSYMH (SEQ ID NO: 57) ; (ii) a VL CDR2 comprising the sequence LASNLES (SEQ ID NO: 55); and (iii) a VL CDR3 comprising the sequence QHSRELPYT (SEQ ID NO: 56).

5. The antibody or the antigen binding fragment of claim 4, wherein the VH region comprises the sequence shown in SEQ ID NO: 5, 85 or a variant with one or several conservative amino acid substitutions in residues that are not within a CDR and/or the VL region comprises the amino acid sequence shown in SEQ ID NO: 3, 6 or a variant thereof with one or several amino acid substitutions in amino acids that are not within a CDR.

6. The antibody or the antigen binding fragment of claim 5, wherein the antibody comprises a light chain comprising the sequence shown in SEQ ID NO: 66 and a heavy chain comprising the sequence shown in SEQ ID NO: 65 or 102 .

7. The antibody or the antigen binding fragment of claim 3, wherein the antibody comprises a VH region produced by the expression vector with ATCC Accession No. PTA-12872.

8. The antibody or the antigen binding fragment of claim 4, wherein the antibody comprises a VL region produced by the expression vector with ATCC Accession No. PTA-12871.

9. An isolated antibody, or an antigen binding fragment thereof, which specifically binds to domain 3 and domain 4 of human Trop-2 (SEQ ID NO:27) with a monovalent antibody binding affinity (K_D) of about 6.5 nM or less as measured by surface plasmon resonance.

10. An isolated antibody, or an antigen binding fragment thereof, which specifically binds to Trop-2, wherein the antibody comprises

(a) a heavy chain variable (VH) region complementary determining regions comprising (i) a VH complementary determining region one (CDR1) comprising the sequence SYWIN (SEQ ID NO: 40), GYTFTSY (SEQ ID NO: 41), or GYTFTSYWIN (SEQ ID NO: 42); (ii) a VH CDR2 comprising the sequence $NIX_1PSDSYSNYNX_2KFKD$ wherein X_1 is Y or F; X_2 is Q or K, or X_1PSDSY wherein X_1 is Y or F (SEQ ID NO: 52); and iii) a VH CDR3 comprising the sequence GSX_1FDY wherein X_1 is S or G (SEQ ID NO: 53); and/or

(b) a light chain variable region (VL) region complementary determining regions comprising (i) a VL CDR1 comprising the sequence RASQTIGTSIH (SEQ ID NO: 59); (ii) a VL CDR2 comprising the sequence YASESIS (SEQ ID NO: 60); and (iii) a VL CDR3 comprising the sequence X_1QSX_2SWPFT wherein X_1 is Q or S; X_2 is N or F (SEQ ID NO:64).

11. An isolated antibody, or an antigen binding fragment thereof, which specifically binds to Trop-2, wherein the antibody comprises:

a VH region comprising a VH CDR1, VH CDR2, and VH CDR3 of the VH sequence shown in SEQ ID NO: 13; and/or

a VL region comprising VL CDR1, VL CDR2, and VL CDR3 of the VL sequence shown in SEQ ID NO: 12.

12. The antibody or the antigen binding fragment of claim 11, wherein the VH region comprises (i) a VH CDR1 comprising the sequence SYWIN (SEQ ID NO: 40), GYTFTSY (SEQ ID NO: 41), or GYTFTSYWIN (SEQ ID NO: 42); (ii) a VH CDR2 comprising the sequence NIFPSDSYSNYNKKFKD (SEQ ID NO: 46) or FPSDSY (SEQ ID NO: 47); and (iii) a VH CDR3 comprising the sequence GSGFDY (SEQ ID NO: 48).

13. The antibody or antigen binding fragment of claim 12, wherein the VL region comprises (i) a VL CDR1 comprising the sequence RASQTIGTSIH (SEQ ID NO: 59); (ii) a VL CDR2 comprising the sequence YASESIS (SEQ ID NO: 60); and (iii) a VL CDR3 comprising the sequence SQSFSWPFT (SEQ ID NO: 62).

14. The antibody or the antigen binding fragment of claim 13, wherein the VH region comprises the sequence shown in SEQ ID NO: 13 or a variant with one or several conservative amino acid substitutions in residues that are not within a CDR and/or the VL region comprises the amino acid sequence shown in SEQ ID NO: 12 or a variant thereof with one or several amino acid substitutions in amino acids that are not within a CDR.

15. The antibody or the antigen binding fragment of claim 14, wherein the antibody comprises a light chain comprising the sequence shown in SEQ ID NO: 68 and a heavy chain comprising the sequence shown in SEQ ID NO: 67.

16. An isolated antibody which specifically binds to Trop-2 and competes with the antibody of claim 2 or 4.

17. The antibody of claim 16, wherein the antibody has a monovalent antibody binding affinity (K_D) of about 6.5 nM or less as measured by surface plasmon resonance.

18. The antibody of any one of claims 1-15, wherein the antibody comprises an acyl donor glutamine-containing tag engineered at a specific site.

19. The antibody of claim 18, wherein the tag comprises an amino acid sequence GGLLQGG (SEQ ID NO: 78), LLQGA (SEQ ID NO:79), or LLQ.

20. The antibody of claim 19, wherein the antibody further comprises an amino acid modification at position 222, 340, or 370.

21. The antibody of claim 20, wherein the amino acid modification is a substitution from lysine to arginine.

22. A conjugate of the antibody or the antigen binding fragment of any one of claims 1-15 and 18-21, wherein the antibody or the antigen binding fragment is conjugated to an agent, wherein the agent is selected from the group consisting of a cytotoxic agent, an immunomodulating agent, an imaging agent, a therapeutic protein, a biopolymer, and an oligonucleotide.

23. The conjugate of claim 22, wherein the agent is a cytotoxic agent.

24. The conjugate of claim 23, wherein the cytotoxic agent is MMAD (Monomethyl Auristatin D) or 0101 (2-methylalanyl-*N*-[(3*R*,4*S*,5*S*)-3-methoxy-1-{(2*S*)-2-[(1*R*,2*R*)-1-methoxy-2-methyl-3-oxo-3-{[(1*S*)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]amino}propyl]pyrrolidin-1-yl}-5-methyl-1-oxoheptan-4-yl]-*N*-methyl-L-valinamide).

25. The conjugate of claim 24, wherein the conjugate comprises the formula: antibody-(acyl donor glutamine-containing tag)-(linker)-(cytotoxic agent).

26. The conjugate of claim 25, wherein the acyl donor glutamine-containing tag comprises an amino acid sequence GGLLQGG (SEQ ID NO: 78), LLQGA (SEQ NO: 79), LLQ, or $LLQX_1X_2X_3X_4X_5$, wherein X_1 is G or P, wherein X_2 is A, G, P, or absent, wherein X_3 is A, G, K, P, or absent, wherein X_4 is G, K, or absent, and wherein X_5 is K or absent (SEQ ID NO: 88), and wherein the linker comprises acetyl-lysine-valine-citrulline-*p*-aminobenzyloxycarbonyl or amino-PEG6-propionyl.

27. The conjugate of claim 26, wherein the conjugate is selected from the group consisting of 1) antibody-LLQGA (SEQ ID NO: 79)- (acetyl-lysine-valine-citrulline-*p*-aminobenzyloxycarbonyl (AcLys-VC-PABC))-0101; 2) antibody-LLQGA (SEQ ID NO: 79)-(AcLys-VC-PABC)-MMAD; 3) antibody-$LLQX_1X_2X_3X_4X_5$ (SEQ ID NO: 88)-(AcLys-VC-PABC)-0101; 4) antibody-$LLQX_1X_2X_3X_4X_5$ (SEQ ID NO: 88)-(AcLys-VC-PABC)-MMAD; 5) antibody –GGLLQGG (SEQ ID NO: 78)- (AcLys-VC-PABC)-0101; and 6) antibody–GGLLQGG (SEQ ID NO: 78)-(AcLys-VC-PABC)-MMAD.

28. The conjugate of claim 27, wherein the conjugate 1) antibody –GGLLQGG (SEQ ID NO: 78)-(AcLys-VC-PABC)-0101; 2) antibody–GGLLQGG (SEQ ID NO: 78)-(AcLys-VC-PABC)-MMAD; 3) antibody-$LLQX_1X_2X_3X_4X_5$ (SEQ ID NO: 88)-(AcLys-VC-PABC)-0101; 4) antibody-$LLQX_1X_2X_3X_4X_5$ (SEQ ID NO: 88)-(AcLys-VC-PABC)-MMAD; 5) antibody-LLQGA (SEQ ID NO: 79)-(AcLys-VC-PABC)-0101; or 6) antibody-LLQGA (SEQ ID NO: 79)- AcLys-VC-PABC-MMAD comprises an amino acid substitution from lysine to arginine at position 222.

29. The conjugate of claim 27, wherein the conjugate 1) antibody-LLQGA (SEQ ID NO: 79)-(AcLys-VC-PABC)-0101; 2) antibody-LLQGA (SEQ ID NO: 79)-

AcLys-VC-PABC-MMAD; 3) antibody-LLQX$_1$X$_2$X$_3$X$_4$X$_5$ (SEQ ID NO: 88)-(AcLys-VC-PABC)-0101; or 4) antibody-LLQX$_1$X$_2$X$_3$X$_4$X$_5$ (SEQ ID NO: 88)-(AcLys-VC-PABC)-MMAD, wherein the amino acid lysine at the C-terminus of heavy chain of the antibody is deleted.

30. The conjugate of claim 24, wherein the conjugate comprises amino acid substitutions at position N297Q and K222R, a linker comprising amino-PEG6-propionyl, and a cytotoxic agent comprising MMAD.

31. A pharmaceutical composition comprising a therapeutically effective amount of the antibody of any one of claims 1-15 or the conjugate of any one of claims 22-30 and a pharmaceutically acceptable carrier.

32. An isolated polynucleotide comprising a nucleotide sequence encoding the antibody of any one of claims 1-15.

33. A vector comprising the polynucleotide of claim 32.

34. An isolated host cell that recombinantly produces the antibody of any one of claims 1-15.

35. A method of producing an antibody, comprising culturing the host cell of claim 34 under conditions that result in production of the antibody, and isolating the antibody from the host cell or culture.

36. A method of treating a condition associated with Trop-2 expression in a subject comprising administering to a subject in need thereof an effective amount of the pharmaceutical composition of claim 31.

37. The method of claim 36, wherein the condition is a cancer.

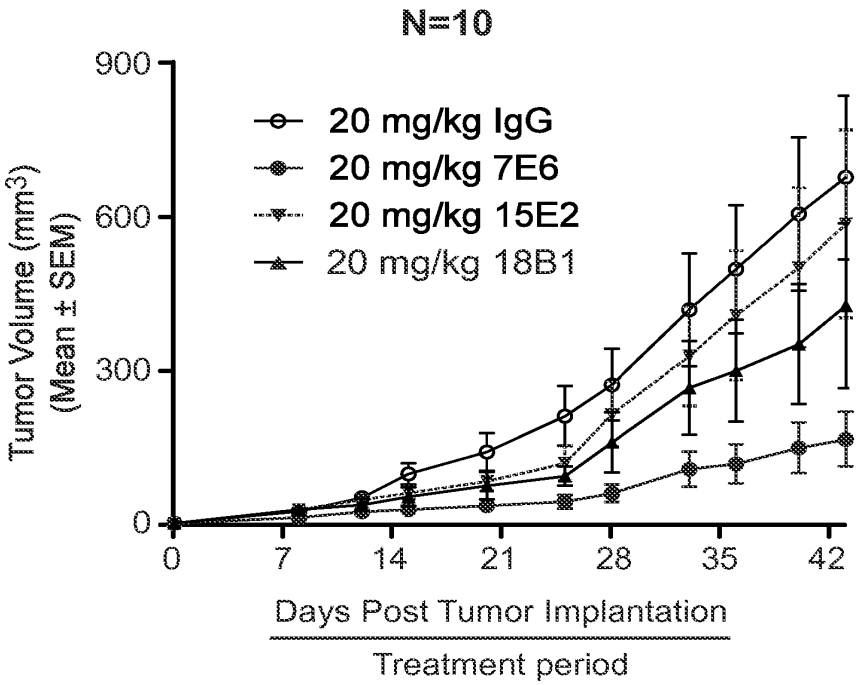
38. The method of claim 37, wherein the cancer is selected from the group consisting of bladder, breast, cervical, choriocarcinoma, colon, esophageal, gastric, glioblastoma, head and neck, kidney, lung, oral, ovarian, pancreatic, prostate, and skin cancer

39. A method of inhibiting tumor growth or progression in a subject who has a Trop-2 expressing tumor, comprising administering to the subject in need thereof an effective amount of the pharmaceutical composition of claim 31 to the subject.

40. A method of inhibiting metastasis of Trop-2 expressing cancer cells in a subject, comprising administering to the subject in need thereof an effective amount of the pharmaceutical composition of claim 31 to the subject.

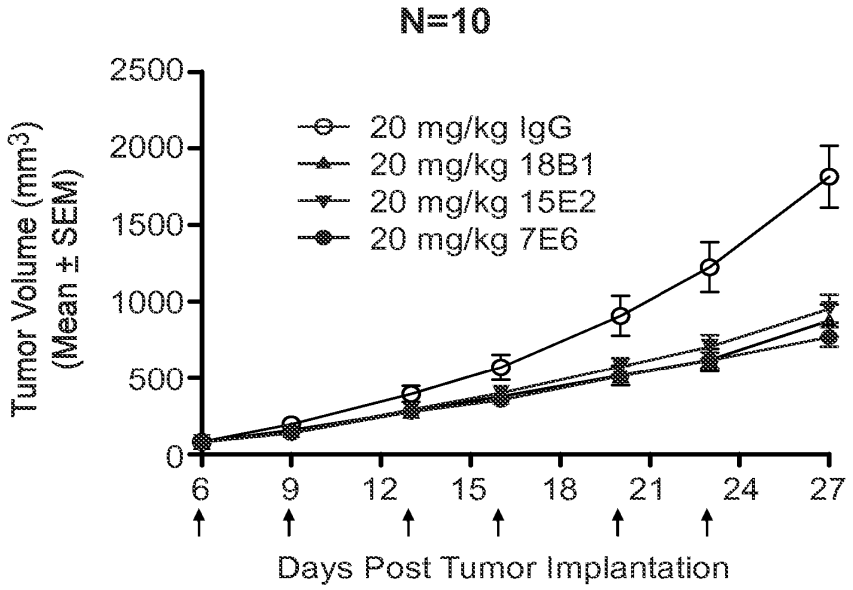
41. A method of induce tumor regression in a subject who has a Trop-2 expressing tumor, comprising administering to the subject in need thereof an effective amount of the pharmaceutical composition of claim 31 to the subject.

Figure 1



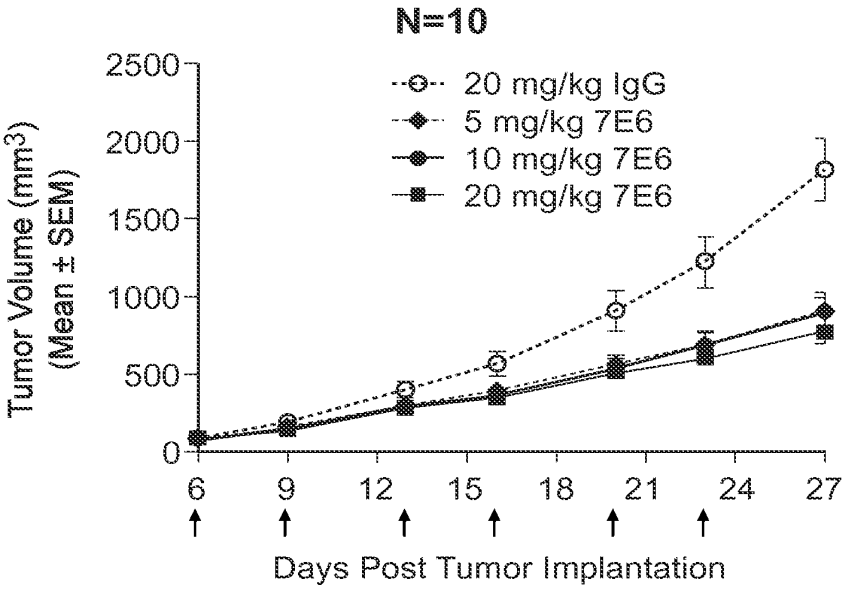
antibody treatment	Mean tumor volume (mm^3) on day 43	TGI (Tumor growth inhibition)
control IgG	673.0	0%
7E6	163.8	76%
15E2	582.0	14%
18B1	423.5	37%

Figure 2A



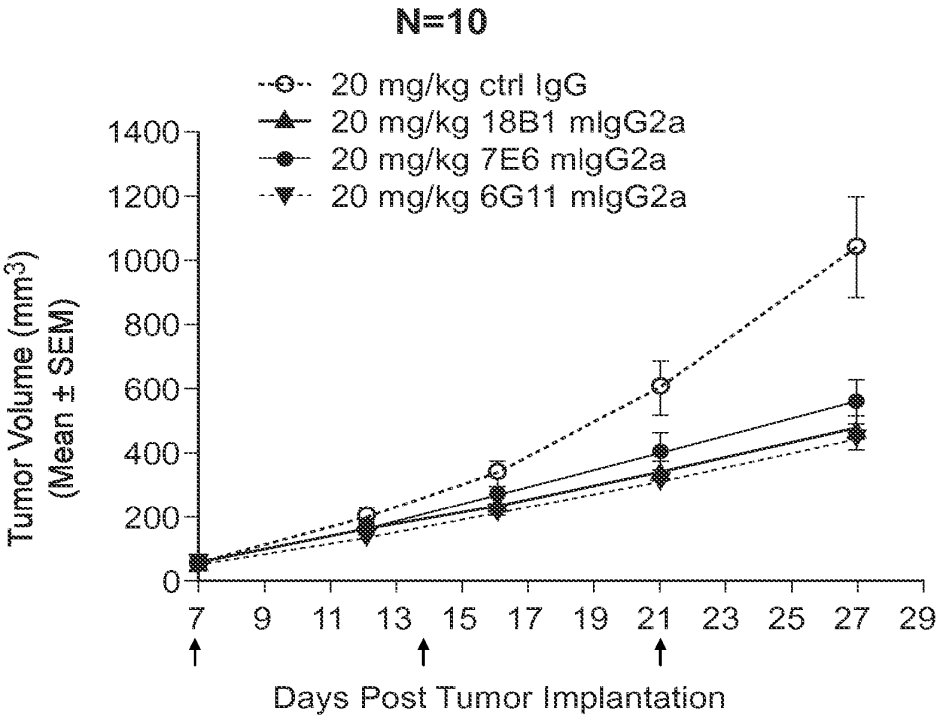
antibody treatment	Mean tumor volume (mm3) on day 27	TGI (Tumor growth inhibition)
control IgG	1814.8	0%
7E6	770.7	58%
15E2	953.6	47%
18B1	875.5	52%

Figure 2B



antibody treatment	Mean tumor volume (mm3) on day 27	TGI (Tumor growth inhibition)
control IgG	1814.8	0%
7E6-5mg/kg	913.9	50%
7E6-10mg/kg	897.7	51%
7E6-20mg/kg	875.5	52%

Figure 2C



antibody treatment	Mean tumor volume (mm3) on day 27	TGI (Tumor growth inhibition)
control IgG	1044.0	0%
6G11	444.8	57%
7E6	561.0	46%
18B1	477.6	54%

Figure 3

antibody	% specific lysis		
	0.01ug/mL	0.10ug/mL	1.00ug/mL
isotope control	1 ± 1.6	2 ± 1.0	2 ± 0.4
7E6	7 ± 1.1	17 ± 1.4	29 ± 1.8
h7E6-WT	7 ± 0.8	18 ± 0.0	32 ± 0.6
h7E6-SVG	9 ± 1.7	26 ± 0.9	35 ± 2.3
h7E6-L	9 ± 1.1	27 ± 1.3	29 ± 2.3
h7E6-SVGL	7 ± 1.8	24 ± 1.0	23 ± 2.1
antl-EGFR	12 ± 2.4	32 ± 1.7	33 ± 0.9

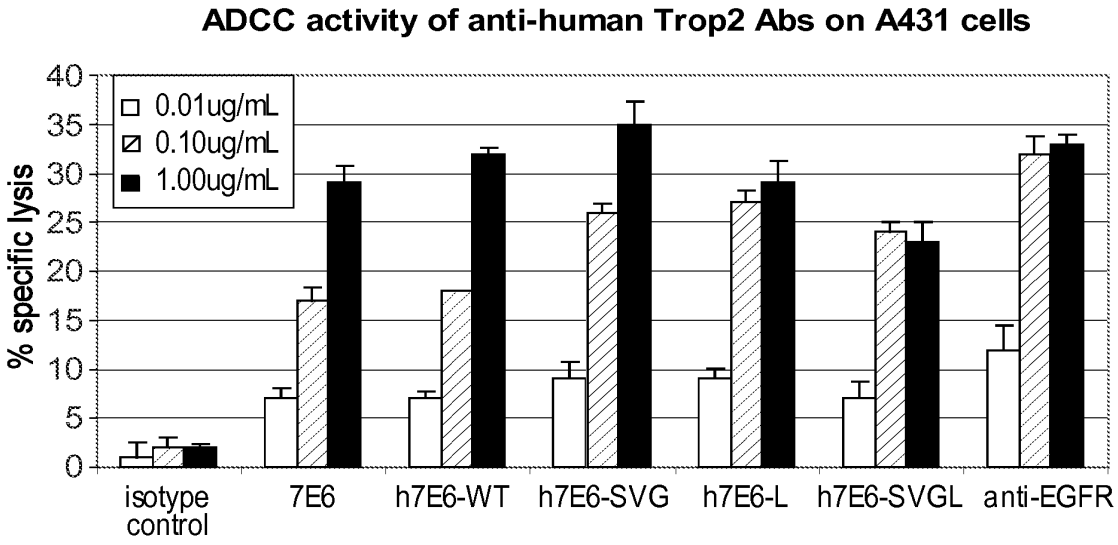


Figure 4

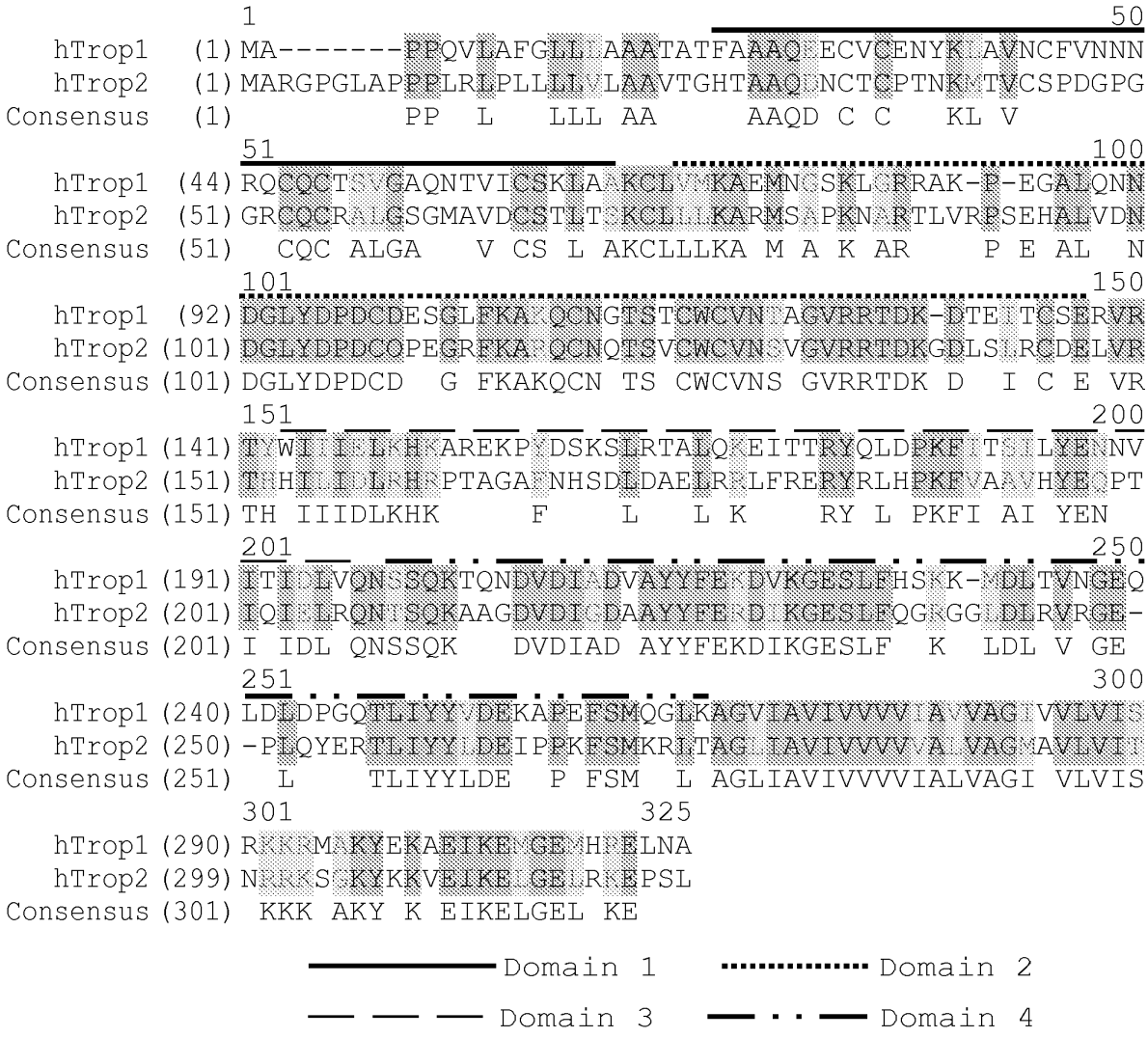

1 50
hTrop1 (1) MA-------PPQVLAFGLLLAAATATFAAAQECVCENYKLAVNCFVNNN
hTrop2 (1) MARGPGLAPPPLRLPLLLLVLAAVTGHTAAQNCTCPTNKMTVCSPDGPG
Consensus (1) PP L LLL AA AAQD C C KL V
51 100
hTrop1 (44) RQCQCTSVGAQNTVICSKLAAKCLVMKAEMNGSKLGRRAK-P-EGALQNN
hTrop2 (51) GRCQCRALGSGMAVDCSTLTSKCLLLKARMSAPKNARTLVRPSEHALVDN
Consensus (51) CQC ALGA V CS L AKCLLLKA M A K AR P E AL N
101 150
hTrop1 (92) DGLYDPDCDESGLFKAKQCNGTSTCWCVNSAGVRRTDK-DTEITCSERVR
hTrop2 (101) DGLYDPDCQPEGRFKAQQCNQTSVCWCVNSVGVRRTDKGDLSLRCDELVR
Consensus (101) DGLYDPDCD G FKAKQCN TS CWCVNS GVRRTDK D I C E VR
151 200
hTrop1 (141) TYWIIILKHRAREKPYDSKSLRTALQKEITTRYQLDPKFITSILYENNV
hTrop2 (151) THHIIDLKHKPTAGAFNHSDLDAEIRKLFRERYRLHPKFVAAVHYEQPT
Consensus (151) TH IIIDLKHK F L L K RY L PKFI AI YEN
201 250
hTrop1 (191) ITIDLVQNSSQKTQNDVDIADVAYYFEKDVKGESLFHSKK-MDLTVNGEQ
hTrop2 (201) IQIILRQNISQKAAGDVDICDAAYYFERDIKGESLFQGKGGIDIRVRGE-
Consensus (201) I IDL QNSSQK DVDIAD AYYFEKDIKGESLF K LDL V GE
251 300
hTrop1 (240) LDLDPGQTLIYYVDEKAPEFSMQGLKAGVIAVIVVVVIAVVAGIVVLVIS
hTrop2 (250) -PLQYERTLIYYIDEIPPKFSMKRITAGIIAVIVVVVIAVAGMAVLVIT
Consensus (251) L TLIYYLDE P FSM L AGLIAVIVVVVIALVAGI VLVIS
301 325
hTrop1 (290) RKKRMAKYEKAEIKEMGEMHRELNA
hTrop2 (299) NRRKSIKYKKVEIKEIGELRKEPSL
Consensus (301) KKK AKY K EIKELGEL KE
Domain 1
Domain 2
Domain 3
Domain 4

Figure 5

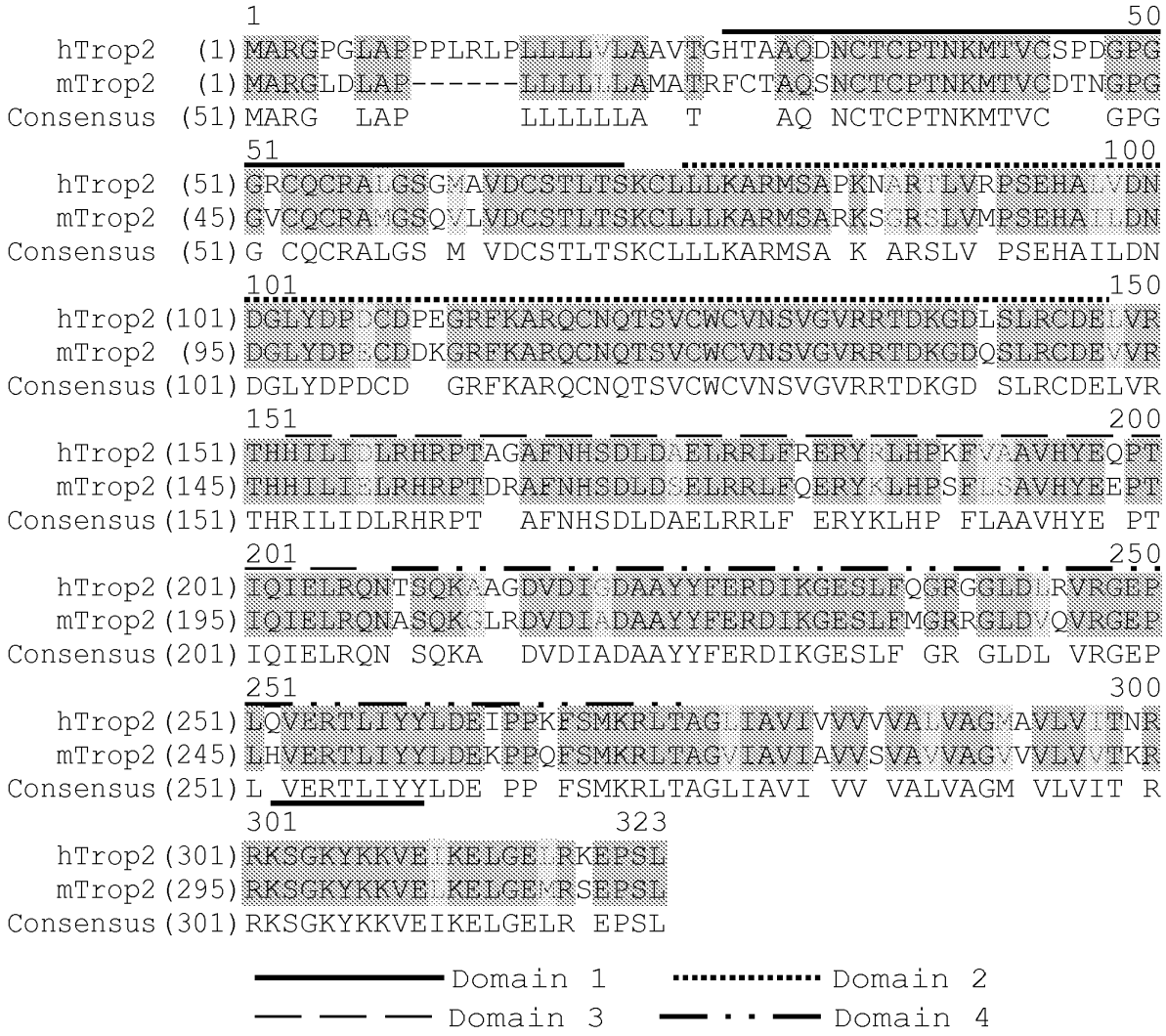

1 50
hTrop2 (1) MARGPGLAPPPLRLPLLLLLVLAAVTGHTAAQDNCTCPTNKMTVCSPDGPG
mTrop2 (1) MARGLDLAP------LLLLLLAMATRFCTAQSNCTCPTNKMTVCDTNGPG
Consensus (51) MARG LAP LLLLLLA T AQ NCTCPTNKMTVC GPG
51 100
hTrop2 (51) GRCQCRALGSGMAVDCSTLTSKCLLLKARMSAPKNARLVRPSEHAIVDN
mTrop2 (45) GVCQCRAMGSQVLVDCSTLTSKCLLLKARMSARKSGRLVMPSEHAILDN
Consensus (51) G CQCRALGS M VDCSTLTSKCLLLKARMSA K ARSLV PSEHAILDN
101 150
hTrop2 (101) DGLYDPDCDPEGRFKARQCNQTSVCWCVNSVGVRRTDKGDLSLRCDELVR
mTrop2 (95) DGLYDPDCDDKGRFKARQCNQTSVCWCVNSVGVRRTDKGDQSLRCDEVVR
Consensus (101) DGLYDPDCD GRFKARQCNQTSVCWCVNSVGVRRTDKGD SLRCDELVR
151 200
hTrop2 (151) THHILILRHRPTAGAFNHSDLDAELRRLFRERYKLHPKFVAAVHYEQPT
mTrop2 (145) THHILILRHRPTDRAFNHSDLDAELRRLFQERYKLHPSFLSAVHYEEPT
Consensus (151) THRILIDLRHRPT AFNHSDLDAELRRLF ERYKLHP FLAAVHYE PT
201 250
hTrop2 (201) IQIELRQNTSQKAAGDVDIADAAYYFERDIKGESLFQGRGGLDLRVRGEP
mTrop2 (195) IQIELRQNASQKSLRDVDIADAAYYFERDIKGESLFMGRRGLDVQVRGEP
Consensus (201) IQIELRQN SQKA DVDIADAAYYFERDIKGESLF GR GLDL VRGEP
251 300
hTrop2 (251) LQVERTLIYYLDEIPPKFSMKRLTAGIAVIVVVVVALVAGMAVLVITNR
mTrop2 (245) LHVERTLIYYLDEKPPQFSMKRLTAGVIAVIAVVSVAVVAGVVLVVTKR
Consensus (251) L VERTLIYYLDE PP FSMKRLTAGLIAVI VV VALVAGM VLVIT R
301 323
hTrop2 (301) RKSGKYKKVEIKELGELRKEPSL
mTrop2 (295) RKSGKYKKVEIKELGELRSEPSL
Consensus (301) RKSGKYKKVEIKELGELR EPSL
Domain 1
Domain 2
Domain 3
Domain 4

Figure 6A



Figure 6B

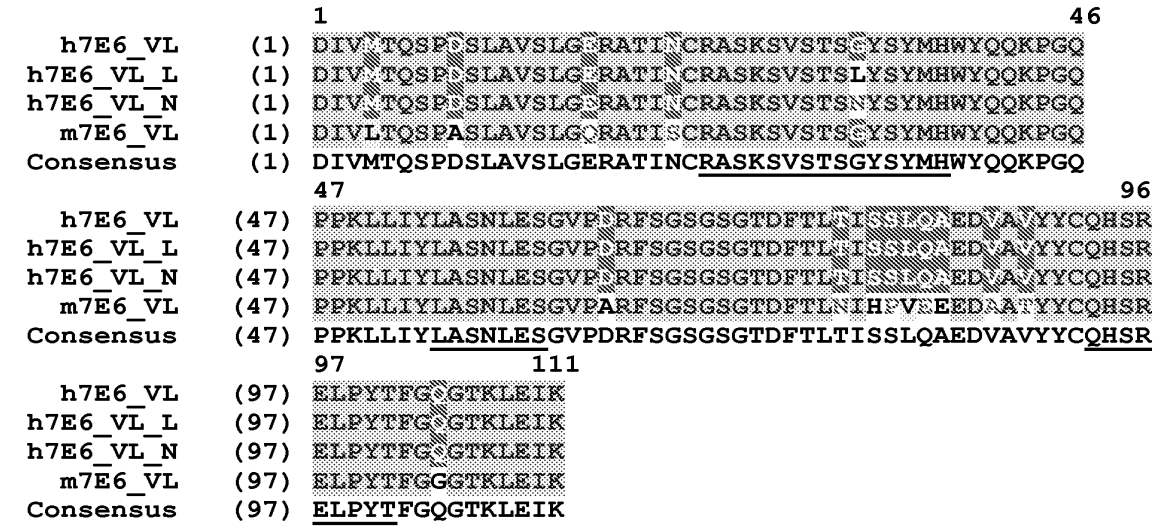


Figure 7A

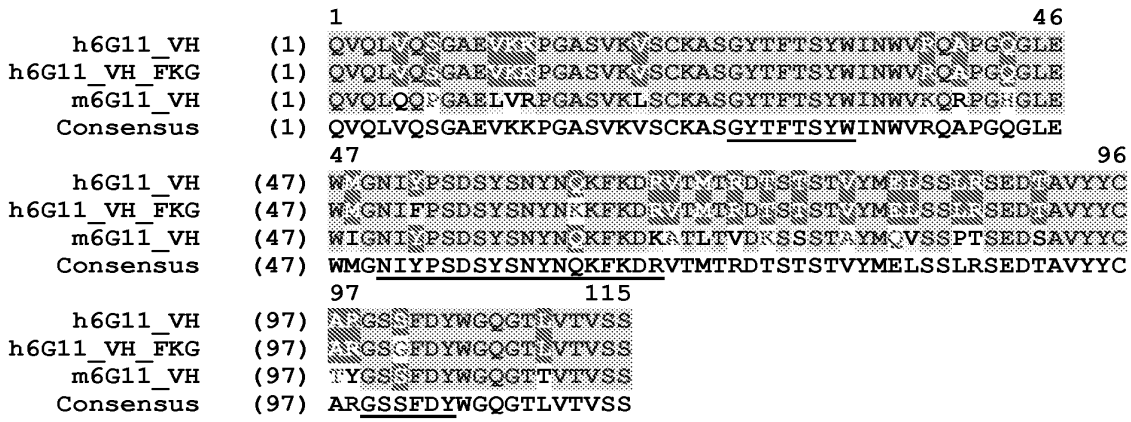


Figure 7B

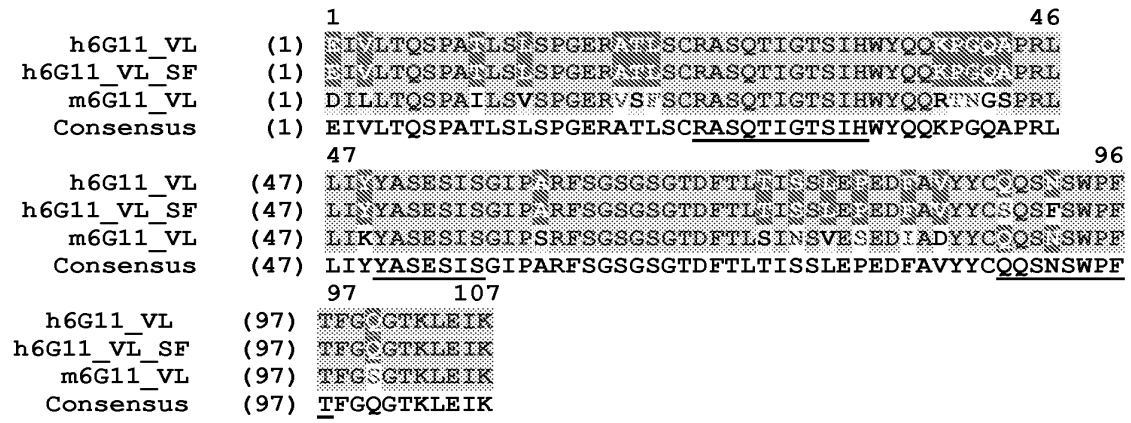
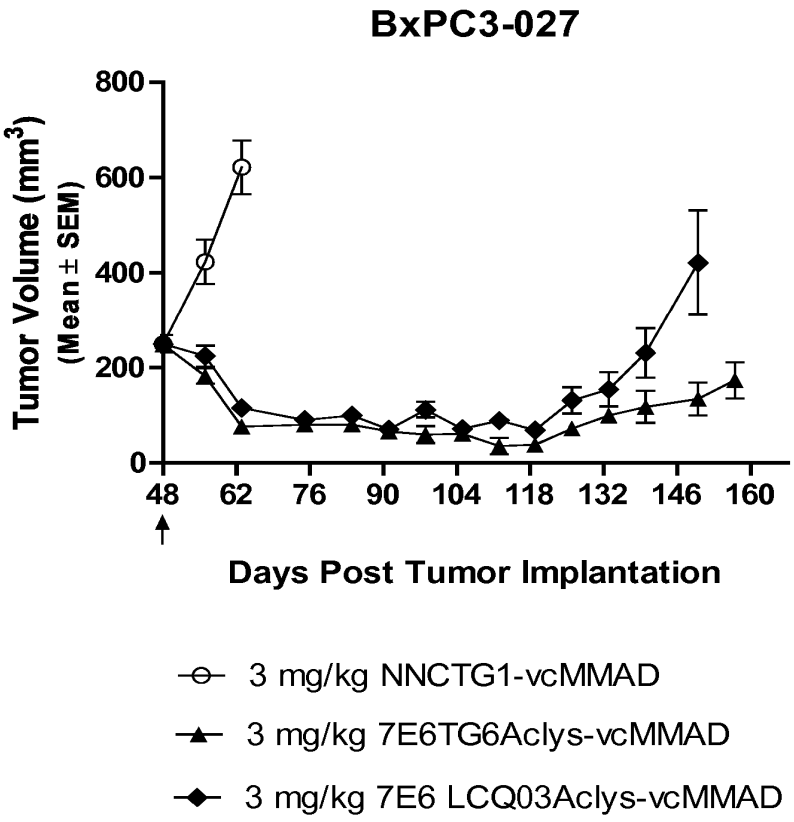


Figure 8


BxPC3-027
Tumor Volume (mm3)
(Mean ± SEM)
800
600
400
200
0
48
62
76
90
104
118
132
146
160
Days Post Tumor Implantation
3 mg/kg NNCTG1-vcMMAD
3 mg/kg 7E6TG6Aclys-vcMMAD
3 mg/kg 7E6 LCQ03Aclys-vcMMAD

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Figure 9A

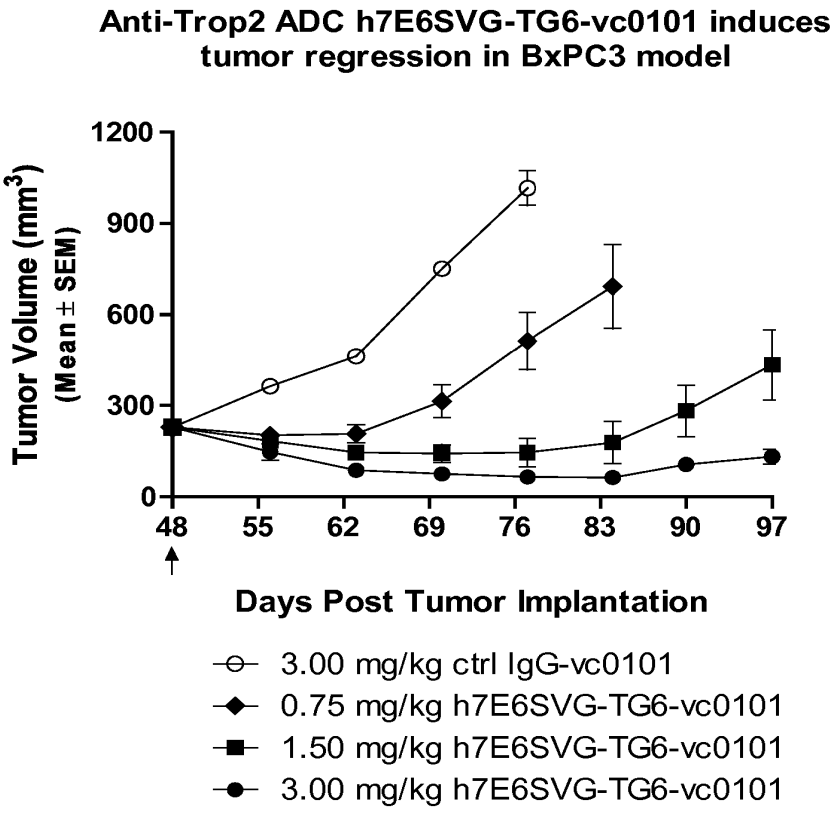


Figure 9B

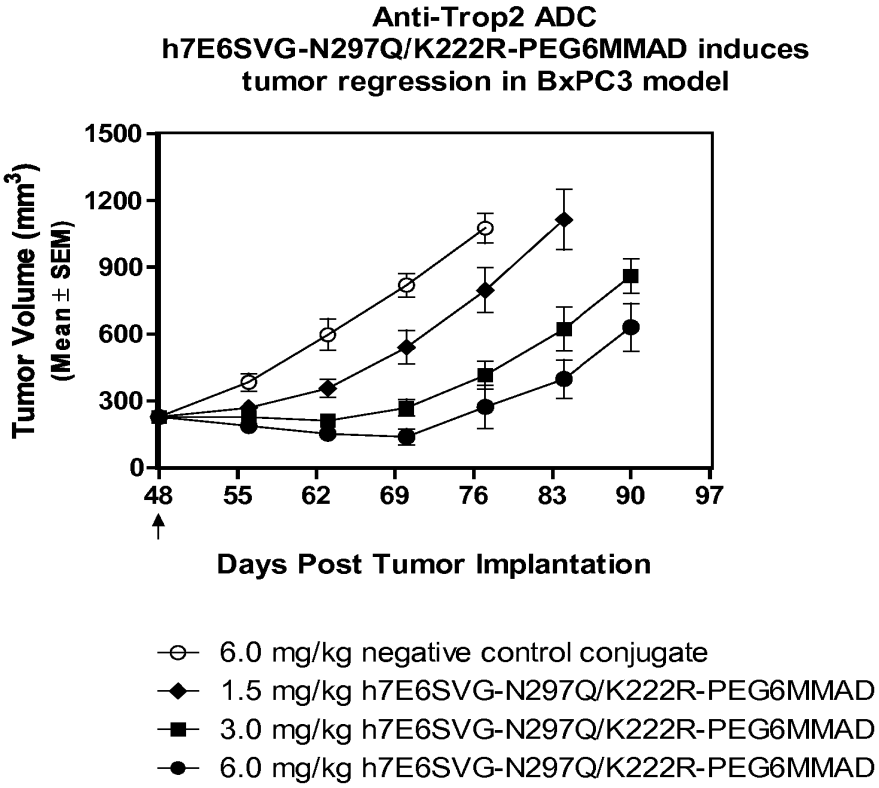

Anti-Trop2 ADC
h7E6SVG-N297Q/K222R-PEG6MMAD induces
tumor regression in BxPC3 model
Tumor Volume (mm3)
(Mean ± SEM)
1500
1200
900
600
300
0
48
55
62
69
76
83
90
97
Days Post Tumor Implantation
6.0 mg/kg negative control conjugate
1.5 mg/kg h7E6SVG-N297Q/K222R-PEG6MMAD
3.0 mg/kg h7E6SVG-N297Q/K222R-PEG6MMAD
6.0 mg/kg h7E6SVG-N297Q/K222R-PEG6MMAD

Figure 9C

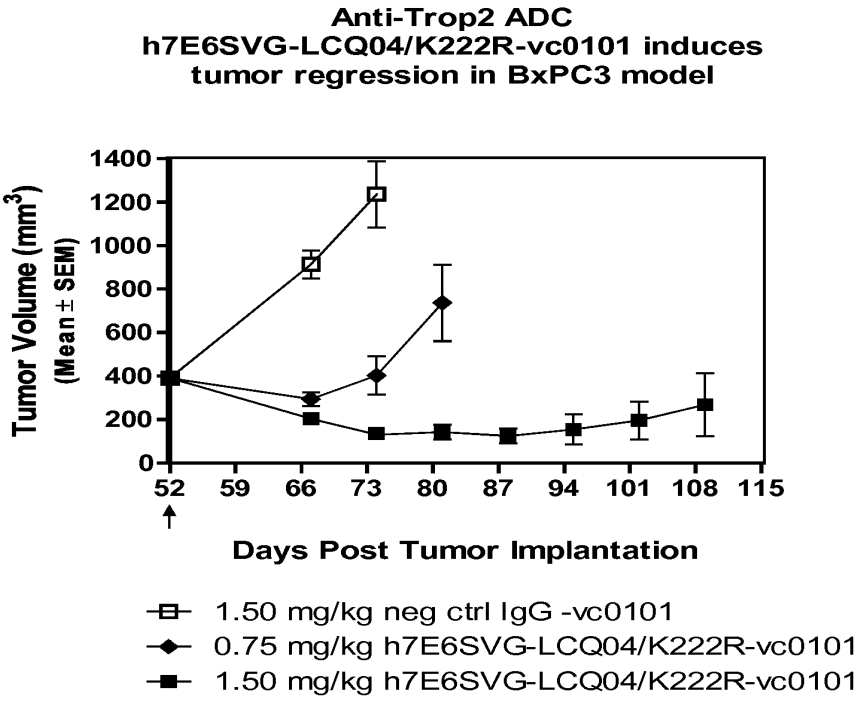

Anti-Trop2 ADC
h7E6SVG-LCQ04/K222R-vc0101 induces
tumor regression in BxPC3 model
Tumor Volume (mm3)
(Mean ± SEM)
1400
1200
1000
800
600
400
200
0
52
59
66
73
80
87
94
101
108
115
Days Post Tumor Implantation
1.50 mg/kg neg ctrl IgG -vc0101
0.75 mg/kg h7E6SVG-LCQ04/K222R-vc0101
1.50 mg/kg h7E6SVG-LCQ04/K222R-vc0101

Figure 10

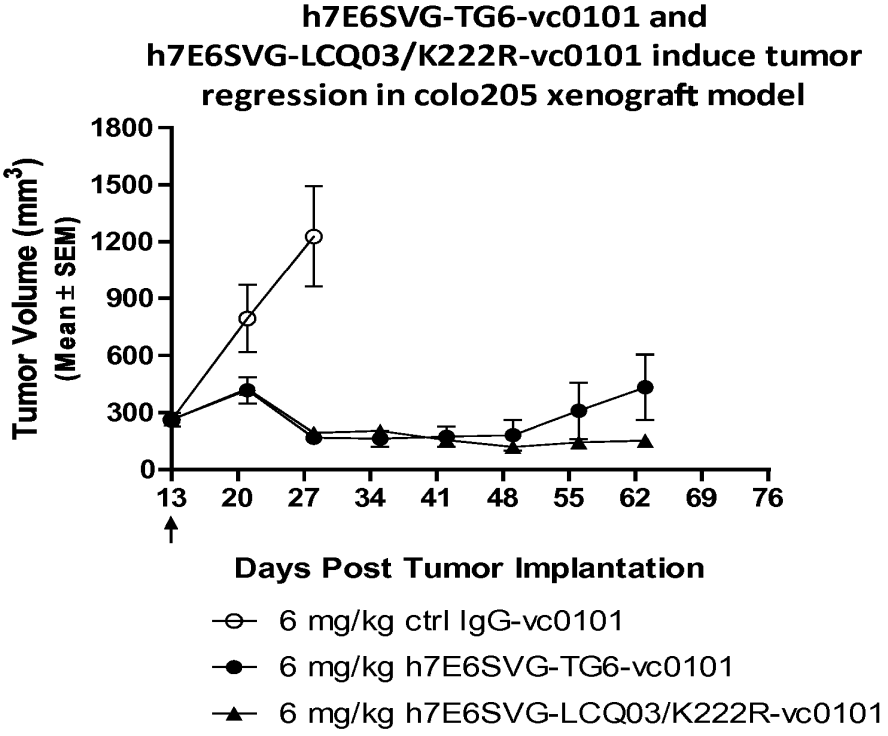

h7E6SVG-TG6-vc0101 and
h7E6SVG-LCQ03/K222R-vc0101 induce tumor
regression in colo205 xenograft model
Tumor Volume (mm3)
(Mean ± SEM)
1800
1500
1200
900
600
300
0
13
20
27
34
41
48
55
62
69
76
Days Post Tumor Implantation
6 mg/kg ctrl IgG-vc0101
6 mg/kg h7E6SVG-TG6-vc0101
6 mg/kg h7E6SVG-LCQ03/K222R-vc0101

Figure 11

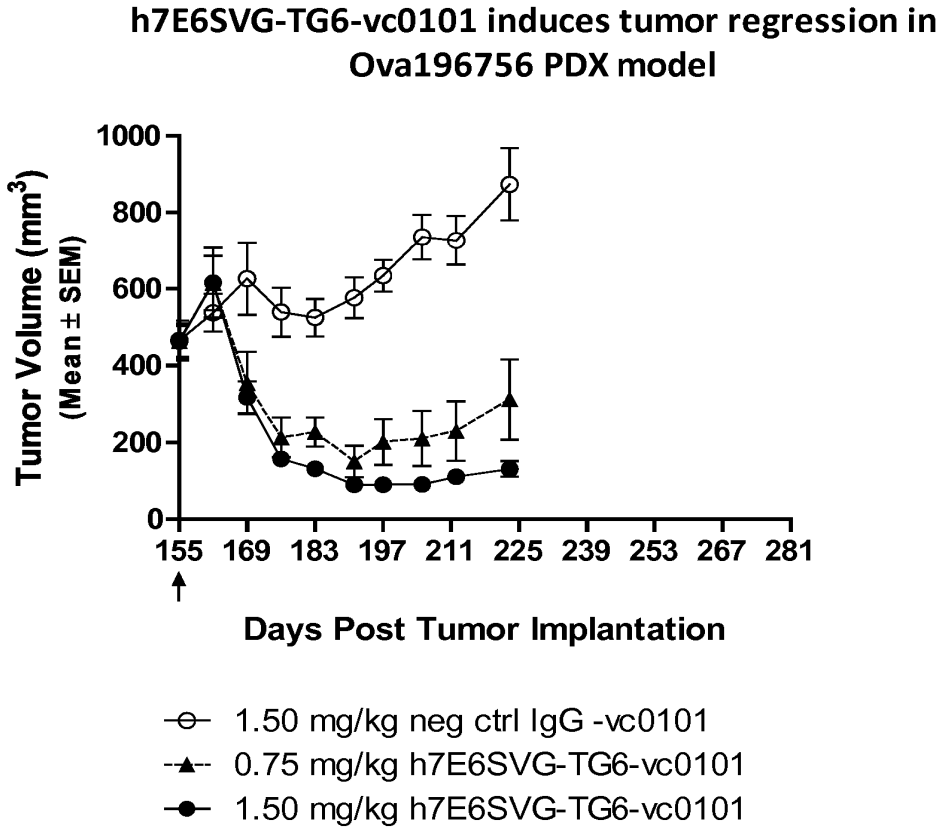

h7E6SVG-TG6-vc0101 induces tumor regression in Ova196756 PDX model
Tumor Volume (mm3) (Mean ± SEM)
1000
800
600
400
200
0
155 169 183 197 211 225 239 253 267 281
Days Post Tumor Implantation
1.50 mg/kg neg ctrl IgG -vc0101
0.75 mg/kg h7E6SVG-TG6-vc0101
1.50 mg/kg h7E6SVG-TG6-vc0101

Figure 12A

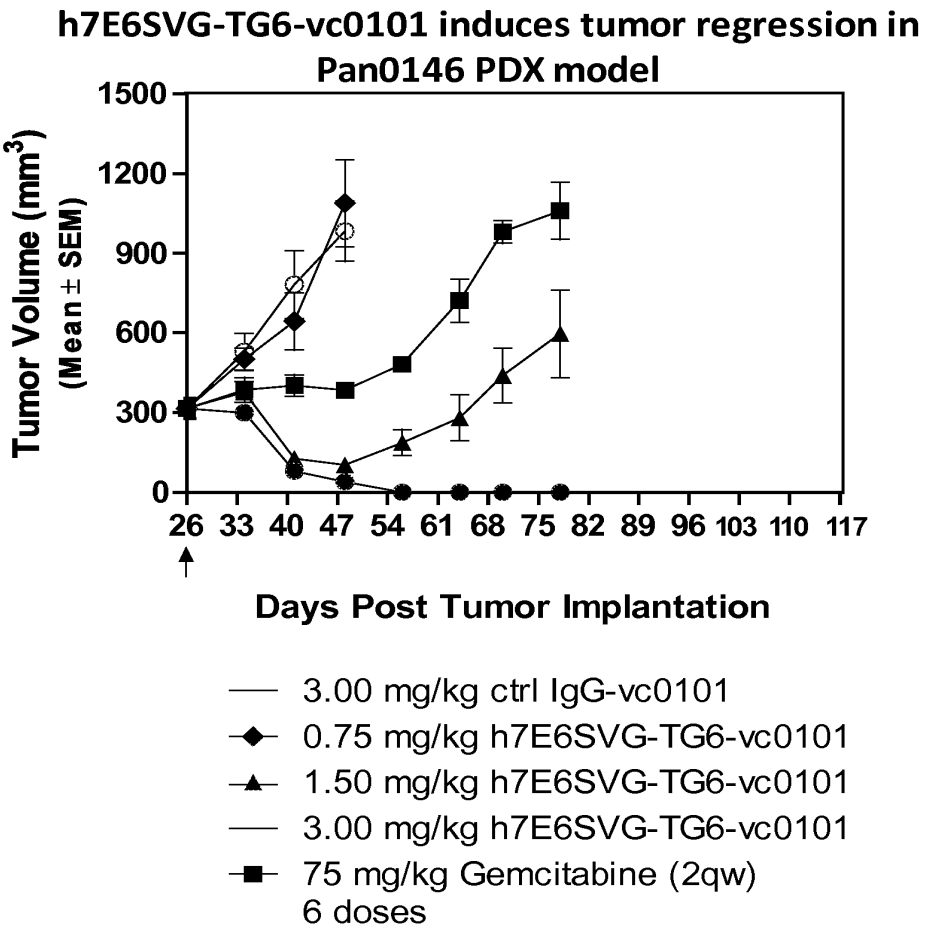

h7E6SVG-TG6-vc0101 induces tumor regression in Pan0146 PDX model
Tumor Volume (mm3) (Mean ± SEM)
1500
1200
900
600
300
0
26 33 40 47 54 61 68 75 82 89 96 103 110 117
Days Post Tumor Implantation
3.00 mg/kg ctrl IgG-vc0101
0.75 mg/kg h7E6SVG-TG6-vc0101
1.50 mg/kg h7E6SVG-TG6-vc0101
3.00 mg/kg h7E6SVG-TG6-vc0101
75 mg/kg Gemcitabine (2qw) 6 doses

Figure 12B

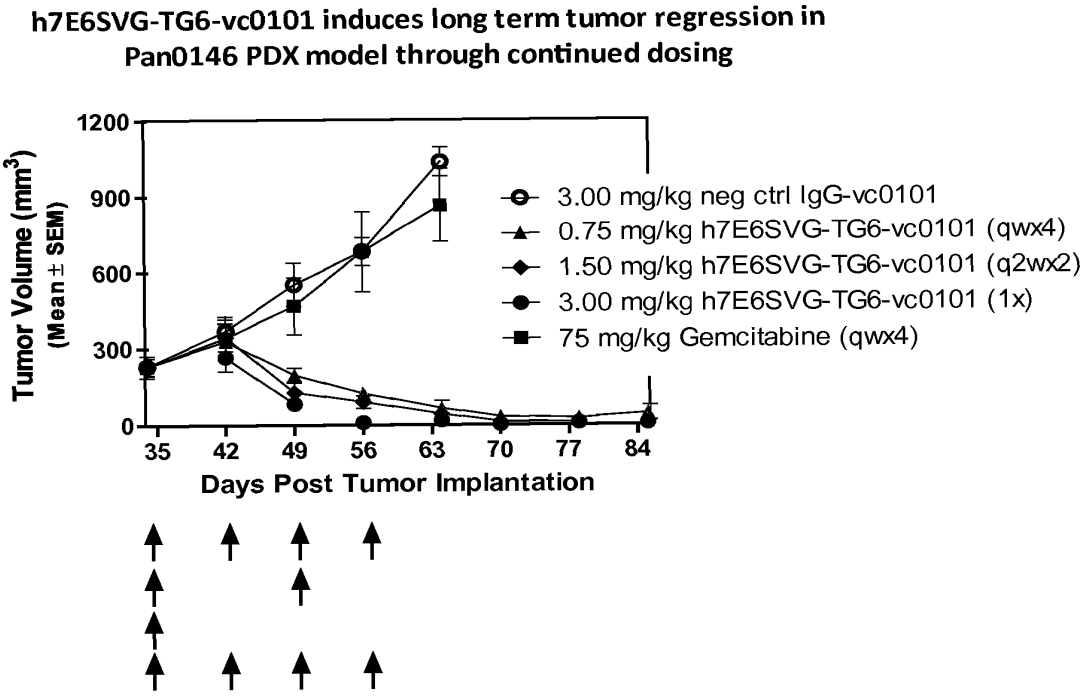

h7E6SVG-TG6-vc0101 induces long term tumor regression in
Pan0146 PDX model through continued dosing
Tumor Volume (mm3)
(Mean ± SEM)
1200
900
600
300
0
35
42
49
56
63
70
77
84
Days Post Tumor Implantation
3.00 mg/kg neg ctrl IgG-vc0101
0.75 mg/kg h7E6SVG-TG6-vc0101 (qwx4)
1.50 mg/kg h7E6SVG-TG6-vc0101 (q2wx2)
3.00 mg/kg h7E6SVG-TG6-vc0101 (1x)
75 mg/kg Gemcitabine (qwx4)

Figure 13

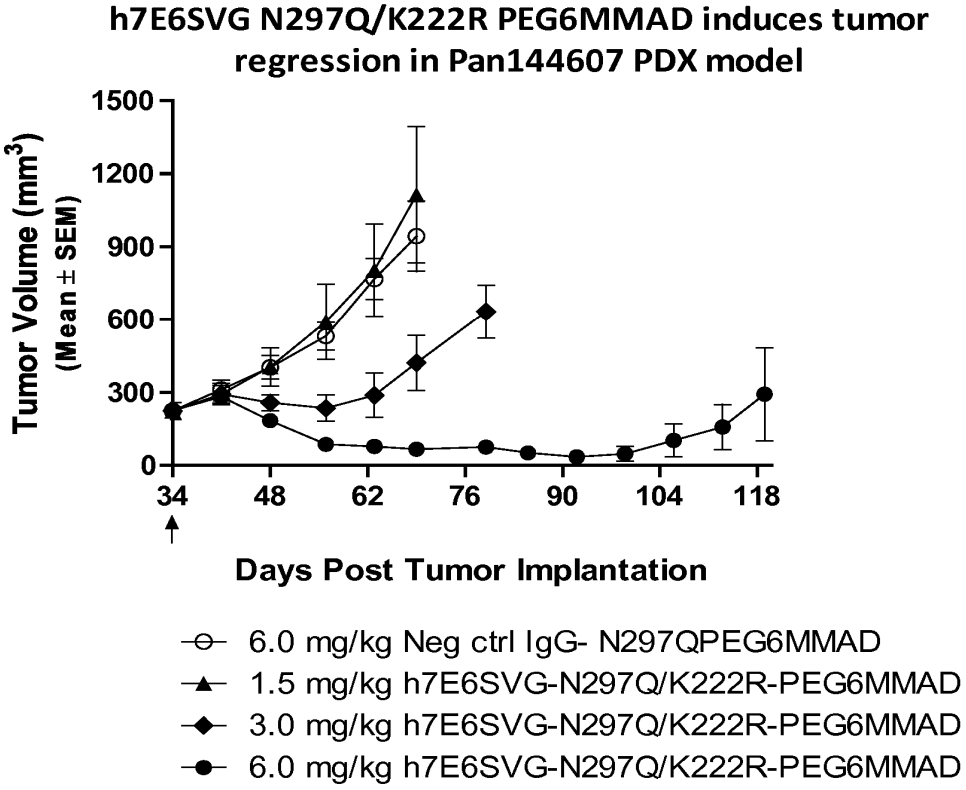
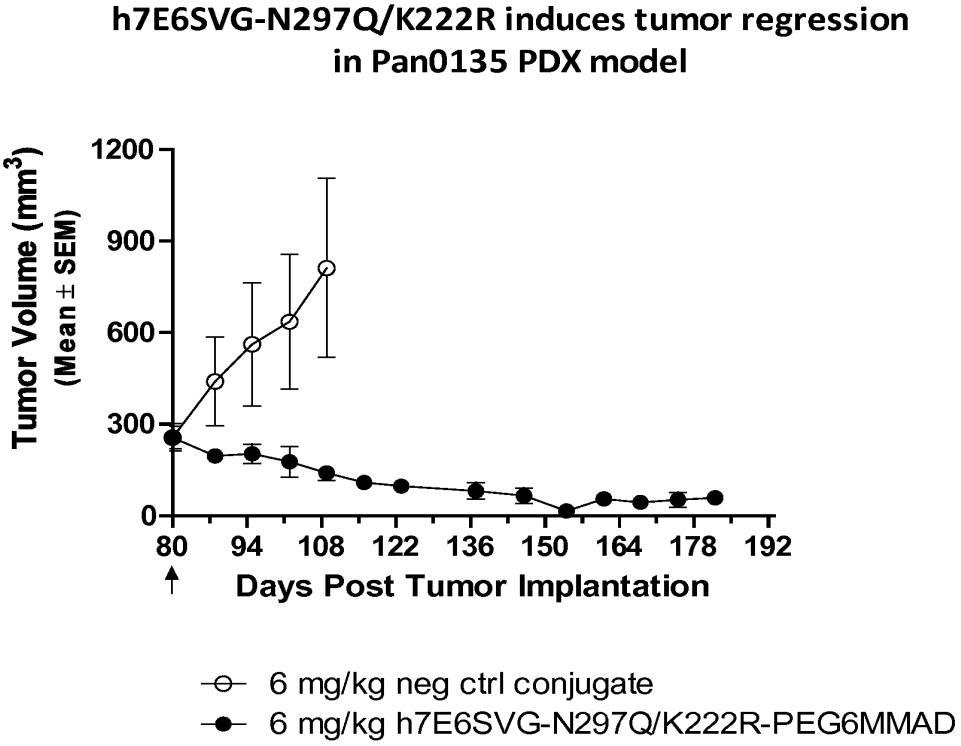

h7E6SVG N297Q/K222R PEG6MMAD induces tumor regression in Pan144607 PDX model
Tumor Volume (mm3) (Mean ± SEM)
1500
1200
900
600
300
0
34
48
62
76
90
104
118
Days Post Tumor Implantation
6.0 mg/kg Neg ctrl IgG- N297QPEG6MMAD
1.5 mg/kg h7E6SVG-N297Q/K222R-PEG6MMAD
3.0 mg/kg h7E6SVG-N297Q/K222R-PEG6MMAD
6.0 mg/kg h7E6SVG-N297Q/K222R-PEG6MMAD

Figure 14


h7E6SVG-N297Q/K222R induces tumor regression
in Pan0135 PDX model
Tumor Volume (mm3)
(Mean ± SEM)
1200
900
600
300
0
80
94
108
122
136
150
164
178
192
Days Post Tumor Implantation
6 mg/kg neg ctrl conjugate
6 mg/kg h7E6SVG-N297Q/K222R-PEG6MMAD

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(71) 出願人: 株式会社リブテック(LIVTECH, INC.) [JP/JP]; 〒2160001 神奈川県川崎市宮前区野川９０７ 帝京大学生物工学研究センター１Ｆ Kanagawa (JP).

(72) 発明者: 中村康司(NAKAMURA, Koji); 〒1830033 東京都府中市分梅町五丁目３５番地の１４ エルデ１０３ Tokyo (JP). 岡村健太郎(OKAMURA, Kentaro); 〒2160035 神奈川県川崎市宮前区馬絹１３２１番地４ カサドＭ１０３ Kanagawa (JP). 田村真紀(TAMURA, Maki); 〒2130015 神奈川県川崎市高津区梶ヶ谷２丁目１番地１３ イルニード７０１ Kanagawa (JP). 柳内浩之(YANAI, Hiroyuki); 〒2230062 神奈川県横浜市港北区日吉本町六丁目９番２２－２Ｃ号 Kanagawa (JP). 菅家徹(KANKE, Toru); 〒2160004 神奈川県川崎市宮前区鷺沼４丁目１番地２ 鷺沼サン・エルサ２０３ Kanagawa (JP). 鶴下直也(TSURUSHITA, Naoya); 94306 カリフォルニア州パロアルト市レッドウッドサークル３７１９番地 California (US). クマールシャンカール(KUMAR, Shankar); 94588 カリフォルニア州プリーザントン市クラールプレイス３９３０番地 California (US).

(74) 代理人: 小林浩, 外(KOBAYASHI, Hiroshi et al.); 〒1040028 東京都中央区八重洲二丁目８番７号福岡ビル９階 阿部・井窪・片山法律事務所 Tokyo (JP).

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— 規則 13 の 2 に基づいて明細書とは別に提出された、寄託された生物材料に関する表示（規則 13 の 2.4(d)(i)及び 48.2(a)(viii)）
— 明細書の別個の部分として表した配列リスト（規則 5.2(a)）

WO 2013/077458 A1

(54) **Title**: ANTI-HUMAN TROP-2 ANTIBODY EXHIBITING ANTITUMOR ACTIVITY IN VIVO

(54) 発明の名称 : in vivo で抗腫瘍活性を有する抗ヒト TROP-2 抗体

(57) **Abstract**: The present invention provides an antibody (particularly a humanized antibody) specifically reacting with hTROP-2 and exhibiting antitumor activity in vivo, a hybridoma producing said antibody, a composite of said antibody and a drug, a pharmaceutical composition for diagnosing or treating tumors, a tumor detection method, and a kit for detecting or diagnosing tumors.

(57) 要約: 本発明は、hTROP-2 と特異的に反応し in vivo で抗腫瘍活性を有する抗体（特にヒト型化抗体）、当該抗体を産生するハイブリドーマ、当該抗体と薬剤との複合体、腫瘍の診断用又は治療用医薬組成物、腫瘍の検出方法、腫瘍の検出用又は診断用キットを提供する。

OnCusp
Ex. 1002

明 細 書

発明の名称

*in vivo*で抗腫瘍活性を有する抗ヒトTROP-2抗体

技術分野

本発明は、抗腫瘍活性を有する抗ヒトTROP-2抗体、特に*in vivo*で抗腫瘍活性を有する抗ヒトTROP-2抗体に関する。また本発明は、当該抗体を産生するハイブリドーマ、及び当該抗体の用途に関する。

背景技術

ヒトTROP-2 (Tacstd2, GA733-1, EGP-1)（以下、hTROP-2とも表記する。）は、種々の上皮細胞癌種において過剰発現していることが知られる、323アミノ酸残基（配列番号２参照）からなる1回膜貫通型の1型細胞膜タンパク質である。以前より、ヒト栄養膜細胞（trophoblasts）と癌細胞に共通する免疫抵抗性に関与する細胞膜タンパク質の存在（非特許文献１）が示唆されていたが、ヒト絨毛癌細胞株BeWoの細胞膜タンパク質に対するマウスモノクローナル抗体（162-25.3, 162-46.2）により認識される抗原分子が特定され、ヒト栄養膜細胞（trophoblasts）に発現する分子の一つとしてTrop-2と命名された（非特許文献２）。後に、同じ分子が、他の研究者によって発見され、胃癌細胞SW948を免疫して得られたマウスモノクローナル抗体GA733によって認識される腫瘍抗原GA733-1（非特許文献３）、非小細胞肺癌細胞免疫によって得られたマウスモノクローナル抗体RS7-3G11により認識される上皮糖タンパク質（epithelial/carcinoma antigen, EGP-1）（非特許文献４）とも呼ばれたが、1995年にTrop-2の遺伝子がクローニングされ、これらが同一の分子であることが確認された（非特許文献５）。また、癌細胞において細胞内カルシウムシグナルを増幅させる機能が明らかにされ（非特許文献６）、tumor-associated calcium signal transducer 2, TACSTD2とも呼ばれる。

hTROP-2遺伝子は、染色体1p32にマッピングされ、約50％の相同性を持つ

GA733-2（TACSTD1、上皮糖タンパク質EGP-2、EpCAM及びTrop-1としても知られている）とともにTACSTD遺伝子ファミリーを構成している（非特許文献７）。hTROP-2タンパク質（323アミノ酸残基；配列番号２）は、およそ36キロダルトンの分子量を有し、親水性のシグナルペプチド（第1～26番目のアミノ酸）、細胞外ドメイン（第27～274番目のアミノ酸）、細胞膜貫通ドメイン（第275～297番目のアミノ酸）ならびに細胞内ドメイン（第298～323番目のアミノ酸）からなる。細胞外ドメインには4つの不均質N結合グリコシル化部位があり、糖鎖付加により見かけ上の分子量は11～13キロダルトン増加する（非特許文献５）。TACSTD遺伝子ファミリーでは、細胞外ドメインに特徴的なthyroglobulin(TY)配列を有し、癌細胞の増殖や浸潤、転移に関与すると考えられている。

これまでに、hTROP-2の生理学的リガンドは同定されておらず、分子機能は明らかにされていないが、腫瘍細胞においてカルシウムシグナルを伝達することが示され（非特許文献６）、また、Ca^{2+}依存性のキナーゼであるプロテインキナーゼC (PKC) により、細胞内セリン303残基がリン酸化されること（非特許文献４）や、細胞内ドメインにPIP2結合配列を有することから、腫瘍細胞におけるシグナル伝達機能が示唆されている（非特許文献８)。

免疫組織化学（IHC）及びフローサイトメトリーなどの解析により、胃癌、肺癌、大腸癌、卵巣癌、乳癌、前立腺癌、膵癌、肝癌、食道癌などの多くの上皮由来癌腫においてhTROP-2の過剰発現が報告されている。これに対し、正常組織における発現は上皮領域の細胞に限定され、発現レベルも癌腫に比較して低いことが示され、TROP-2の腫瘍形成への関与が示唆されている（特許文献１～３及び９）。

また、臨床検体におけるバイオマーカーとしてのhTROP-2の発現は、大腸癌（非特許文献１０及び１１）、膵臓癌（非特許文献１２）や口腔癌（非特許文献１３）の悪性度と相関し、hTROP-2が高発現している場合には癌の転移や再発が有意に高いことが示されている。また、cDNAマイクロアレイ技術を使用した大規模遺伝子発現分析において、正常な卵巣上皮と比較して卵巣の重篤な乳頭状腺癌に最も高度に過剰発現している遺伝子群のひとつとして同定された（非特許

文献１４）。

さらに、近年、腫瘍形成におけるhTROP-2の重要な役割が大腸癌細胞を用いたモデルにより証明された（非特許文献１５）。hTROP-2の発現が腫瘍細胞の足場非依存的細胞増殖を促進し、免疫不全マウスの皮下に移植した癌細胞の腫瘍形成及び増殖に必要であることから、機能的な腫瘍抗原として新たな治療標的となる可能性を示した。

これまでに、いくつかの抗hTROP-2抗体について抗腫瘍効果の検討が報告されている。RS7抗体（特許文献１）について、放射性物質で標識した抗体を用いた*in vivo*モデルにおいて試験され、ヌードマウス異種移植モデルにおける抗腫瘍活性が示されているが、抗体単独（裸抗体）での抗腫瘍効果は報告されていない。

この他に、細胞毒素を結合させた抗hTROP-2モノクローナル抗体BR110（特許文献２）による、ヒト癌細胞株H3619、H2987、MCF-7、H3396及びH2981に対する*in vitro*実験での細胞障害性が報告されているが、*in vivo*データでは裸抗体又はBR110の免疫複合体について開示されているものはなかった。

近年、ヒト卵巣癌組織をマウスに免疫することで得られたハイブリドーマ細胞株AR47A6.4.2あるいはAR52A301.5 により産生される単離モノクローナル抗体が、hTROP-2と結合し、裸抗体として初めて、*in vitro*での細胞障害性に加え、ヌードマウス異種移植モデルにおける抗腫瘍活性を示すことが報告された（特許文献３及び４）。しかしながら、これら文献中では、膵臓癌細胞株BxPC-3及びPL45、前立腺癌細胞PC-3、乳癌細胞MCF-7並びに大腸癌細胞Colo205を移植したマウス異種移植モデルにおいて抗体単独での抗腫瘍効果が示されているが、BxPC-3細胞移植において治療効果が示されている以外は、抗体の予防的投与により腫瘍形成及び増殖を部分的に（約40～60%）抑制することが示されているに留まり、また、必要とされる抗体量も、20mg/kg程度と非常に高用量であった。

以上の従来の知見から、抗hTROP-2抗体の抗腫瘍抗体としての可能性が示唆されているが、同時に、抗hTROP-2抗体のすべてが裸抗体として抗体単独で*in vivo*において抗腫瘍効果を示すものではなく、抗体の結合部位や親和性、モノクローナル抗体の性質により、hTROP-2に対する作用が異なることを示している。

特許文献１：米国特許第6653104号
特許文献２：米国特許第5840854号
特許文献３：米国特許第7420040号
特許文献４：米国特許第7420041号
非特許文献１：Faulk WP, et al., Proc. Natl. Acad. Sci. U S A, 75(4), p. 1947-1951 (1978)
非特許文献２：Lipinski M, et al., Proc. Natl. Acad. Sci. U S A, 78(8), p. 5147-5150 (1981)
非特許文献３：Linnenbach AJ, et al., Proc. Natl. Acad. Sci. U S A, 86(1), p. 27-31 (1989)
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非特許文献５：Fornaro M, et al., Int. J. Cancer, 62(5), p. 610-618 (1995)
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非特許文献１０：Ohmachi T et al., Clin. Cancer Res., 12(10), p. 3057-3063 (2006)
非特許文献１１：Fang YJ, et al., Int. J. Colorectal Dis., 24(8), p. 875-884 (2009)
非特許文献１２：Fong D, et al., Br. J. Cancer, 99(8), p. 1290-1295 (2008)
非特許文献１３：Fong D, et al., Mod. Pathol., 21(2), p. 186-191 (2008)
非特許文献１４：Santin AD, et al., Int. J. Cancer, 112(1), p. 14-25 (2004)
非特許文献１５：Wang J, et al., Mol. Cancer Ther., 7(2), p. 280-285 (2008)

発明の概要

このような状況下において、*in vivo*において高い抗腫瘍活性を有する抗

hTROP-2抗体（抗hTROP-2モノクローナル抗体）、具体的には、裸抗体として抗体単独で*in vivo*における抗腫瘍効果を有し、しかも低用量で当該効果を有する抗hTROP-2抗体、特に当該抗体であってヒト型化抗体であるもの等の開発が望まれていた。

本発明は、上記状況を考慮してなされたもので、以下に示す、抗hTROP-2抗体（抗hTROP-2モノクローナル抗体）、当該抗体を産生するハイブリドーマ、当該抗体の断片、当該抗体等と薬剤との複合体（イムノコンジュゲート）、腫瘍の診断用又は治療用医薬組成物、腫瘍の検出方法、腫瘍の検出用又は診断用キット等を提供するものである。

（１）Ｈ鎖Ｖ領域のアミノ酸配列が配列番号９２又は９８で示されるアミノ酸配列からなり、Ｌ鎖Ｖ領域のアミノ酸配列が配列番号９３で示されるアミノ酸配列からなるヒトTROP-2に対する抗体。

上記(1)の抗体は、そのＨ鎖Ｖ領域のＣＤＲ１～３のアミノ酸配列が、それぞれ順に、配列番号３６～３８で示されるアミノ酸配列であり、及び／又は、抗体のＬ鎖Ｖ領域のＣＤＲ１～３のアミノ酸配列が、それぞれ順に、配列番号４１～４３で示されるアミノ酸配列である。

（２）Ｈ鎖Ｖ領域のアミノ酸配列が配列番号９４又は９５で示されるアミノ酸配列からなり、Ｌ鎖Ｖ領域のアミノ酸配列が配列番号９６で示されるアミノ酸配列からなる、ヒトTROP-2に対する抗体。

上記(2)の抗体は、そのＨ鎖Ｖ領域のＣＤＲ１～３のアミノ酸配列が、それぞれ順に、配列番号６６～６８で示されるアミノ酸配列であり、及び／又は、抗体のＬ鎖Ｖ領域のＣＤＲ１～３のアミノ酸配列が、それぞれ順に、配列番号７１～７３で示されるアミノ酸配列である。

上記(1)及び(2)の抗体は、例えば、ヒト型化抗体であるものが挙げられる。

上記(1)及び(2)の抗体は、例えば、*in vivo*で抗腫瘍活性を有するものが挙げられる。

上記(1)及び(2)の抗体は、例えば、5～20mg/kg体重の投与量で50％以上の腫瘍成長阻害活性を示すものが挙げられる。ここで、当該腫瘍成長阻害活性を示すための投与回数は、例えば、多くても１週間に１回である。

上記(1)及び(2)の抗体は、例えば、10mg/kg体重の単回投与で50％以上の腫瘍成長阻害活性を示すものが挙げられる。

上記(1)及び(2)の抗体は、例えば、2種以上のヒト腫瘍細胞株に対して抗腫瘍活性を有するものが挙げられる。

上記(1)及び(2)の抗体は、例えば、解離定数（Kd値）が1.0×10^{-10} M以下であるものが挙げられる。

上記(1)及び(2)の抗体は、例えば、モノクローナル抗体であるものが挙げられる。

ここで、上記腫瘍としては、例えば、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌からなる群より選ばれる少なくとも１種が挙げられ、なかでもヒト膵癌、ヒト大腸癌、ヒト乳癌、ヒト肺癌及びヒト卵巣癌からなる群より選ばれる少なくとも１種が好ましく挙げられる。

また、上記腫瘍としては、例えば、再発癌又は転移癌が挙げられる。

また、上記腫瘍細胞株としては、例えば、ヒト膵癌細胞株PK-59、ヒト膵癌細胞株BxPC-3、ヒト膵癌細胞株KP-3L、ヒト膵癌細胞株KP-2、ヒト膵癌細胞株PK-1、ヒト膵癌細胞株PK-45H、ヒト膵癌細胞株PK-45P、ヒト膵癌細胞株TCC-PAN2、ヒト膵癌細胞株SUIT-2、ヒト大腸癌細胞株CACO-2、ヒト大腸癌細胞株SW480、ヒト大腸癌細胞株DLD-1、ヒト大腸癌細胞株HCT 116、ヒト乳癌細胞株JIMT-1、ヒト乳癌細胞株HCC1143、ヒト乳癌細胞株MCF-7、ヒト乳癌細胞株MBA-MD-468、ヒト前立腺癌細胞株DU145及びヒト前立腺癌細胞株PC-3、ヒト肺癌細胞株Calu-3、ヒト卵巣癌細胞株SK-OV-3、及びヒト胆管癌細胞株TFK-1からなる群より選ばれる少なくとも2種が挙げられ、なかでも、ヒト膵癌細胞株PK-59、ヒト膵癌細胞株BxPC-3、ヒト大腸癌細胞株SW480、ヒト肺癌細胞株Calu-3、ヒト乳癌細胞株MBA-MD-468及びヒト卵巣癌細胞株SK-OV-3からなる群より選ばれる少なくとも2種が好ましく挙げられる。

（３）上記(1)及び(2)の抗体に由来する抗体断片。

上記(3)の抗体断片は、例えば、配列番号９２または９８で示されるアミノ酸配列、及び／又は、配列番号９３で示されるアミノ酸配列を含むものや、あるいは、配列番号９４又は９５で示されるアミノ酸配列、及び／又は、配列番号９６

で示されるアミノ酸配列を含むものが挙げられる。

（４）上記(1)及び(2)の抗体と、抗腫瘍活性及び／又は殺細胞活性を有する物質とを含む、抗体－薬剤複合体。

（５）上記(3)の抗体断片と、抗腫瘍活性及び／又は殺細胞活性を有する物質とを含む、抗体断片－薬剤複合体。

上記(4)及び(5)の複合体において、腫瘍としては、例えば、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌からなる群より選ばれる少なくとも１種が挙げられ、なかでもヒト膵癌、ヒト大腸癌、ヒト乳癌、ヒト肺癌及びヒト卵巣癌からなる群より選ばれる少なくとも１種が好ましく挙げられる。また、腫瘍としては、例えば、再発癌又は転移癌も挙げられる。

（６）上記(1)及び(2)の抗体、上記(3)の抗体断片、及び上記(4)及び(5)の複合体からなる群より選ばれる少なくとも１種を含む、医薬組成物。

上記(6)の医薬組成物は、例えば、腫瘍の治療に用いるものや、体重減少の副作用を有しないものや、腫瘍の診断に用いるものが挙げられる。ここで、腫瘍としては、例えば、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌からなる群より選ばれる少なくとも１種が挙げられ、なかでもヒト膵癌、ヒト大腸癌、ヒト乳癌、ヒト肺癌及びヒト卵巣癌からなる群より選ばれる少なくとも１種が好ましく挙げられる。また、腫瘍としては、例えば、再発癌又は転移癌も挙げられる。

（７）上記(1)及び(2)の抗体、上記(3)の抗体断片、及び上記(4)及び(5)の複合体からなる群より選ばれる少なくとも１種を含む、腫瘍治療剤。

上記(7)の腫瘍治療剤は、例えば、体重減少の副作用を有しないものが挙げられる。ここで、腫瘍としては、例えば、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌からなる群より選ばれる少なくとも１種が挙げられ、なかでもヒト膵癌、ヒト大腸癌、ヒト乳癌、ヒト肺癌及びヒト卵巣癌からなる群より選ばれる少なくとも１種が好ましく挙げられる。

（８）上記(1)及び(2)の抗体、上記(3)の抗体断片、及び上記(4)及び(5)の複合体からなる群より選ばれる少なくとも１種を含む、腫瘍診断剤。

上記(8)の腫瘍診断剤において、腫瘍としては、例えば、ヒト膵癌、ヒト前立

腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌からなる群より選ばれる少なくとも１種が挙げられ、なかでもヒト膵癌、ヒト大腸癌、ヒト乳癌、ヒト肺癌及びヒト卵巣癌からなる群より選ばれる少なくとも１種が好ましく挙げられる。

（９）上記(1)及び(2)の抗体、上記(3)の抗体断片、及び上記(4)及び(5)の複合体からなる群より選ばれる少なくとも１種と、生体から採取された試料とを反応させ、反応した抗体及び／又は抗体断片のシグナルを検出することを特徴とする、腫瘍の検出方法。

上記(9)の腫瘍の検出方法において、腫瘍としては、例えば、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌からなる群より選ばれる少なくとも１種が挙げられ、なかでもヒト膵癌、ヒト大腸癌、ヒト乳癌、ヒト肺癌及びヒト卵巣癌からなる群より選ばれる少なくとも１種が好ましく挙げられる。

（１０）上記(1)及び(2)の抗体、上記(3)の抗体断片、及び上記(4)及び(5)の複合体からなる群より選ばれる少なくとも１種を含む、腫瘍の治療用、診断用又は検出用キット。

上記(10)の腫瘍の治療用、診断用又は検出用キットにおいて、腫瘍としては、例えば、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌からなる群より選ばれる少なくとも１種が挙げられ、なかでもヒト膵癌、ヒト大腸癌、ヒト乳癌、ヒト肺癌及びヒト卵巣癌からなる群より選ばれる少なくとも１種が好ましく挙げられる。

（１１）上記(1)及び(2)の抗体をコードするポリヌクレオチド。

（１２）上記(3)の抗体断片をコードするポリヌクレオチド。

（１３）上記(11)又は(12)のポリヌクレオチドを含む組換えベクター。

（１４）上記(13)の組換えベクターを含む形質転換体。

図面の簡単な説明

図１は、抗 hTROP-2 モノクローナル抗体(K5-70)の、抗原親和性（Kd：解離定数）測定を示す図である。 Abt: Antibody (total), Agf: Antigen (free)

図２は、抗 hTROP-2 モノクローナル抗体を産生するハイブリドーマ培養上清の、HuH-7 細胞（hTROP-2 陰性）と HuH-7-hTROP-2 細胞との反応性を示す図である。黒塗りのヒストグラムは HuH-7 細胞を示し、黒のラインのヒストグラムは HuH-7-hTROP-2 細胞を示す。

図３は、抗 hTROP-2 モノクローナル抗体の、内在的に hTROP-2 を細胞表面に発現している、ヒト膵臓癌細胞株（PK-59 細胞）との反応性を示す図である。黒塗りのヒストグラムは二次抗体（PE 標識抗マウス IgG)のみ、黒ラインのヒストグラムは、各抗 hTROP-2 モノクローナル抗体と反応させたものを示す

図４は、抗 hTROP-2 モノクローナル抗体の、内在的に hTROP-2 を細胞表面に発現している、ヒト膵臓癌細胞株（BxPC-3 細胞）との反応性を示す図である。黒塗りのヒストグラムは二次抗体（PE 標識抗マウス IgG)のみ、黒ラインのヒストグラムは、各抗 hTROP-2 モノクローナル抗体と反応させたものを示す。

図５は、抗 hTROP-2 モノクローナル抗体(K5-70)の、ヒト膵臓癌細胞株との反応性を示す図である。黒塗りのヒストグラムは二次抗体（PE 標識抗マウス IgG)のみ、黒ラインのヒストグラムは、抗 hTROP-2 モノクローナル抗体と反応させたものを示す。

図６は、抗 hTROP-2 モノクローナル抗体(K5-70)の、ヒト大腸癌細胞株（Colo320, CACO2, SW480, DLD1, CW2, HCT 116), ヒト乳癌（JIMT-1, HCC1143)および ヒト前立腺癌細胞株（PC-3, DU145)との反応性を示す図である。黒塗りのヒストグラムは二次抗体（PE 標識抗マウス IgG)のみ、黒ラインのヒストグラムは、抗 hTROP-2 モノクローナル抗体と反応させたものを示す。

図７は、抗 hTROP-2 モノクローナル抗体の、マウス TROP-2 との交差反応性を調べた図である。細胞は、CHO-K1 細胞に、マウス TROP-2 遺伝子を一過性発現させた細胞を用い、陽性対照抗体として、マウス TROP-2 と交差性を示す T2-102 抗体（マウス IgG1)を用いた。黒塗りのヒストグラムは二次抗体（PE 標識抗マウス IgG)のみ、黒ラインのヒストグラムは、各抗 hTROP-2 モノクローナル抗体と反応させたものを示す。

図８は、抗 hTROP-2 モノクローナル抗体の、ヒト EpCAM/TROP-1 との交差反応性を調べた図である。細胞は、CHO-K1 細胞にヒト EpCAM/TROP-1 遺伝

子を一過性発現させた細胞を用い、陽性対照抗体として、PE 標識抗ヒト EpCAM モノクローナル抗体（ベクトンディッキンソン）を用いた。黒塗りのヒストグラムは二次抗体（PE 標識抗マウス IgG)のみ、黒ラインのヒストグラムは、各抗 hTROP-2 モノクローナル抗体と反応させたものを示す。

図９は、抗 hTROP-2 抗体（T6-16, T5-86, K5-70, K5-107)のヒト膵臓癌細胞株 (PK-59 細胞)の細胞増殖阻害活性を示す図である。mIgG はコントロール抗体(マウス IgG), YY01:市販の抗 hTROP-2 抗体(Santa Cruz)を示す。白カラム：0μg/mL, 灰色カラム：0.1μg/mL, 黒カラム：1μg/mL。抗体添加をしていない（0μg/mL)時の値に対する ratio で表した。エラーバーは標準偏差を示す。
*P < 0.05, **P < 0.01（by Student's t-test）

図１０は、抗 hTROP-2 抗体（T6-16, K5-70)のヒト膵臓癌細胞株 (PK-59 細胞)のスクラッチアッセイを示す図である。

A. PK-59 細胞のスクラッチ領域の写真の代表例を示す。Day 0: スクラッチ直後の代表例を示す。mIgG (Day 1): スクラッチ後、コントロール抗体（マウス IgG、1μg/mL）を培地に添加し、1 日後（24 時間後）の写真。K5-70 (Day 1): スクラッチ後、K5-70 抗体（1μg/mL）を培地に添加し、1 日後（24 時間後）の写真。T6-16 (Day 1): スクラッチ後、T6-16 抗体（1μg/mL）を培地に添加し、1 日後（24 時間後）の写真。写真中に示した矢印は、スクラッチ領域の幅を示した。

B. 画像解析ソフト(Scion Image)でスクラッチ領域の面積を解析し、その値を基に、コントロール抗体(mIgG) 添加群の Day0 を基準値 1 として、各被検抗体における値を算出した。

*P <0.05, **P < 0.01（by Student's t-test）

図１１は、ヒト膵臓癌細胞株, PK-59 細胞の幹細胞マーカーの発現を示す FACS の図である。 A: PK-59 細胞における EpCAM の発現を示す FACS の図である。黒塗りのヒストグラムは二次抗体（PE 標識抗マウス IgG)のみ、黒ラインは、抗ヒト EpCAM 抗体（ベクトンディッキンソン）と反応させた場合のヒストグラムを示す。B, C: PK-59 細胞における P-glycoprotein/MCR1 (B), および ABCG2 (C)の発現を示す FACS の図である。青のヒストグラムは、二次抗体

のみ、赤のヒストグラムが抗ヒト P-glycoprotein/MDR1 抗体(BD ファーミンジェン)（B), 抗ヒト ABCG2 抗体（BD ファーミンジェン）（C)と反応させた場合を示す。 D: PK-59 細胞を膵臓癌幹細胞マーカーである FITC 標識抗ヒト CD24 抗体（BD ファーミンジェン）と PE 標識抗ヒト CD44 抗体（BD ファーミンジェン）で二重染色した FACS の図である。D の図中の数字は、それぞれの画分の細胞の存在比率を示す。

図１２は、PK-59細胞を用いたxenograft treatmentモデルにおいて、新規な抗hTROP-2モノクローナル抗体クローンK5-70 (マウスIgG2a)の抗腫瘍活性を評価した図である。

A： コントロール群（●：マウスIgG）とK5-70抗体(10mg/kg 体重)投与群(○)の腫瘍成長を経時的に表した（平均値±標準偏差）。矢印は、抗体投与期間を表す。* P < 0.05, ** P < 0.01（by Student's t-test）

B： Aの試験において、21日目(Day21)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。各プロット上に示す数値は、平均値±標準偏差。 ** P < 0.01（by Student's t-test）

図１３は、PK-59細胞を用いたxenograft treatmentモデルにおける、A. クローンK5-107, B. クローンT6-16, C. クローンK5-116-2-1の抗腫瘍活性を評価した図である。●はコントロール群（マウスIgG）、○は抗hTROP-2抗体(10mg/kg 体重)投与群を示す。グラフ中の矢印は抗体投与期間を表し、各プロット上に示す数値は、平均値±標準偏差。* P < 0.05（by Student's t-test）

図１４は、PK-59細胞を用いたxenograft preventionモデルにおける、クローンK5-70 (A)、クローンT6-16 (B) 及び,クローンK5-116-2-1 (C) の抗腫瘍活性を評価した図である。●はコントロール群（マウスIgG）、○は抗hTROP-2抗体(10mg/kg 体重)投与群を示す。グラフ中の矢印は抗体投与期間を表し、各プロット上に示す数値は、平均値±標準偏差。** P < 0.01（by Student's t-test）

図１５は、BxPC-3細胞を用いたxenograft prevention及びtreatmentモデルにおける、クローンK5-70の抗腫瘍活性を評価した図である。A： preventionモデルにおけるコントロール群（●：マウスIgG）とK5-70抗体(10mg/kg 体重)投与群（○）の腫瘍成長を経時的に表した（平均値±標準偏差）。矢印は、抗体投与

期間を表す。** P < 0.01（by Student's t-test）B： treatmentモデルにおけるコントロール群（●：マウスIgG）とK5-70抗体(10mg/kg 体重)投与群(○)の腫瘍成長を経時的に表した（平均値±標準偏差）。矢印は、抗体投与期間を表す。* P < 0.05（by Student's t-test）

図１６は、PK-59 細胞を用いた xenograft prevention モデルにおける、クローン K5-70 の用量依存的な抗腫瘍活性を示した図である。腫瘍体積は平均値±標準偏差で表した。

A：コントロール群（●：マウス IgG）と各用量での K5-70 抗体投与群(□：1mg/kg 体重、△：5mg/kg 体重）の腫瘍成長を経時的に表す（平均値±標準偏差）。矢印は、抗体投与期間を表す。* P < 0.05（by Student's t-test），** P < 0.01（by Student's t-test）

B：A の試験において、17 日目(Day17)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。各プロット上に示す数値は、平均値±標準偏差。 ** P < 0.01（by Student's-t-test）

図１７は、実験に使用したヒト／マウスキメラTROP-2タンパク質の模式図である。SP：シグナル配列、TY domain：サイログロブリンタイプ１領域、TM：膜貫通領域、C：細胞内領域 黒の塗りつぶしで表している領域はhTROP-2由来のポリペプチドである。白抜きで表している領域は、マウスTROP-2由来のポリペプチドである。キメラタンパク質の模式図の上段に示してある数字は、マウスTROP-2タンパク質の、下段はhTROP-2タンパク質のアミノ酸番号をそれぞれ示している。

図１８は、ヒト／マウスキメラTROP-2を用いた抗hTROP-2モノクローナル抗体結合領域の同定の結果を示す図である。ヒト／マウスキメラTROP2-C（hmTROP2-C）及びマウス／ヒトキメラTROP2-D（mhTROP2-D）タンパク質を恒常的に発現するHEK293細胞を用い、図中に示した抗hTROP-2モノクローナル抗体との反応性を検討した。陰性コントロールはmouse IgG2bを用いた。

図１９は、抗hTROP-2モノクローナル抗体の抗体結合領域の同定の結果を示す図である。

hTROP-2遺伝子及び各ヒト／マウスキメラTROP-2遺伝子をHEK293細胞に導

入し、一過性に発現させた細胞を用いてFACS解析を行った。(A) K5-70、K5-107、T5-86およびK5-116-2-1抗体について、hTROP-2（上段）、hmTROP-2-A（中段）及びhmTROP-2-B（下段）との反応性を検討した。mouse IgG2bは陰性コントロールとして用いた。(B) T6-4およびT6-16抗体について、hTROP-2（上段）、mhTROP-2-E（中段）及びmhTROP-2-F（下段）との反応性を検討した。mouse IgG2bは陰性コントロールとして用いた。

図２０は、ヒト正常組織におけるhTROP-2の発現を示す図である。抗hTROP-2モノクローナル抗体 クローンK5-63-17を用いてヒト正常組織アレイの免疫染色を行った。(A) 皮膚、(B) 食道、(C) 腎臓（皮質）、(D) 腎臓（髄質）、(E) 膵臓、(F) 前立腺、(G) 膀胱、(H) 扁桃腺、(I) 心臓、(J) 肝臓 （倍率：×200）

図２１は、癌組織でのhTROP-2の発現を示す図である。抗hTROP-2モノクローナル抗体 クローンK5-63-17を用いてヒト癌組織アレイの免疫染色を行った。(A) 乳癌、(B) 肺癌、(C) 食道癌、(D) 胃癌、(E) 膵臓癌、(F) 大腸癌、(G) 膀胱癌、(H) 前立腺癌、(I) 卵巣癌 （倍率：×100）

図２２は、PK-59細胞を用いたxenograft preventionモデルにおける、クローンK5-70の単回投与による抗腫瘍活性を示した図である。

A. コントロール群（●：マウスIgG）とK5-70抗体(10mg/kg 体重)投与群（○）の腫瘍形成を経時的に表す（平均値±標準偏差）。矢印は、抗体投与を表す。* $P < 0.05$（by Student's t-test）, ** $P < 0.01$（by Student's t-test）.

B. Aの試験において、28日目(Day 28)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。** $P < 0.01$（by Student's t-test）.

C. コントロール群（●：マウスIgG）とK5-70抗体(10mg/kg 体重)投与群（○）の、各個体の腫瘍形成を経時的に表す。矢印は抗体投与を示す。

図２３は、ヒト大腸癌細胞SW480細胞を用いたxenograft treatmentモデルにおける、クローンK5-70の抗腫瘍活性を示す図である。

A： コントロール群（●：マウスIgG）とK5-70抗体(10mg/kg 体重)投与群（○）の腫瘍形成を経時的に表した（平均値±標準偏差）。矢印は、抗体投与期間を表す。** $P < 0.01$（by Student's t-test）

B： Aの試験において、44日目(Day 44)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。** P < 0.01（by Student’s t-test)

図２４は、SW480細胞を用いたxenograft treatmentモデルにおける、クローンK5-116-2-1の抗腫瘍活性を示す図である。

A： コントロール群（●：マウスIgG）とK5-116-2-1抗体(10mg/kg 体重)投与群（○）の腫瘍形成を経時的に表した（平均値±標準偏差）。矢印は、抗体投与期間を表す。** P < 0.01 (by Student’s t-test)

B： Aの試験において、42日目(Day 42)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。** P < 0.01（by Student’s t-test)

図２５は、SW480細胞を用いたxenograft treatmentモデルにおける、クローンT6-16の抗腫瘍活性を示す図である。

A： コントロール群（●：マウスIgG）とT6-16抗体(10mg/kg 体重)投与群（○）の腫瘍形成を経時的に表した（平均値±標準偏差）。矢印は、抗体投与期間を表す。* P < 0.05 (by Student’s t-test)

B： Aの試験において、42日目(Day 42)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。* P < 0.05（by Student’s t-test)

図２６は、SW480細胞を用いたxenograft treatmentモデルにおける、クローンK5-70の用量依存的な抗腫瘍活性を示す図である。

A： コントロール群（●：マウスIgG）とK5-70抗体投与群（○：1mg/kg 体重, △：5mg/kg 体重, □：10mg/kg 体重）の腫瘍形成を経時的に表した（平均値±標準偏差）。矢印は、抗体投与期間を表す。*P < 0.05 (by Student’s t-test)

B： Aの試験において、42日目(Day 42)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。* P < 0.05（by Student’s t-test)

図２７は、. SW480細胞を用いたxenograft treatmentモデルにおける、クローンK5-70の抗腫瘍活性を示す図である。

A: 1週間に1回の投与間隔でのK5-70抗体の抗腫瘍活性を示す図である。コントロール群（●：マウス IgG）とK5-70抗体(10mg/kg)投与群（○)の腫瘍形成を経時的に表した（平均値±標準偏差）。矢頭（Day 10, 17, 24, 31, 38)は、K5-

70抗体の投与を示す。*P < 0.05 by Student's t-test.

B: 10日に1回 (q10d)、または2週間に1回 (q14d)の投与間隔でのK5-70抗体の抗腫瘍活性を示す図である。コントロール群（●：マウス IgG、10mg/kg）とK5-70抗体投与群（○：q10d、10 mg/kg, △：q14d、10 mg/kg) の腫瘍形成を経時的に表した（平均値±標準偏差）。黒塗りの矢頭（▼; Day 9, 19, 29)と白塗りの矢頭（▽； Day 9, 23, 37)は、K5-70抗体の投与を示す。*P < 0.05, **P < 0.01 by Student's t-test.

図２８は、SW480細胞を用いたxenograft treatmentモデルにおける、クローンT6-16の用量依存的な抗腫瘍活性を示す図である。

A： コントロール群（●：マウス IgG）とT6-16抗体投与群（○：1mg/kg 体重, △：5mg/kg 体重, □：10mg/kg 体重）の腫瘍形成を経時的に表した（平均値±標準偏差）。矢印は、抗体投与期間を表す。**P < 0.01 (by Student's t-test)

B： Aの試験において、43日目(Day 43)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。** P < 0.01（by Student's t-test)

図２９は、SW480細胞を用いたxenograft treatmentモデルにおける、クローンT6-16の抗腫瘍活性を示す図である。コントロール群（●：マウス IgG 10 mg/kg 体重）、T6-16抗体(10 mg/kg 体重）投与群（○：q7d,△：q10d)の腫瘍形成を経時的に表した（平均値±標準偏差）。矢頭（Day 10, 17, 24, 31, 38)、及び矢印（Day 10, 20, 30, 40)は、T6-16抗体の投与を示す。コントロール群は3日に1回投与を行った。*P < 0.05, **P < 0.01 by Student's t-test.

図３０は、ヒト前立腺癌細胞DU-145細胞を用いたxenograft preventionモデルにおける、クローンK5-70の抗腫瘍活性を示す図である。

A： コントロール群（●：マウス IgG）とK5-70抗体(10mg/kg 体重)投与群（○）の腫瘍形成を経時的に表した（平均値±標準偏差）。矢印は、抗体投与期間を表す。* P < 0.05 (by Student's t-test)

B： Aの試験において、40日目(Day 40)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。* P < 0.05（by Student's t-test)

図３１は、PK-59細胞の肝転移モデルを用いたクローンK5-70の転移抑制活性

を示す図である。

A： コントロール群（●：マウス IgG）とB： K5-70抗体(10mg/kg 体重)投与群の細胞移植6週間後の摘出肝臓像を示す。矢印は、肝転移巣を示す。

図３２は、SW480細胞を用いたxenograftモデルにおける塩酸イリノテカン投与後の癌再発モデルにおけるK5-70の抗腫瘍活性を示す図である。無処置群（◆）、と塩酸イリノテカン(40mg/kg 体重）投与後K5-70抗体（○：10 mg/kg 体重)、あるいはマウスIgG投与群（●： 10 mg/kg 体重)の腫瘍形成を経時的に表した図である（平均値±標準偏差）。矢頭（Day 11, 14, 17)は、塩酸イリノテカンの投与を示す。K5-70抗体あるいはマウスIgGはDay 20より3日に1回投与を行った。矢印は抗体投与期間を示す。*P < 0.05, **P < 0.01 by Student’s t-test.

図３３は、クローンK5-70 H鎖可変領域（VH）のcDNA塩基配列（配列番号３４）と推定アミノ酸配列（配列番号３５）を示す図である。シグナルペプチドはイタリックで表している。二重下線を引いたグルタミン（Q）は、成熟ペプチドのN末端アミノ酸残基を示す。CDR配列（下線；*IYWIN, NIYPSDSYTNYNQKFKD, TSMADY*）は、Kabatら(Sequences of Proteins of Immunological Interests, Fifth edition, NIH Publication No. 91-3242, U.S. Department of Health and Human Services, 1991) の定義に従って決定した。クローンK5-70 VHのCDR1～3のアミノ酸配列は、それぞれ順に、配列番号３６～３８に示す。

図３４は、クローンK5-70 L鎖可変領域（VL）のcDNA塩基配列（配列番号３９）と推定アミノ酸配列（配列番号４０）を示す図である。シグナルペプチドはイタリックで表している。二重下線を引いたアスパラギン酸（D）は、成熟ペプチドのN末端アミノ酸残基を示す。CDR配列（下線; *RASQSIGTSIH, YASESIS, QQSNSWPFT*）は、Kabatら(前掲；U.S. Department of Health and Human Services, 1991) の定義に従って決定した。クローンK5-70 VLのCDR1～3のアミノ酸配列は、それぞれ順に、配列番号４１～４３に示す。

図３５は、クローンK5-107 H鎖可変領域（VH）のcDNA塩基配列（配列番号４４）と推定アミノ酸配列（配列番号４５）を示す図である。シグナルペプチドはイタリックで表している。二重下線を引いたグルタミン（Q）は、成熟ペプチ

ドのN末端アミノ酸残基を示す。CDR配列（下線；*SYWMH, NIYPGGGYTNYDEKFKS, SSVFDY*）は、Kabatら(前掲；U.S. Department of Health and Human Services, 1991) の定義に従って決定した。クローンK5-107 VHのCDR1～3のアミノ酸配列は、それぞれ順に、配列番号４６～４８に示す。

図３６は、クローンK5-107 L鎖可変領域（VL）のcDNA塩基配列（配列番号４９）と推定アミノ酸配列（配列番号５０）を示す図である。シグナルペプチドはイタリックで表している。二重下線を引いたアスパラギン酸（D）は、成熟ペプチドのN末端アミノ酸残基を示す。CDR配列（下線；*RASQNIGTSIH, YASESIS, QQSNSWPFT*）は、Kabatら(前掲；U.S. Department of Health and Human Services, 1991) の定義に従って決定した。クローンK5-107 VLのCDR1～3のアミノ酸配列は、それぞれ順に、配列番５１０～５３に示す。

図３７は、クローンK5-116-2-1 H鎖可変領域（VH）のcDNA塩基配列（配列番号５４）と推定アミノ酸配列（配列番号５５）を示す図である。シグナルペプチドはイタリックで表している。二重下線を引いたグルタミン（Q）は、成熟ペプチドのN末端アミノ酸残基を示す。CDR配列（下線；*SYWIT, NIYPSDSYTNYNQKFRD, LFDY*）は、Kabatら(前掲；U.S. Department of Health and Human Services, 1991) の定義に従って決定した。クローンK5-116-2-1 VHのCDR1～3のアミノ酸配列は、それぞれ順に、配列番号５６～５８に示す。

図３８は、クローンK5-116-2-1 L鎖可変領域（VL）のcDNA塩基配列（配列番号５９）と推定アミノ酸配列（配列番号６０）を示す図である。シグナルペプチドはイタリックで表している。二重下線を引いたアスパラギン酸（D）は、成熟ペプチドのN末端アミノ酸残基を示す。CDR配列（下線；*RASQSIGTSIH, YASESIS, QQSNSWPFT*）は、Kabatら(前掲；U.S. Department of Health and Human Services, 1991) の定義に従って決定した。クローンK5-116-2-1 VLのCDR1～3のアミノ酸配列は、それぞれ順に、配列番号６１～６３に示す。

図３９は、クローンT6-16 H鎖可変領域（VH）のcDNA塩基配列（配列番号６４）と推定アミノ酸配列（配列番号６５）を示す図である。シグナルペプチド

はイタリックで表している。二重下線を引いたグルタミン酸（E）は、成熟ペプチドのN末端アミノ酸残基を示す。CDR配列（下線；*DYNMH, YIYPYNGGTGYNQRFKS, EDYGSSPSYAMDY*）は、Kabatら(前掲；U.S. Department of Health and Human Services, 1991) の定義に従って決定した。クローンT6-16 VHのCDR1～3のアミノ酸配列は、それぞれ順に、配列番号６６～６８に示す。

図４０は、クローンT6-16 L鎖可変領域（VL）のcDNA塩基配列（配列番号６９）と推定アミノ酸配列（配列番号７０）を示す図である。シグナルペプチドはイタリックで表している。二重下線を引いたアスパラギン酸（D）は、成熟ペプチドのN末端アミノ酸残基を示す。CDR配列（下線; *RSSQSLVHGNGNTYLH, KVSNRFS, SQTTHVPT*）は、Kabatら(前掲；U.S. Department of Health and Human Services, 1991) の定義に従って決定した。クローンT6-16 VLのCDR1～3のアミノ酸配列は、それぞれ順に、配列番号７１～７３に示す。

図４１は、クローン K5-70 の H 鎖可変領域（K5-70 VH）のアミノ酸配列（配列番号３５）、ヒト型化 K5-70 の H 鎖可変領域（HuK5-70 VH）のアミノ酸配列（配列番号７５）、及びヒト型化抗体作製に用いたアクセプター（Genbank accession No. DA980102；配列番号８４）の H 鎖可変領域（DA980102 VH）のアミノ酸配列（配列番号８５）についての、アライメントを示す図である（なお、図中のいずれのアミノ酸配列も、各々の H 鎖可変領域のアミノ酸配列の一部（具体的には、シグナルペプチド部分を除いた成熟タンパク質部分のアミノ酸配列）である。）。

K5-70 VH中にアンダーラインで示したアミノ酸配列はKabatら(前掲；U.S. Department of Health and Human Services, 1991)により定義されたCDR配列を表している。また、アミノ酸配列上部に記された番号は上記Kabatらの定義に従ったアミノ酸の位置番号を示している。DA980102 VH中の各CDR配列は、図中では－－－と表記し、記載を省略している。HuK5-70 VH中のアンダーラインで示したアミノ酸は、CDRの構造維持に重要であると予想されるため、K5-70 VHの配列を維持した。また、HuK5-70 VH中の二重下線で示したアミノ酸は、対応するDA980102 VHのアミノ酸（メチオニン（M））がこの位置では稀にし

か認められないものであり、抗原性を低下させる目的で同様のサブグループに属する典型的なアミノ酸であるロイシン（L）に置換した。

図４２は、クローンK5-70のL鎖可変領域（K5-70 VL）のアミノ酸配列（配列番号４０）、ヒト型化K5-70のL鎖可変領域（HuK5-70 VL）のアミノ酸配列（配列番号７７）、及びヒト型化抗体作製に用いたアクセプター（Genbank accession No. L41174；配列番号８６）のL鎖可変領域（L41174 VL）のアミノ酸配列（配列番号８７；Genbank accession No. AAA64877）についての、アライメントを示す図である（なお、図中のいずれのアミノ酸配列も、各々のL鎖可変領域のアミノ酸配列の一部（具体的には、シグナルペプチド部分を除いた成熟タンパク質部分のアミノ酸配列）である。）。

K5-70 VL中にアンダーラインで示したアミノ酸配列はKabatら(前掲；U.S. Department of Health and Human Services, 1991)により定義されたCDR配列を表している。また、アミノ酸配列上部に記された番号は上記Kabatらの定義に従ったアミノ酸の位置番号を示している。L41174 VL中の各CDR配列は、図中ではーーーと表記し、記載を省略している。HuK5-70 VL中のアンダーラインで示したアミノ酸は、CDRの構造維持に重要であると予想されるため、K5-70 VLの配列を維持した。

図４３は、クローンT6-16のH鎖可変領域（T6-16 VH）のアミノ酸配列（配列番号６５）、２種類のヒト型化T6-16のH鎖可変領域（HuT6-16 VH1、HuT6-16 VH2）のアミノ酸配列（それぞれ順に、配列番号７９、配列番号８１）、及びヒト型化抗体作製に用いたアクセプター（Genbank accession No. DA935238；配列番号８８）のH鎖可変領域（DA935238 VH）のアミノ酸配列（配列番号８９）についての、アライメントを示す図である（なお、図中のいずれのアミノ酸配列も、各々のH鎖可変領域のアミノ酸配列の一部（具体的には、シグナルペプチド部分を除いた成熟タンパク質部分のアミノ酸配列）である。）。

HuT6-16 VH中にアンダーラインで示したアミノ酸配列はKabatら(前掲；U.S. Department of Health and Human Services, 1991)により定義されたCDR配列を表している。また、アミノ酸配列上部に記された番号は上記Kabatらの定義に従ったアミノ酸の位置番号を示している。DA935238 VH中の各CDR配列は、図

中ではーーーと表記し、記載を省略している。HuT6-16 VH1およびHuT6-16 VH2中のアンダーラインで示したアミノ酸は、CDRの構造維持に重要であると予想されるため、T6-16 VHの配列を維持した。また、HuT6-16 VH1中の73番目の位置にあるリジン（K）は、HuT6-16 VH2ではアクセプター配列であるDA935238 VHに由来するスレオニン（T）に置換した。

図４４は、クローンT6-16のL鎖可変領域（T6-16 VL）のアミノ酸配列（配列番号７０）、ヒト型化T6-16のL鎖可変領域（HuT6-16 VL）のアミノ酸配列（配列番号８３）、及びヒト型化抗体作製に用いたアクセプター（Genbank accession No. M99608；配列番号９０）のL鎖可変領域（M99608 VL）のアミノ酸配列（配列番号９１；Genbank accession No. AAA60341）についての、アライメントを示す図である（なお、図中のいずれのアミノ酸配列も、各々のL鎖可変領域のアミノ酸配列の一部（具体的には、シグナルペプチド部分を除いた成熟タンパク質部分のアミノ酸配列）である。）。

T6-16 VL中にアンダーラインで示したアミノ酸配列はKabatら(前掲；U.S. Department of Health and Human Services, 1991)により定義されたCDR配列を表している。また、アミノ酸配列上部に記された番号は上記Kabatらの定義に従ったアミノ酸の位置番号を示している。M99608 VL中の各CDR配列は、図中ではーーーと表記し、記載を省略している。

図４５は、HuK5-70 VH の遺伝子配列（配列番号７４）及びアミノ酸配列（配列番号７５）を示す図である。

各行の上段は遺伝子配列（cDNA配列）、下段はアミノ酸配列を示す。当該アミノ酸配列中、シグナルペプチド部分には破線の下線を、各CDR配列（CDR1～3）には実線の下線を引いている（当該シグナルペプチド部分を除いた成熟タンパク質部分のみのアミノ酸配列は、配列番号９２に示す）。なお、HuK5-70 VH遺伝子の5'末端にはEcoRI部位（GAA TTC）およびKozak配列（ACC ACC）、3'末端にはNhe I部位（GCT AGC）を付加した。

図４６は、HuK5-70 VL の遺伝子配列（配列番号７６）及びアミノ酸配列（配列番号７７）を示す図である。

各行の上段は遺伝子配列（cDNA配列）、下段はアミノ酸配列を示す。当該ア

ミノ酸配列中、シグナルペプチド部分には破線の下線を、各CDR配列（CDR1～3）には実線の下線を引いている（当該シグナルペプチド部分を除いた成熟タンパク質部分のみのアミノ酸配列は、配列番号９３に示す）。なお、HuK5-70 VL遺伝子の5'末端にはAge I部位（ACC GGT）およびKozak配列（ACC ACC）、3'末端にはBsiWI 部位（CGT ACG）を付加した。

図４７は、HuT6-16 VH1 の遺伝子配列（配列番号７８）及びアミノ酸配列（配列番号７９）を示す図である。

各行の上段は遺伝子配列（cDNA配列）、下段はアミノ酸配列を示す。当該アミノ酸配列中、シグナルペプチド部分には破線の下線を、各CDR配列（CDR1～3）には実線の下線を引いている（当該シグナルペプチド部分を除いた成熟タンパク質部分のみのアミノ酸配列は、配列番号９４に示す）。なお、HuT6-16 VH1遺伝子の5'末端にはEcoRI部位（GAA TTC）およびKozak配列（ACC ACC）、3'末端にはNhe I部位（GCT AGC）を付加した。

図４８は、HuT6-16 VH2 の遺伝子配列（配列番号８０）及びアミノ酸配列（配列番号８１）を示す図である。

各行の上段は遺伝子配列（cDNA配列）、下段はアミノ酸配列を示す。当該アミノ酸配列中、シグナルペプチド部分には破線の下線を、各CDR配列（CDR1～3）には実線の下線を引いている（当該シグナルペプチド部分を除いた成熟タンパク質部分のみのアミノ酸配列は、配列番号９５に示す）。なお、HuT6-16 VH2遺伝子の5'末端にはEcoRI部位（GAA TTC）およびKozak配列（ACC ACC）、3'末端にはNhe I部位（GCT AGC）を付加した。

図４９は、HuT6-16 VL の遺伝子配列（配列番号８２）及びアミノ酸配列（配列番号８３）を示す図である。

各行の上段は遺伝子配列（cDNA配列）、下段はアミノ酸配列を示す。当該アミノ酸配列中、シグナルペプチド部分には破線の下線を、各CDR配列（CDR1～3）には実線の下線を引いている（当該シグナルペプチド部分を除いた成熟タンパク質部分のみのアミノ酸配列は、配列番号９６に示す）。なお、HuT6-16 VL遺伝子の5'末端にはAge I部位（ACC GGT）およびKozak配列（ACC ACC）、3'末端にはBsiWI 部位（CGT ACG）を付加した。

図５０は、HuK5-70 抗体、HuT6-16-1 抗体および HuT6-16-2 抗体の発現を確認した結果を示す図である。

(A) 293F 細胞に発現ベクターpFUSE-CHIg-HuK5-70 および pFUSE2-CLIg-HuK5-70 を導入し、培養上清中の HuK5-70 抗体の発現をウエスタンブロットにより検討した。レーン 1 は遺伝子導入を行っていない 293F 細胞の培養上清（ネガティブコントロール）、レーン 2 は上記発現ベクターを導入した 293F 細胞の培養上清。HuK5-70 抗体の重鎖および軽鎖タンパク質はビオチン標識した抗ヒト IgG F(ab')^2 抗体で検出した。

(B) 293F 細胞に発現ベクターpFUSE-CHIg-HuT6-16-1 および pFUSE2-CLIg-HuT6-16（レーン 3）、pFUSE-CHIg-HuT6-16-2 および pFUSE2-CLIg-HuT6-16（レーン 4）の組み合わせでそれぞれ導入し、HuT6-16-1 抗体および HuT6-16-2 抗体の発現をウエスタンブロットにより検討した。HuT6-16-1 抗体および HuT6-16-2 抗体の重鎖タンパク質はビオチン標識した抗ヒト IgG Fc 抗体で、HuT6-16-1 抗体および HuT6-16-2 抗体の軽鎖タンパク質はビオチン標識した抗ヒト IgG F(ab')^2 抗体でそれぞれ検出した。

図５１は、精製した HuK5-70 抗体、HuT6-16-1 抗体および HuT6-16-2 抗体のクマシー染色の結果を示す図である。

精製した HuK5-70 抗体（レーン 1）、HuT6-16-1 抗体（レーン 2）および HuT6-16-2 抗体（レーン 3）それぞれ 1μg を SDS-PAGE により展開し、クマシー染色を行った。

図５２は、フローサイトメーターを用いた HuK5-70 抗体、HuT6-16-1 抗体および HuT6-16-2 抗体の抗原結合能の解析結果を示す図である。

図に示した各抗体を用いてHEK293-hTROP-2細胞（A）およびPK-59細胞（B）に対する反応性をFACS解析により検討した。ネガティブコントロールは二次抗体のみとし（黒塗り）、各抗体の反応性をグレーラインで示した。

図５３は、ELISA 法を用いた HuK5-70 抗体の抗原結合能の測定結果を示す図である。

抗原固相化ELISA法によりK5-70抗体およびHuK5-70抗体の抗原結合能について検討した。▲はK5-70抗体、●はHuK5-70抗体の測定結果を表す。

図５４は、ELISA 法を用いた HuT6-16-1 抗体および HuT6-16-2 抗体の抗原結合能の測定結果を示す図である。

抗原固相化ELISA法によりT6-16抗体、HuT6-16-1抗体およびHuT6-16-2抗体の抗原結合能について検討した。▲はT6-16抗体、●はHuT6-16-1抗体、■はHuT6-16-2抗体の測定結果を表す。

図５５は、ヒト大腸癌細胞SW480細胞を用いたxenograft treatmentモデルにおける、ヒト型化抗hTROP-2抗体（HuK5-70抗体）の抗腫瘍活性を示す図である。

A： コントロール群（●： PBS）とHuK5-70抗体(10mg/kg 体重)投与群（○）の腫瘍形成を経時的に表した（平均値±標準偏差）。矢印は、抗体投与期間を表す。* P < 0.05, ** P < 0.01（by Student's t-test）

B： Aの試験において、39日目(Day 39)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。* P < 0.05（by Student's t-test）

図５６は、ヒト大腸癌細胞SW480細胞を用いたxenograft treatmentモデルにおける、ヒト型化抗hTROP-2抗体（HuK5-70）の用量依存的な抗腫瘍活性を示す図である。

A： コントロール群（●： PBS）とHuK5-70抗体投与群（○；1mg/kg 体重, △；5mg/kg 体重, □；10mg/kg 体重）の腫瘍形成を経時的に表した（平均値±標準偏差）。矢印は、抗体投与期間を表す。 ** P < 0.01（by Student's t-test）

B： Aの試験において、癌細胞移植から48日目(Day 48)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。* * P < 0.01（by Student's t-test）

図５７は、ヒト大腸癌細胞SW480細胞を用いたxxenograft treatmentモデルにおける、ヒト型化抗hTROP-2抗体（HuT6-16-2）の用量依存的な抗腫瘍活性を示す図である。

A： コントロール群（●： PBS）とHuT6-16-2抗体投与群（○；1mg/kg 体重, △；5mg/kg 体重, □；10mg/kg 体重）の腫瘍形成を経時的に表した（平均値±標準偏差）。矢印は、抗体投与期間を表す。 ** P < 0.01（by Student's t-

test）

B： Aの試験において、癌細胞移植から48日目(Day 48)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。* P < 0.05（by Student's t-test）

図５８は、ヒト卵巣癌細胞SK-OV-3細胞を用いたxenograft treatmentモデルにおける、マウス抗hTROP-2抗体（K5-70およびT6-16）の抗腫瘍活性を示す図である。

A： コントロール群（●： PBS）、K5-70抗体 (10mg/kg 体重)投与群（○）、およびT6-16抗体 (10mg/kg 体重)投与群（△）の腫瘍形成を経時的に表した（平均値±標準偏差）。矢印は、抗体投与期間を表す。 ** P < 0.01（by Student's t-test）

B： Aの試験において、癌細胞移植から56日目(Day 56)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。* P < 0.05（by Student's t-test）

図５９は、ヒト乳癌細胞MDA-MB-468細胞を用いたxxenograft treatmentモデルにおける、マウス抗hTROP-2抗体（K5-70およびT6-16）の抗腫瘍活性を示す図である。

A： コントロール群（●： PBS）、K5-70抗体 (10mg/kg 体重)投与群（○）、およびT6-16抗体 (10mg/kg 体重)投与群（△）の腫瘍形成を経時的に表した（平均値±標準偏差）。矢印は、抗体投与期間を表す。 ** P < 0.01（by Student's t-test）

B： Aの試験において、癌細胞移植から54日目(Day 54)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。* P < 0.05, ** P < 0.01（by Student's t-test）

図６０は、ヒト肺癌細胞Calu-3細胞を用いたxenograft treatmentモデルにおける、マウス抗hTROP-2抗体（K5-70およびT6-16）の抗腫瘍活性を示す図である。

A： コントロール群（●： PBS）、K5-70抗体 (10mg/kg 体重)投与群（○）、およびT6-16抗体 (10mg/kg 体重)投与群（△）の腫瘍形成を経時的に表し

た（平均値±標準偏差）。矢印は、抗体投与期間を表す。*P < 0.05, ** P < 0.01 (by Student's t-test)

B： Aの試験において、癌細胞移植から41日目(Day 41)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。* P < 0.05, ** P < 0.01 (by Student's t-test)

図６１は、ヒト胆管癌細胞TFK-1細胞を用いたxenograft preventionモデルにおける、マウス抗hTROP-2抗体K5-70の抗腫瘍活性を示す図である。

A： コントロール群（●： PBS）、およびK5-70抗体 (10mg/kg 体重)投与群（○）の腫瘍形成を経時的に表した（平均値±標準偏差）。矢印は、抗体投与期間を表す。** P < 0.01 (by Student's t-test)

B： Aの試験において、癌細胞移植から31日目(Day 31)（実験最終日）の時点での各マウスにおける腫瘍重量をプロットした図である。** P < 0.01 (by Student's t-test)

図６２は、HuK5-70およびHuT6-16-2抗体の結合活性を、低濃度抗原固相化ELISAを用いて検討した結果である。96穴プレートを0.1μg/mLのリコンビナントhTACSTD2-Fc-Hisタンパク質でコーティングし、20μg/mLから2倍ずつの希釈系列を調製した被検抗体（K5-70、HuK5-70、T6-16およびHuT6-16-2抗体）を反応させた。(A)はK5-70(▲)およびHuK5-70(●)抗体を、(B)はT6-16(▲)およびHuT6-16-2抗体(●)をそれぞれ被験抗体として使用した。

図６３は、hTROP-2のK5-70およびHuK5-70抗体に対する結合活性を示すELISAの結果である。96穴プレートにanti-mouse IgG (γ chain specific)およびanti-human IgG1 (Fcγ specific)を介してK5-70およびHuK5-70抗体を固相化し、5μg/mLから2倍希釈系列を調製したhTROP-2-EC-Hisタンパク質を反応させた。hTROP-2-EC-Hisタンパク質の結合は、抗Hisタグ抗体を用いて検出した。(▲)K5-70抗体、 (●) HuK5-70抗体

図６４は、遺伝子合成により作製したK5-70 VH遺伝子の塩基配列（上段；配列番号９９）とK5-70 VHのアミノ酸配列（下段；配列番号３５）を示す図である。当該塩基配列においては、5’末端にEcoRI部位(GAA TTC)およびKozak配列（ACC ACC）を付加し、3’末端にNheI部位(GCT AGC)を付加している。当

該アミノ酸配列は、一文字表記で示しており、N末端側のシグナルペプチドはイタリック体で表している。二重下線を引いたグルタミン（Q）は、成熟ペプチドのN末端アミノ酸残基を示す。CDR配列（下線；*IYWIN, NIYPSDSYTNYNQKFKD, TSMADY*）は、Kabatら(Sequences of Proteins of Immunological Interests, Fifth edition, NIH Publication No. 91-3242, U.S. Department of Health and Human Services, 1991) の定義に従って決定した。クローンK5-70 VHのCDR1～3のアミノ酸配列は、それぞれ順に、配列番号３６～３８に示す。

図６５は、遺伝子合成により作製したK5-70 VL遺伝子の塩基配列（上段；配列番号１００）とK5-70 VLのアミノ酸配列（下段；配列番号４０）を示す図である。当該塩基配列においては、5’末端にAgeI部位(ACC GGT)およびKozak配列（ACC ACC）を付加し、3’末端にBsiWI部位(CGT ACG)を付加している。当該アミノ酸配列は、一文字表記で示しており、N末端のシグナルペプチドはイタリック体で表している。二重下線を引いたアスパラギン酸（D）は、成熟ペプチドのN末端アミノ酸残基を示す。CDR配列（下線; *RASQSIGTSIH, YASESIS, QQSNSWPFT*）は、Kabatら(前掲；U.S. Department of Health and Human Services, 1991) の定義に従って決定した。クローンK5-70 VLのCDR1～3のアミノ酸配列は、それぞれ順に、配列番号４１～４３に示す。

図６６は、ChK5-70、HuK5-70、HuVH/MuVL（HuK5-70抗体のVLをK5-70抗体のVLに置換したもの）およびMuVH/HuVL（HuK5-70抗体のVH をK5-70抗体のVHに置換したもの）抗体のhTROP-2に対する結合活性を示した図である。96穴プレートを0.1μg/mLのリコンビナントhTACSTD2-Fc-Hisタンパク質でコーティングし、被検抗体（ChK5-70、HuK5-70、HuVH/MuVLおよびMuVH/HuVL抗体）を一過性発現させた細胞の培養上清を1、0.1、0.01、0.001μg/mLの抗体濃度になるように希釈し反応させた。(▲)ChK5-70抗体、(△)MuVH/HuVL抗体、(○)HuVH/MuVL 抗体、(●)HuK5-70抗体

図６７は、HuK5-70 VHおよびそのアミノ酸置換変異体のアミノ酸配列を示した図である。アミノ酸は一文字表記で示した。各アミノ酸置換変異体では、HuK5-70 VHのアミノ酸と同じアミノ酸については “-“で示し、置換したア

ミノ酸についてのみ一文字表記で示した。配列上部の数字はアミノ酸番号を示す（Kabatら1991）。

図６８は、ChK5-70、HuK5-70、HuK5-70 VH A40R（HuK5-70抗体のVHの40番目のアラニンをアルギニンに置換した変異体）およびHuK5-70 VH R44G抗体（HuK5-70抗体のVHの44番目のアルギニンをグリシンに置換した変異体）のhTROP-2に対する結合活性を示した図である。96穴プレートを0.1μg/mLのリコンビナントhTACSTD2-Fc-Hisタンパク質でコーティングし、被検抗体（ChK5-70、HuK5-70、HuK5-70 VH A40RおよびHuK5-70 VH R44G抗体）を一過性発現させた細胞の培養上清を0.5μg/mLから2倍希釈系列（6点）を作製し反応させた。(▲)ChK5-70抗体、(△)HuK5-70 VH R44G抗体、(○) HuK5-70 VH A40R抗体、(●)HuK5-70抗体

図６９は、遺伝子合成により作製したHuK5-70 VH R44G遺伝子の塩基配列（上段）とアミノ酸配列（下段）を示す。5’末端にEcoRI部位(GAA TTC)およびKozak配列（ACC ACC）、3’末端にNheI部位(GCT AGC)を付加した。アミノ酸配列は一文字表記で示した。N末端のシグナルペプチドはイタリック体で示し、成熟VHのN末端側のアミノ酸（Q：グルタミン）を二重下線で示し、CDR配列（Kabatら、1991）を下線で示した。

図７０は、精製HuK5-70-2抗体のSDS-PAGEの図である。HuK5-70-2抗体1μgを還元条件下で11%SDS-PAGEゲルで展開した。レーン1: 分子量マーカー（プレシジョンPlusデュアルスタンダード (BIO-RAD)），レーン2: HuK5-70-2抗体。図の左側の数値は分子量を示す。

図７１は、K5-70、HuK5-70およびHuK5-70-2抗体のhTROP-2に対する結合活性を示した図である。96穴プレートを0.1μg/mLのリコンビナントhTACSTD2-Fc-Hisタンパク質でコーティングし、精製した被検抗体（K5-70、HuK5-70およびHuK5-70-2抗体）を1μg/mLから2倍希釈系列（10点）を作製し反応させた。(■)K5-70抗体、(△)HuK5-70-2抗体、 (●)HuK5-70抗体

図７２Ａは、ヒト型化抗hTROP-2抗体（白カラム: HuK5-70, 黒カラム: HuT6-16-2)を用いたADCC活性を示す図である。詳しくは、ヒト大腸癌細胞株SW480に、HuK5-70抗体及びHuT6-16-2抗体 (いずれも0, 0.1, 0.3, 1, 3, 10μ

g/mL)と健常人末梢血単核球を加え、６時間培養し、培養上清中に放出されるLDHの活性を測定することによりADCC活性を測定した結果を示す（平均値±標準誤差（N=3), ：エフェクター／ターゲット（E/T）＝40)。 抗体濃度0は抗体非添加を示す。*P< 0.05, **P < 0.01(by Student’s t-test)

図７２Ｂは、ヒト型化抗hTROP-2抗体（白カラム: HuK5-70, 灰色カラム: HuK5-70-2, 黒カラム: HuT6-16-2)を用いたADCC活性を示す図である。詳しくは、ヒト膵臓癌細胞株（PK-59)に、HuK5-70抗体, HuK5-70-2抗体及びHuT6-16-2抗体（いずれも0, 0.3, 1, 3, 10, 30μg/mL)と健常人末梢血単核球を加え、６時間培養し、培養上清中に放出されるLDHの活性を測定することによりADCC活性を測定した結果を示す（平均値±標準誤差（N=3), ：エフェクター／ターゲット（E/T）＝40)。抗体濃度0は抗体非添加であることを示す。**P < 0.01 (byStudent’s t-test)

図７２Ｃは、ヒト型化抗hTROP-2抗体（白カラム: HuK5-70, 灰色カラム: HuK5-70-2, 黒カラム: HuT6-16-2)を用いたADCC活性を示す図である。詳しくは、ヒト前立腺癌細胞株（PC-3)に、HuK5-70抗体, HuK5-70-2抗体及びHuT6-16-2抗体（いずれも0, 0.3, 1, 3, 10, 30μg/mL)と健常人末梢血単核球を加え、６時間培養し、培養上清中に放出されるLDHの活性を測定することによりADCC活性を測定した結果を示す（平均値±標準誤差（N=3), ：エフェクター／ターゲット（E/T）＝40)。抗体濃度0は抗体非添加であることを示す。**P < 0.01 (by Student’s t-test)。

発明を実施するための形態

以下、本発明を詳細に説明する。本発明の範囲はこれらの説明に拘束されることはなく、以下の例示以外についても、本発明の趣旨を損なわない範囲で適宜変更し実施し得る。

なお、本明細書は、本願優先権主張の基礎となる米国仮出願61/562,672号明細書（2011年11月22日出願）の全体を包含する。また、本明細書において引用された全ての刊行物、例えば先行技術文献、及び公開公報、特許公報その他の特許文献は、参照として本明細書に組み込まれる。

１．本発明の概要

ヒトTROP-2（hTROP-2）は、前述したように、全長323アミノ酸残基からなる１回膜貫通型の１型膜タンパク質である。そして、hTROP-2遺伝子及びその遺伝子産物は、種々の癌細胞において発現していることが知られている。

前述したように、*in vivo* において高い抗腫瘍活性を有する抗 hTROP-2 抗体（抗 hTROP-2 モノクローナル抗体）などの開発が望まれていた状況の下、本発明者は、極めて多数のクローンからスクリーニングを行うことにより、*in vivo* で高い抗腫瘍活性を有するクローンを取得することに成功した。具体的には、本発明は、*in vivo* において hTROP-2 の細胞外領域を特異的に認識するモノクローナル抗体、特にピコモルオーダー（pM）の高い親和性を示すモノクローナル抗体を提供するものである。本発明の抗体は、裸抗体の単独投与で、既存の抗 hTROP-2 抗体と比較して、より少ない用量（例えば 1/20 投与量）で顕著な腫瘍成長阻害活性を有し、かつ複数のヒト癌細胞を用いた担癌マウス治療モデルにおいて顕著な腫瘍成長阻害活性を示す、抗 hTROP-2 モノクローナル抗体（特にヒト型化抗体）である点で、極めて有用なものである。

２．抗hTROP-2抗体の作製

（１）抗原の調製

hTROP-2のアミノ酸配列（配列番号２）の情報は、例えばNCBI（GenBank）のウェブサイト（http://www.ncbi.nlm.nih.gov/）に「Accession number : NP 002344」として公表されている。なお、hTROP-2のアミノ酸配列をコードする塩基配列（配列番号１）の情報は、同ウェブサイトに「Accession number : NM 002353」として公表されている。

抗原としては、hTROP-2のアミノ酸配列の少なくとも一部（全部又は一部）を含むポリペプチド又はペプチド（単にペプチドともいう）を使用することができ、好ましくは、hTROP-2の細胞外領域のアミノ酸配列の少なくとも一部（全部又は一部）を含むペプチドを使用することができる。hTROP-2の細胞外領域（シグナルペプチドを含む）は、配列番号２で示されるアミノ酸配列のうちの第

1番目～第274番目のアミノ酸を含む領域（シグナルペプチド：第1番目～第26番目のアミノ酸）のことを言う。ここで、抗原に用いるペプチドにつき、上記「アミノ酸配列の少なくとも一部」とは、長さが特に限定されるわけではないが、例えば、配列番号２で示されるアミノ酸配列のうちの第1番目～第145番目のアミノ酸を含む領域や、当該アミノ酸配列のうちの第146番目～第274番目のアミノ酸を含む領域などが好ましい。

抗原とするペプチドの作製方法は、化学合成でも、大腸菌などを用いる遺伝子工学的手法による合成でもよく、当業者に周知の方法を用いることができる。

ペプチドの化学合成を行う場合は、ペプチドの合成の周知方法によって合成することができる。また、その合成は、固相合成法及び液相合成法のいずれをも適用することができる。市販のペプチド合成装置（例えば、島津製作所製：PSSM-8など）を使用してもよい。

ペプチドを遺伝子工学的に合成する場合は、まず、当該ペプチドをコードするDNAを設計し合成する。当該設計及び合成は、例えば、全長hTROP-2遺伝子を含むベクター等を鋳型とし、所望のDNA領域を合成し得るように設計したプライマーを用いて、PCR法により行うことができる。そして、上記DNAを適当なベクターに連結することによってタンパク質発現用組換えベクターを得、この組換えベクターを目的遺伝子が発現し得るように宿主中に導入することによって形質転換体を得る（Sambrook J. et al., Molecular Cloning, A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press, 2001）。

ベクターには、宿主微生物で自律的に増殖し得るファージ又はプラスミドが使用される。さらに、動物ウイルス、昆虫ウイルスベクターを用いることもできる。組換えベクターの作製は、精製されたDNAを適当な制限酵素で切断し、適当なベクターDNAの制限酵素部位等に挿入してベクターに連結すればよい。形質転換に使用する宿主としては、目的の遺伝子を発現できるものであれば特に限定されるものではない。例えば、細菌（大腸菌、枯草菌等）、酵母、動物細胞（COS細胞、CHO細胞等）、昆虫細胞又は昆虫が挙げられる。ヤギ等の哺乳動物を宿主として使用することも可能である。宿主への組換えベクターの導入方法は公知である。

そして、前記形質転換体を培養し、その培養物から抗原として使用されるペプチドを採取する。「培養物」とは、(a)培養上清、(b)培養細胞若しくは培養菌体又はその破砕物のいずれをも意味するものである。

培養後、目的ペプチドが菌体内又は細胞内に生産される場合には、菌体又は細胞を破砕することによりペプチドを抽出する。また、目的ペプチドが菌体外又は細胞外に生産される場合には、培養液をそのまま使用するか、遠心分離等により菌体又は細胞を除去する。その後、ペプチドの単離精製に用いられる一般的な生化学的方法、例えば硫酸アンモニウム沈殿、ゲル濾過、イオン交換クロマトグラフィー、アフィニティークロマトグラフィー等を単独で又は適宜組み合わせて用いることにより、目的のペプチドを単離精製することができる。

本発明においては、無細胞合成系を用いた*in vitro*翻訳により抗原となるペプチドを得ることもできる。この場合は、RNAを鋳型にする方法とDNAを鋳型にする方法（転写／翻訳）の２通りの方法を用いることができる。無細胞合成系としては、市販のシステム、例えばExpressway™システム（インビトロジェン社）、PURESYSTEM（登録商標；ポストゲノム研究所）、TNTシステム（登録商標；プロメガ社）等を用いることができる。

上記のごとく得られたペプチドは、適当なキャリアタンパク質、例えば牛血清アルブミン(BSA)、キーホールリンペットヘモシアニン(KLH)、ヒトチログロブリン、ニワトリガンマグロブリン等に結合することも可能である。

また抗原は、hTROP-2のアミノ酸配列（配列番号２）又は前述したその部分配列において１又は複数のアミノ酸が欠失、置換又は付加されたアミノ酸配列からなるペプチドであってもよい。例えば、hTROP-2のアミノ酸配列又はその部分配列のうち１又は複数個（好ましくは１個又は数個（例えば１個～10個、さらに好ましくは１個～５個））のアミノ酸が欠失しており、１又は複数個（好ましくは１個又は数個（例えば１個～10個、さらに好ましくは１個～５個））のアミノ酸が他のアミノ酸で置換されており、あるいは、１又は複数個（好ましくは1個又は数個（例えば１個～10個、さらに好ましくは１個～５個））の他のアミノ酸が付加されたアミノ酸配列からなるペプチドを使用することもできる。

本発明において、細胞等に導入するための遺伝子としては、hTROP-2タンパク質若しくはその部分断片又はこれらの変異型のタンパク質又は断片をコードする遺伝子が挙げられる。そのような遺伝子としては、例えば、配列番号１に示される塩基配列又はその部分配列を有するものを使用することができる。

また、細胞等に導入するための遺伝子としては、配列番号１に示される塩基配列に相補的な配列とストリンジェントな条件下でハイブリダイズし且つhTROP-2活性を有するタンパク質をコードする塩基配列、又はその部分配列を使用することも可能である。

「ストリンジェントな条件」とは、ハイブリダイズさせた後の洗浄時の条件であって、バッファーの塩（ナトリウム）濃度が10～500mMであり、温度が42℃～72℃、好ましくは、上記塩濃度が50～300mMであり、温度が55～68℃での条件をいう。

遺伝子に変異を導入するには、Kunkel法やGapped duplex法等の公知手法により、例えば部位特異的突然変異誘発法を利用した変異導入用キット、例えばGeneTailor™ Site-Directed Mutagenesis System（インビトロジェン社製）、TaKaRa Site-Directed Mutagenesis System（Mutan-K、Mutan-Super Express Km等：タカラバイオ社製）を用いて行うことができる。

（２）ポリクローナル抗体の作製

調製した抗原を、免疫のため哺乳動物に投与する。哺乳動物は特に限定はされず、例えばラット、マウス及びウサギなどを挙げることができ、なかでもマウスが好ましい。

抗原の動物一匹あたりの投与量は、アジュバントの有無により適宜設定することができる。アジュバントとしては、フロイント完全アジュバント(FCA)、フロイント不完全アジュバント(FIA)、水酸化アルミニウムアジュバント等が挙げられる。免疫は、主として静脈内、足蹠、皮下、腹腔内等に注入することにより行うことができる。また、免疫の間隔については、特に限定されず、数日から数週間間隔、好ましくは１週間間隔で、１～10回、好ましくは２～３回免疫を行う。そして、最終の免疫日から３～７日後に、酵素免疫測定法（ELISA又はEIA）や放射性免疫測定法（RIA）等で抗体価を測定し、所望の抗体価を示した日に採血

して、抗血清を得ることができる。上記抗体の採取方法において、抗体の精製が必要とされる場合は、硫安塩析法、イオン交換クロマトグラフィー、ゲル濾過クロマトグラフィー、アフィニティークロマトグラフィー等の公知の方法を適宜選択して、又はこれらを組み合わせることにより、精製することができる。その後は、抗血清中のポリクローナル抗体の反応性をELISA法などで測定する。

（3）モノクローナル抗体の作製

(3-1) 抗体産生細胞の採取

本発明の抗hTROP-2抗体は、限定はされないが、モノクローナル抗体であることが好ましい。

調製した抗原を、免疫のため哺乳動物、例えばラット、マウス及びウサギなどに投与する。抗原の動物一匹あたりの投与量は、アジュバントの有無により適宜設定することができる。アジュバントとしては上記と同様である。免疫手法も前記と同様である。そして、最終の免疫日から１～60日後、好ましくは１～14日後に、抗体産生細胞を採取する。抗体産生細胞としては、脾臓細胞、リンパ節細胞及び末梢血細胞などが挙げられるが、なかでもリンパ節細胞及び脾臓細胞が好ましい。

(3-2) 細胞融合

ハイブリドーマ（抗体産生細胞株）を得るため、抗体産生細胞とミエローマ細胞との細胞融合を行う。抗体産生細胞と融合させるミエローマ細胞として、マウスなどの動物の一般に入手可能な株化細胞を用いることができる。用いる細胞株としては、薬剤選択性を有し、未融合の状態ではHAT選択培地（ヒポキサンチン、アミノプテリン及びチミジンを含む）で生存できず、抗体産生細胞と融合した状態でのみ生存できる性質を有するものが好ましい。

ミエローマ細胞としては、例えば、P3-X63-Ag8.653、P3-X63-Ag8(X63)、P3-X63-Ag8.U1(P3U1)、P3/NS I/1-Ag4-1(NS1) 及びSp2/0-Ag14(Sp2/0)等のマウスミエローマ細胞株が挙げられる。ミエローマ細胞の選択は、抗体産生細胞との適合性を適宜考慮して行うことができる。

次いで、ミエローマ細胞と抗体産生細胞とを細胞融合させる。細胞融合は、血清を含まないDMEM及びRPMI-1640培地などの動物細胞用培地中で、1×10^{6}～

1×10^7個／mLの抗体産生細胞と2×10^5～2×10^6個／mLのミエローマ細胞とを混合する。抗体産生細胞とミエローマ細胞との細胞比（抗体産生細胞：ミエローマ細胞）は、限定はされないが、通常、１：１～１０：１とすることが好ましく、より好ましくは３：１である。次に、細胞融合促進剤の存在下で融合反応を行う。細胞融合促進剤として、例えば、平均分子量1,000～6,000ダルトン(D)のポリエチレングリコールなどを使用することができる。また、電気刺激（例えばエレクトロポレーション）を利用した市販の細胞融合装置を用いて、抗体産生細胞とミエローマ細胞とを融合させることもできる。

(3-3) ハイブリドーマの選別及びクローニング

細胞融合処理後の細胞から目的とするハイブリドーマを選別する。その方法として、細胞懸濁液を、例えばウシ胎児血清含有RPMI-1640培地などに適当に希釈後、マイクロタイタープレート上に播き、各ウェルに選択培地を加え、以後適当に選択培地を交換して培養を行う。その結果、選択培地で培養開始後、14日前後から生育してくる細胞をハイブリーマとして得ることができる。

次に、増殖してきたハイブリドーマの培養上清中に、hTROP-2に反応する抗体が存在するか否かをスクリーニングする。ハイブリドーマのスクリーニングは、通常の方法に従えばよく、特に限定はされない。例えば、ハイブリドーマとして生育したウェルに含まれる培養上清の一部を採取し、ELISA、EIA及びRIAなどによってスクリーニングすることができる。

融合細胞のクローニングは、限界希釈法等により行うことができる。hTROP-2に強い反応性を示す抗体をフローサイトメトリー等により判定し、これを産生するハイブリドーマを選択し、クローンとして樹立する。

(3-4) モノクローナル抗体の採取

樹立したハイブリドーマを培養し、得られる培養物からモノクローナル抗体を採取する方法として、通常の細胞培養法、又は腹水形成法等を採用することができる。「培養」とは、ハイブリドーマを培養皿又は培養ボトル中で生育させること、あるいはハイブリドーマを下記のように動物の腹腔内で増殖させることを意味する。

細胞培養法においては、ハイブリドーマを10%ウシ胎児血清含有RPMI-1640培地、MEM培地又は無血清培地等の動物細胞培養培地中で、通常の培養条件（例えば37℃、５％CO_2濃度）で７～14日間培養し、その培養上清から抗体を取得することができる。

腹水形成法の場合は、ミエローマ細胞由来の哺乳動物と同種系動物の腹腔内にハイブリドーマを約１x10^7個投与し、ハイブリドーマを大量に増殖させる。そして、２～３週間後に腹水を採取することが好ましい。

上記抗体の採取方法において、抗体の精製が必要とされる場合は、硫安塩析法、イオン交換クロマトグラフィー、ゲル濾過、アフィニティークロマトグラフィー等の公知の方法を適宜選択して、又はこれらを組み合わせることにより精製することができる。

(3-5) 抗腫瘍活性を有するクローンの選別

本発明の抗hTROP-2抗体は、*in vivo*で抗腫瘍活性を有する抗体である。

ここで、「抗腫瘍活性」とは、腫瘍細胞（癌細胞）を死滅させる活性又は腫瘍成長を阻害する活性を意味する。抗腫瘍活性としては、例えば、癌細胞の増殖阻害活性や腫瘍血管新生阻害活性が好ましく挙げられる。また、本発明の抗体が抗腫瘍活性を発揮しうるヒト腫瘍（腫瘍細胞）の種類としては、hTROP-2の発現が確認されている公知の各種ヒト腫瘍が挙げられ、特に限定はされないが、例えば、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌などの各種ヒト腫瘍のうちの１種又は２種以上が好ましく挙げられ、より好ましくはヒト膵癌、ヒト大腸癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌及びヒト肺癌のうちの１種又は２種以上であり、さらに好ましくはヒト膵癌及び／又はヒト大腸癌である。さらに、上記腫瘍の種類としては、再発癌や転移癌であってもよく、本発明の抗体はこれらに腫瘍に対しても優れた抗腫瘍活性を発揮し得る。

*in vivo*での抗腫瘍活性の確認は、例えば、所望の腫瘍細胞をマウスの皮下に移植した担癌マウス（担癌動物治療モデル）を用い、このマウスに前記のとおり得られた抗体を投与することにより行うことができる。この場合、抗体の投与は、腫瘍細胞の移植直後から行ってもよいし（preventionモデル）、移植に腫瘍が所

定の体積になったのを確認してから行ってもよい（treatmentモデル）。投与方法は、限定はされないが、例えば、3日、1週間,10日又は2週間に1回あるいは単回（1回のみ）で、5～20mg/kg体重、腹腔内投与等とすることができる。preventionモデルの場合は、腫瘍形成頻度と腫瘍体積により抗腫瘍活性の有無及びレベルを評価することができる。treatmentモデルの場合は、腫瘍体積により抗腫瘍活性の有無及びレベルを評価することができる。

本発明において、*in vivo*で抗腫瘍活性を有する抗hTROP-2抗体としては、例えば、H鎖V領域のCDR1～3のアミノ酸配列が、それぞれ順に、配列番号３６～３８で示されるアミノ酸配列であり、及び／又は、L鎖V領域のCDR1～3のアミノ酸配列が、それぞれ順に、配列番号４１～４３で示されるアミノ酸配列であるものが、好ましく挙げられる。当該H鎖V領域としては、例えば、配列番号３５で示されるアミノ酸配列からなるものが好ましく、当該L鎖V領域としては、例えば、配列番号４０で示されるアミノ酸配列からなるものが好ましい。

また本発明の抗hTROP-2抗体の他の態様としては、例えば、H鎖V領域のCDR1～3のアミノ酸配列が、それぞれ順に、配列番号４６～４８で示されるアミノ酸配列であり、及び／又は、L鎖V領域のCDR1～3のアミノ酸配列が、それぞれ順に、配列番号５１～５３で示されるアミノ酸配列であるものが、好ましく挙げられる。当該H鎖V領域としては、例えば、配列番号４５で示されるアミノ酸配列からなるものが好ましく、当該L鎖V領域としては、例えば、配列番号５０で示されるアミノ酸配列からなるものが好ましい。

同様に、本発明の抗hTROP-2抗体の他の態様としては、例えば、H鎖V領域のCDR1～3のアミノ酸配列が、それぞれ順に、配列番号５６～５８で示されるアミノ酸配列であり、及び／又は、L鎖V領域のCDR1～3のアミノ酸配列が、それぞれ順に、配列番号６１～６３で示されるアミノ酸配列であるものが、好ましく挙げられる。当該H鎖V領域としては、例えば、配列番号５５で示されるアミノ酸配列からなるものが好ましく、当該L鎖V領域としては、例えば、配列番号６０で示されるアミノ酸配列からなるものが好ましい。

同様に、本発明の抗hTROP-2抗体の他の態様としては、例えば、H鎖V領域のCDR1～3のアミノ酸配列が、それぞれ順に、配列番号６６～６８で示される

アミノ酸配列であり、及び／又は、Ｌ鎖Ｖ領域のCDR1～3のアミノ酸配列が、それぞれ順に、配列番号７１～７３で示されるアミノ酸配列であるものが、好ましく挙げられる。当該H鎖Ｖ領域としては、例えば、配列番号６５で示されるアミノ酸配列からなるものが好ましく、当該Ｌ鎖Ｖ領域としては、例えば、配列番号７０で示されるアミノ酸配列からなるものが好ましい。

本発明において、*in vivo*で抗腫瘍活性を有する抗 hTROP-2 抗体としては、より具体的には、例えば、受領番号が FERM BP-11251 であるハイブリドーマにより産生される抗 hTROP-2 モノクローナル抗体（クローン名：K5-70）、受領番号が FERM BP-11252 であるハイブリドーマにより産生される抗 hTROP-2 モノクローナル抗体（クローン名：K5-107）、受領番号が FERM BP-11253 であるハイブリドーマにより産生される抗 hTROP-2 モノクローナル抗体（クローン名：K5-116-2-1）、受領番号が FERM BP-11346 であるハイブリドーマにより産生される抗 hTROP-2 モノクローナル抗体（クローン名：T6-16）及び受領番号が FERM BP-11254 であるハイブリドーマにより産生される抗 hTROP-2 モノクローナル抗体（クローン名：T5-86）などが好ましく挙げられる。

ここで、受領番号が FERM BP-11251 であるハイブリドーマは、「Mouse-Mouse Hybridoma K5-70」と称し 2010 年 5 月 12 日付で、受領番号が FERM BP-11252 であるハイブリドーマは、「Mouse-Mouse Hybridoma K5-107」と称し 2010 年 5 月 12 日付で、受領番号が FERM BP-11253 であるハイブリドーマは、「Mouse-Mouse Hybridoma K5-116-2-1」と称し 2010 年 5 月 12 日付で、受領番号が FERM BP-11346 であるハイブリドーマは、「Mouse-Mouse Hybridoma T6-16」と称し 2011 年 3 月 1 日付で、受領番号が FERM BP-11254 であるハイブリドーマは、「Mouse-Mouse Hybridoma T5-86」と称し 2010 年 5 月 12 日付で、いずれも独立行政法人産業技術総合研究所 特許生物寄託センター（〒305-8566 茨城県つくば市東 1-1-1 中央第 6）に寄託されたものである。

さらに、本発明の抗hTROP-2抗体としては、例えば、受領番号がFERM BP-11251、FERM BP-11252、FERM BP-11253、FERM BP-11346又はFERM BP-11254であるハイブリドーマにより産生されるモノクローナル抗体が結合（

認識）する部位（例えばエピトープ）に結合する抗hTROP-2抗体も好ましく挙げられる。エピトープとしては、下記(3-6)項において例示するものが好ましく挙げられる。

(3-6) 抗hTROP-2抗体のエピトープ

本発明の抗hTROP-2抗体のエピトープ（抗原決定基）は、抗原であるhTROP-2の少なくとも一部であればよく限定はされないが、例えば、配列番号２に示されるhTROP-2のアミノ酸配列中の、第252番目～第260番目のアミノ酸からなる領域を除いた領域の少なくとも一部であることが好ましく、より好ましくは、第1番目～第69番目のアミノ酸からなる領域の少なくとも一部又は第100番目～第274番目のアミノ酸からなる領域（第252番目～第260番目のアミノ酸からなる領域を除く）の少なくとも一部であり、さらに好ましくは第27番目～第69番目のアミノ酸からなる領域の少なくとも一部又は第109番目～第206番目のアミノ酸からなる領域の少なくとも一部である。上記エピトープとして、特に好ましくは、配列番号２に示されるhTROP-2のアミノ酸配列中の、第43番目～第65番目のアミノ酸からなる領域、第152番目～第165番目のアミノ酸からなる領域、第171番目～第183番目のアミノ酸からなる領域、第109番目～第120番目のアミノ酸からなる領域、第193番目～第206番目のアミノ酸からなる領域、及び第43番目～第56番目のアミノ酸からなる領域、並びにこれらを含む部分であり、中でも特に好ましいのは、第43番目～第65番目のアミノ酸からなる領域、第152番目～第165番目のアミノ酸からなる領域、第171番目～第183番目のアミノ酸からなる領域、及び第109番目～第120番目のアミノ酸からなる領域、並びにこれらを含む部分である。当該領域を認識する（当該領域又はそれを含む部分と結合する）抗hTROP-2抗体は、例えば、腫瘍細胞内へのインターナリゼーション活性が高く、後述するイムノコンジュゲート等の用途に極めて有用なものである。

(3-7) 抗hTROP-2抗体の特性

本発明の抗hTROP-2抗体は、前述の通り、低用量で高い*in vivo*抗腫瘍活性を有する抗体である。具体的には、担癌動物モデルに対して、20mg/kg体重以下（好ましくは10mg/kg体重以下、より好ましくは5mg/kg体重以下、さらに好ましくは1mg/kg体重以下）の投与量（裸抗体）で50％以上（好ましくは80％以上、

より好ましくは90％以上、さらに好ましくは95％以上、特に好ましくはほぼ100％（例えば98％以上又は99％以上））の腫瘍成長阻害活性を示すものであることが好ましい。

ここで、腫瘍成長阻害活性（％）は、例えば、下記式により算出することができる。

腫瘍成長阻害活性（％）＝ 100－〔（抗体投与群の腫瘍体積又は腫瘍重量）÷（対照群の腫瘍体積又は腫瘍重量）〕×100

また、本発明の抗hTROP-2抗体は、2種以上のヒト腫瘍細胞株に対して抗腫瘍活性を有するものであることが好ましい。ヒト腫瘍細胞株としては、限定はされないが、例えば、各種ヒト膵癌細胞株、ヒト前立腺癌細胞株、ヒト大腸癌細胞株、ヒト乳癌細胞株、ヒト卵巣癌細胞株、ヒト肺癌細胞株及びヒト胆管癌細胞株からなる群より選ばれる少なくとも2種が挙げられ、具体的には、ヒト膵癌細胞株PK-59、ヒト膵癌細胞株BxPC-3、ヒト膵癌細胞株KP-3L、ヒト膵癌細胞株KP-2、ヒト膵癌細胞株PK-1、ヒト膵癌細胞株PK-45H、ヒト膵癌細胞株PK-45P、ヒト膵癌細胞株TCC-PAN2、ヒト膵癌細胞株SUIT-2、ヒト大腸癌細胞株CACO-2、ヒト大腸癌細胞株SW480、ヒト大腸癌細胞株DLD-1、ヒト大腸癌細胞株HCT116、ヒト乳癌細胞株JIMT-1、ヒト乳癌細胞株HCC1143、ヒト乳癌細胞株MCF-7、ヒト乳癌細胞株MDA-MB-468、ヒト前立腺癌細胞株DU145、ヒト前立腺癌細胞株PC-3、ヒト卵巣癌細胞株SK-OV-3、ヒト肺癌細胞株Calu-3、及びヒト胆管癌細胞株TFK-1からなる群より選ばれる少なくとも2種が好ましく挙げられる。中でも、前記2種以上のヒト腫瘍細胞株としては、ヒト膵癌細胞株PK-59、ヒト膵癌細胞株BxPC-3、ヒト大腸癌細胞株SW480、ヒト肺癌細胞株Calu-3、ヒト乳癌細胞株MBA-MD-468、及びヒト卵巣癌細胞株SK-OV-3からなる群より選ばれる少なくとも2種がより好ましく挙げられる。

さらに、本発明の抗hTROP-2抗体は、解離定数（Kd値）が1.0×10^{-10} M以下であることが好ましく、より好ましくは1.0×10^{-11} M以下であり、さらに好ましくは1.0×10^{-12} M以下である。ここで、抗体の結合能（親和性）は、例えば、スキ

ャッチャード解析やBiacoreと呼ばれる表面プラズモン共鳴センサーにより、解離定数(Kd値)、解離速度定数(Kdiss [1/Sec])、結合速度定数(Kass [1/M.Sec])として測定することができる。Biacore装置としては、例えばBiacore 3000、Biacore 2000、Biacore X、Biacore J、Biacore Q（いずれもBiacore社）などが挙げられる。抗体は、解離定数（Kd値）が小さい値であるほど結合能（親和性）が高いという点で好ましい。Kd値は、Kdiss及びKassの２つのパラメーターにより決定され、例えば、式：Kd[M]＝Kdiss/Kass により表すことができる。なお、Kd値の算出方法については、後述する実施例（具体的には実施例10）に記載の方法も好ましく採用することができる。

（4）遺伝子組換え抗体、及び抗体断片

(4-1) 遺伝子組換え抗体

本発明の抗hTROP-2抗体の好ましい態様の一つとして、遺伝子組換え抗体が挙げられる。遺伝子組換え抗体としては、限定はされないが、例えば、キメラ抗体、ヒト型化抗体 及びヒト抗体等が挙げられる。

キメラ抗体（すなわちヒト型キメラ抗体）は、マウス由来抗体の可変領域をヒト由来の定常領域に連結（接合）した抗体であり（Proc. Natl. Acad. Sci. U.S.A. 81, 6851-6855, (1984) 等を参照）、キメラを作製する場合は、そのように連結した抗体が得られるよう、遺伝子組換え技術によって容易に構築できる。

ヒト型化抗体を作製する場合は、マウス抗体の可変領域から相補性決定領域（CDR）をヒト可変領域に移植して、フレームワーク領域（FR）はヒト由来のものでCDRはマウス由来のものからなる、再構成した可変領域を作製する（いわゆるCDRグラフティング（CDR移植））。次に、これらのヒト型化された再構成ヒト可変領域をヒト定常領域に連結する。このようなヒト型化抗体の作製法は、当分野において周知である（Nature, 321, 522-525 (1986)；J. Mol. Biol., 196, 901-917 (1987)；Queen C et al., Proc. Natl. Acad. Sci. USA, 86: 10029-10033 (1989)；特表平4-502408号公報（特許第2828340号公報；クイーンら）等を参照）。

ここで、本発明のヒト型化抗hTROP-2抗体に用いることができるマウス由来のCDR配列としては、限定はされないが、例えば、H鎖V領域(VH)のCDR1～3

として（それぞれ順に）配列番号３６～３８に示されるアミノ酸配列や配列番号６６～６８で示されるアミノ酸配列が、L鎖V領域(VL)のCDR1～3として（それぞれ順に）配列番号４１～４３に示されるアミノ酸配列や配列番号７１～７３で示されるアミノ酸配列が、好ましく挙げられる。

また、ヒト型化された再構成ヒト可変領域のうち、H鎖V領域のアミノ酸配列としては、例えば、配列番号９２で示されるアミノ酸配列（配列番号３６～３８に示されるアミノ酸配列からなるCDR1～3を含む；さらにシグナルペプチドを含むアミノ酸配列は配列番号７５で示される）、配列番号９８で示されるアミノ酸配列（配列番号３６～３８に示されるアミノ酸配列からなるCDR1～3を含む；さらにシグナルペプチドを含むアミノ酸配列は配列番号９７で示される）、配列番号９４で示されるアミノ酸配列（配列番号６６～６８に示されるアミノ酸配列からなるCDR1～3を含む；さらにシグナルペプチドを含むアミノ酸配列は配列番号７９で示される）、及び配列番号９５で示されるアミノ酸配列（配列番号６６～６８に示されるアミノ酸配列からなるCDR1～3を含む；さらにシグナルペプチドを含むアミノ酸配列は配列番号８１で示される）が好ましく挙げられる。ここで、上記配列番号９８で示されるH鎖V領域のアミノ酸配列は、上記の配列番号９２で示されるH鎖V領域のアミノ酸配列の第４４番目のアルギニン(R)をグリシン(G)に置換した改変型のアミノ酸配列である。

同様に、ヒト型化された再構成ヒト可変領域のうち、L鎖V領域のアミノ酸配列としては、例えば、配列番号９３で示されるアミノ酸配列（配列番号４１～４３に示されるアミノ酸配列からなるCDR1～3を含む；さらにシグナルペプチドを含むアミノ酸配列は配列番号７７で示される）、及び配列番号９６で示されるアミノ酸配列（配列番号７１～７３に示されるアミノ酸配列からなるCDR1～3を含む；さらにシグナルペプチドを含むアミノ酸配列は配列番号８３で示される）が好ましく挙げられる。

ここで、本発明のヒト型化抗hTROP-2抗体の態様としては、例えば、(i) H鎖Ｖ領域のアミノ酸配列が配列番号９２で示されるアミノ酸配列からなり、かつ、Ｌ鎖Ｖ領域のアミノ酸配列が配列番号９３で示されるアミノ酸配列からなるものや、(ii) H鎖Ｖ領域のアミノ酸配列が配列番号９８で示されるアミノ酸配列から

なり、かつ、Ｌ鎖Ｖ領域のアミノ酸配列が配列番号９３で示されるアミノ酸配列からなるものが好ましい。なかでも、Ｈ鎖Ｖ領域のアミノ酸配列が配列番号９８で示されるアミノ酸配列である上記(ii)のヒト型化抗hTROP-2抗体は、アビディティー（Avidity）（すなわち２つの抗原結合アームの運動能に関するフレキシビィリティー）がより向上したものであり、抗原結合活性が高いものであるため、特に好ましい。

同様に、本発明のヒト型化抗hTROP-2抗体の他の態様としては、例えば、(iii) Ｈ鎖Ｖ領域のアミノ酸配列が配列番号９４で示されるアミノ酸配列からなり、かつ、Ｌ鎖Ｖ領域のアミノ酸配列が配列番号９６で示されるアミノ酸配列からなるものや、(iv) Ｈ鎖Ｖ領域のアミノ酸配列が配列番号９５で示されるアミノ酸配列からなり、かつ、Ｌ鎖Ｖ領域のアミノ酸配列が配列番号９６で示されるアミノ酸配列からなるものが好ましい。

ヒト抗体（完全ヒト抗体）は、一般にV領域の抗原結合部位である超可変領域（Hyper Variable region）、V領域のその他の部分及び定常領域の構造が、ヒトの抗体と同じ構造を有するものである。但し、超可変部位は他の動物由来であってもよい。ヒト抗体を作製する技術も公知であり、ヒトに共通の遺伝子配列については遺伝子工学的手法によって作製する方法が確立されている。ヒト抗体は、例えば、ヒト抗体のH鎖及びL鎖の遺伝子を含むヒト染色体断片を有するヒト抗体産生マウスを用いた方法（Tomizuka, K.et al., Nature Genetics, (1977)16, 133-143; Kuroiwa, Y.et.al., Nuc. Acids Res., (1998)26, 3447-3448; Yoshida, H.et.al., Animal Cell Technology: Basic and Applied Aspects,(1999)10, 69-73 (Kitagawa, Y.,Matuda, T. and Iijima, S. eds.), Kluwer Academic Publishers; Tomizuka, K.et.al., Proc. Natl. Acad. Sci. USA, (2000)97, 722-727 等を参照）や、ヒト抗体ライブラリーより選別したファージディスプレイ由来のヒト抗体を取得する方法（Wormstone, I. M.et.al, Investigative Ophthalmology & Visual Science., (2002)43 (7), 2301-8; Carmen, S. et.al., Briefings in Functional Genomics and Proteomics,(2002)1 (2), 189-203; Siriwardena, D. et.al., Opthalmology, (2002)109 (3), 427-431 等を参照）により取得することができる。

上記キメラ抗体、ヒト型抗体及びヒト抗体は、抗体Fc領域におけるN-グリコ

シド結合複合型糖鎖が該糖鎖の還元末端のN-アセチルグルコサミンにフコースが結合していない糖鎖であるものが好ましく、詳しくは、該フコースの１位がN-グリコシド結合複合型糖鎖還元末端のN-アセチルグルコサミンの６位にα結合していない糖鎖を抗体分子のFc領域に有する遺伝子組換え抗体分子からなる抗体が挙げられる。このような抗体であれば、ADCC活性を飛躍的に向上させることができる。なお、この点（抗体Fc領域におけるN-グリコシド結合複合型糖鎖の特徴）は、前述したポリクローナル抗体及びモノクローナル抗体についても同様に好ましい。

(4-2) 抗体断片

本発明の抗hTROP-2抗体の断片（部分断片）も、本発明の抗体に含まれるものとする。ここで、本発明の抗体断片は、本発明の抗hTROP-2抗体と同様に、hTROP-2に対する結合活性を有するもの（すなわちhTROP-2に結合し得るもの）であって *in vivo*で抗腫瘍活性を有するものである。

当該抗体の断片としては、抗hTROP-2ポリクローナル抗体又は抗hTROP-2モノクローナル抗体の一部分の領域（すなわち、本発明の抗hTROP-2抗体に由来する抗体断片）を意味し、例えば、Fab、Fab'、$F(ab')_2$、Fv（variable fragment of antibody）、一本鎖抗体（H鎖、L鎖、H鎖V領域、及びL鎖V領域等）、scFv、diabody（scFv二量体）、dsFv（ジスルフィド安定化V領域）、並びに、相補性決定領域（complementarity determining region：CDR）を少なくとも一部に含むペプチド等が挙げられる。

Fabは、抗体分子をタンパク質分解酵素パパインで処理して得られる断片のうち、H鎖のN末端側約半分とL鎖全体とがジスルフィド結合で結合した、分子量約5万の抗原結合活性を有する抗体断片である。また、抗体のFabをコードするDNAを、原核生物用発現ベクター又は真核生物用発現ベクターに挿入し、該ベクターを原核生物又は真核生物へ導入することにより発現させ、Fabを製造することもできる。

$F(ab')_2$は、抗体分子をタンパク質分解酵素ペプシンで処理して得られる断片のうち、Fabがヒンジ領域のジスルフィド結合を介して結合されたものよりやや大きい、分子量約10万の抗原結合活性を有する抗体断片である。また、後述す

るFabをチオエーテル結合あるいはジスルフィド結合させて、作製することもできる。

Fab'は、上記F(ab')$_2$のヒンジ領域のジスルフィド結合を切断した、分子量約5万の抗原結合活性を有する抗体断片である。また、抗体のFab'断片をコードするDNAを、原核生物用発現ベクター又は真核生物用発現ベクターに挿入し、該ベクターを原核生物又は真核生物へ導入することにより発現させ、Fab'を製造することもできる。

scFvは、１本のH鎖V領域（VH）と１本のL鎖V領域（VL）とを適当なペプチドリンカー（P）を用いて連結した、VH-P-VLないしはVL-P-VHポリペプチドで、抗原結合活性を有する抗体断片である。scFvは、抗体のVHおよびVLをコードするcDNAを取得し、scFvをコードするDNAを構築して、該DNAを原核生物用発現ベクター又は真核生物用発現ベクターに挿入し、該発現ベクターを原核生物又は真核生物へ導入することにより発現させて、製造することができる。

diabodyは、scFvが二量体化した抗体断片で、二価の抗原結合活性を有する抗体断片である。二価の抗原結合活性は、同一であることもできるし、一方を異なる抗原結合活性とすることもできる。diabodyは、抗体のVHおよびVLをコードするcDNAを取得し、scFvをコードするDNAをPのアミノ酸配列の長さが８残基以下となるように構築して、該DNAを原核生物用発現ベクター又は真核生物用発現ベクターに挿入し、該発現ベクターを原核生物又は真核生物へ導入することにより発現させて、製造することができる。

dsFvは、VHおよびVL中のそれぞれ１アミノ酸残基をシステイン残基に置換したポリペプチドを、該システイン残基間のジスルフィド結合を介して結合させたものをいう。システイン残基に置換するアミノ酸残基は、Reiterらにより示された方法（Protein Engineering, 7, 697-704, 1994）に従って、抗体の立体構造予測に基づいて選択することができる。dsFvは、抗体のVHおよびVLをコードするcDNAを取得し、dsFvをコードするDNAを構築して、該DNAを原核生物用発現ベクター又は真核生物用発現ベクターに挿入し、該発現ベクターを原核生物又は真核生物へ導入することにより発現させて、製造することができる。

CDRを含むペプチドは、VHのCDR（CDR1～3）及びVLのCDR（CDR1～3

）の中の少なくとも１領域以上を含んで構成され、VHのCDRをすべて含むもの、及びVLのCDRをすべて含むものがより好ましく、VH及びVLのCDR（計6領域）をすべて含むものが特に好ましい。CDRのアミノ酸配列としては、例えば、前述した配列番号３６～３８、４１～４３、４６～４８、５１～５３、５６～５８、６１～６３、６６～６８及び７１～７３に示されるアミノ酸配列が好ましく挙げられる。複数のCDRを含むペプチドは、直接又は適当なペプチドリンカーを介して結合させることができる。CDRを含むペプチドは、抗体のVHおよびVLのCDRをコードするDNAを構築し、該DNAを原核生物用発現ベクター又は真核生物用発現ベクターに挿入して、該発現ベクターを原核生物又は真核生物へ導入することにより発現させて、製造することができる。また、CDRを含むペプチドは、Fmoc法（フルオレニルメチルオキシカルボニル法）及びtBoc法（t-ブチルオキシカルボニル法）等の化学合成法によって製造することもできる。

本発明の抗体断片としては、そのままの形状でN-グリコシド結合複合型糖鎖が該糖鎖の還元末端のN-アセチルグルコサミンにフコースが結合していない糖鎖である抗体Fc領域の一部または全部を含む抗体断片でもよく、また、上述した抗体断片とN-グリコシド結合複合型糖鎖が該糖鎖の還元末端のN-アセチルグルコサミンにフコースが結合していない糖鎖である抗体Fc領域の一部または全部との融合タンパク質であってもよい。このような抗体断片であれば、ADCC活性を飛躍的に向上させることができるため、好ましい。

以下、本明細書中の説明においては、上述した抗体断片も、本発明の抗hTROP-2抗体に含まれるものとする。

３．ポリヌクレオチド、組換えベクター及び形質転換体

本発明においては、上述した本発明の抗hTROP-2抗体やその抗体断片をコードするポリヌクレオチド（遺伝子、DNA）も提供することができる。当該ポリヌクレオチドとしては、具体的には、上述した本発明の抗hTROP-2抗体や抗体断片の例示として示した各アミノ酸配列をコードする塩基配列を含むポリヌクレオチドであることが好ましい。また、本発明のポリヌクレオチドは、本発明の抗hTROP-2抗体や抗体断片をコードするポリヌクレオチドのみからなるものであ

ってもよいし、当該ポリヌクレオチドを一部に含み、その他に遺伝子発現に必要な公知の塩基配列（転写プロモーター、SD配列、Kozak配列、ターミネーター等）をも含むものであってもよく、限定はされない。

本発明の抗hTROP-2抗体や抗体断片をコードするポリヌクレオチドとしては、翻訳後の個々のアミノ酸に対応するコドンは、特に限定はされず、転写後、ヒト等の哺乳類において一般的に用いられているコドン（好ましくは使用頻度の高いコドン）を示すヌクレオチドDNAを含むものであってもよいし、また、大腸菌や酵母等の微生物や、植物等において一般的に用いられているコドン（好ましくは使用頻度の高いコドン）を示すヌクレオチドDNAを含むものであってもよい。

本発明においては、上記本発明のポリヌクレオチドを含む組換えベクターや、当該組換えベクターを含む形質転換体を提供することもできる。

組換えベクターとして用いる発現ベクターに組込むポリヌクレオチド（遺伝子、DNA）には、必要に応じて、予め、上流に転写プロモーター、SD配列（宿主が原核細胞の場合）及びKozak配列（宿主が真核細胞の場合）を連結しておいてもよいし、下流にターミネーターを連結しておいてもよく、その他、エンハンサー、スプライシングシグナル、ポリA付加シグナル、選択マーカー等を連結しておくこともできる。なお、上記転写プロモーター等の遺伝子発現に必要な各要素は、初めから当該ポリヌクレオチドに含まれていてもよいし、もともと発現ベクターに含まれている場合はそれを利用してもよく、各要素の使用態様は特に限定されない。

発現ベクターに当該ポリヌクレオチドを組込む方法としては、例えば、制限酵素を用いる方法や、トポイソメラーゼを用いる方法など、公知の遺伝子組換え技術を利用した各種方法が採用できる。また、発現ベクターとしては、例えば、プラスミドDNA、バクテリオファージDNA、レトロトランスポゾンDNA、レトロウイルスベクター、人工染色体DNAなど、本発明の抗hTROP-2抗体やその抗体断片をコードするポリヌクレオチド（遺伝子、DNA）を保持し得るものであれば、限定はされず、使用する宿主細胞に適したベクターを適宜選択して使用することができる。

次いで、構築した上記組換えベクターを宿主に導入して形質転換体を得、これを培養することにより、本発明の抗hTROP-2抗体やその抗体断片を発現させることができる。なお、本発明で言う「形質転換体」とは宿主に外来遺伝子が導入されたものを意味し、例えば、宿主にプラスミドDNA等を導入すること（形質転換）で外来遺伝子が導入されたもの、並びに、宿主に各種ウイルス及びファージを感染させること（形質導入）で外来遺伝子が導入されたものが含まれる。

宿主としては、上記組換えベクターが導入された後、本発明の抗hTROP-2抗体やその抗体断片を発現し得るものであれば、限定はされず、適宜選択することができるが、例えば、ヒトやマウス等の各種動物細胞、各種植物細胞、細菌、酵母、植物細胞等の公知の宿主が挙げられる。

動物細胞を宿主とする場合は、例えば、ヒト繊維芽細胞、ヒト胎児腎細胞、HEK293細胞、293F細胞、CHO細胞、サル細胞COS-7、Vero、マウスL細胞、ラットGH3、ヒトFL細胞等が用いられる。また、Sf9細胞、Sf21細胞等の昆虫細胞を用いることもできる。

細菌を宿主とする場合、例えば、大腸菌、枯草菌等が用いられる。

酵母を宿主とする場合は、例えば、サッカロミセス・セレビシエ（*Saccharomyces cerevisiae*）、シゾサッカロミセス・ポンベ（*Schizosaccharomyces pombe*）等が用いられる。

植物細胞を宿主とする場合は、例えば、タバコBY-2細胞等が用いられる。

形質転換体を得る方法は、限定はされず、宿主と発現ベクターとの種類の組み合わせを考慮し、適宜選択することができるが、例えば、電気穿孔法、リポフェクション法、ヒートショック法、PEG法、リン酸カルシウム法、DEAEデキストラン法、並びに、DNAウイルスやRNAウイルス等の各種ウイルスを感染させる方法などが好ましく挙げられる。

得られる形質転換体においては、組換えベクターに含まれるポリヌクレオチドのコドン型は、用いた宿主のコドン型と一致していても異なっていてもよく、限定はされない。

4．抗体－薬剤複合体の作製

上記本発明の抗hTROP-2抗体を用いたイムノコンジュゲート等として、当該抗体と、抗腫瘍活性及び／又は殺細胞活性を有する物質（化合物等）とを含む、抗体－薬剤複合体を提供することができる。なお、予め、当該抗体分子と、抗腫瘍活性及び／又は殺細胞活性を有する物質とを、それぞれ調製したのち、これらを複合化させて得られたものは、一般に、イムノコンジュゲートと称される。また、遺伝子組換え技術を用い、抗腫瘍活性及び／又は殺細胞活性を有する物質としてのタンパク質トキシンを、遺伝子上で抗体遺伝子と連結させて、１つのタンパク質（融合タンパク質）として発現させて得られたものは、一般に、イムノトキシンと称される。

抗腫瘍活性を有する物質としては、例えば、ドキソルビシン、カリケアマイシン、マイトマイシン C、Auristatin E、放射性同位体（RI）などが挙げられる。殺細胞活性を有する物質としては、例えば、サポリン、リシン、緑膿菌外毒素、ジフテリアトキシン、放射性同位体（RI）等が挙げられ、なかでもサポリン及び緑膿菌外毒素が好ましく用いられる。なお、抗腫瘍活性及び／又は殺細胞活性を有する RI としては、限定はされないが、例えば、^{90}Y、^{111}In、^{125}I、^{3}H、^{35}S、^{14}C、^{186}Re、^{188}Re、^{189}Re、^{177}Lu、^{67}Cu、^{212}Bi、^{213}Bi、^{211}At、^{198}Au、^{224}Ac、^{126}I、^{133}I、^{77}Br、^{113m}In、^{95}Ru、^{97}Ru、^{103}Ru、^{105}Ru、^{107}Hg、^{203}Hg、^{94m}Tc、^{121m}Te、^{122m}Te、^{125m}Te、^{165}Tm、^{167}Tm、^{168}Tm、^{111}Ag、^{197}Pt、^{109}Pd、^{32}P、^{33}P、^{47}Sc、^{153}Sm、^{177}Lu、^{105}Rh、^{142}Pr、^{143}Pr、^{161}Tb、^{166}Ho、^{199}Au、^{57}Co、^{58}Co、^{51}Cr、^{59}Fe、^{18}F、^{75}Se、^{201}Tl、^{225}Ac、^{76}Br、^{86}Y、^{169}Yb、^{166}Dy、^{212}Pb 及び ^{223}Ra 等が挙げられる。

抗体－薬剤複合体の作製方法としては、限定はされないが、例えば、ジスルフィド結合やヒドラゾン結合によって抗体と薬剤とをカップリングする方法などが挙げられる。

上記本発明の抗hTROP-2抗体は、hTROP-2を発現する標的腫瘍細胞内へのインターナリゼーション活性に優れたものである。そのため、予め、抗腫瘍活性及び／又は殺細胞活性を有する物質を複合化させておくことにより、これら物質を腫瘍細胞に直接かつ高選択的に作用させることができる。本発明の抗体－薬剤複合体は、標的腫瘍細胞への薬剤送達能に極めて優れたものである。

なお、細胞内へのインターナリゼーション活性は、抗体をローダミン等により蛍光標識し、細胞内への移行挙動及び抗体の局在性について蛍光顕微鏡等を用いて観察することにより評価することができる。

また本発明においては、抗体－薬剤複合体において、抗体の代わりに前述の抗体断片を用いた抗体断片－薬剤複合体を提供することもできる。抗体断片－薬剤複合体の詳細については、上述の抗体－薬剤複合体の説明を適宜適用することができる。

以下、本明細書中の説明においては、抗体断片－薬剤複合体も、本発明の抗体－薬剤複合体に含まれるものとする。

5．医薬組成物

本発明の抗hTROP-2抗体及び抗体－薬剤複合体は、医薬組成物に含まれる有効成分として有用である。

当該医薬組成物は、腫瘍の治療用及び／又は診断用の医薬組成物として有用である。特に、本発明の抗hTROP-2抗体、及び該抗体を含む抗体－薬剤複合体は、抗腫瘍活性として優れた腫瘍成長阻害活性を有するものであるため、腫瘍の治療用に使用されることが好ましい。すなわち、本発明の抗hTROP-2抗体及び抗体－薬剤複合体は、腫瘍治療剤及び腫瘍診断剤に含まれる有効成分として有用なものである。なお、本発明において、上記腫瘍の治療には、腫瘍の成長阻害及び成長抑制の意味も含むものとし、具体的には、例えば腫瘍治療剤であれば、腫瘍の成長阻害剤及び成長抑制剤の形態も含むものとする。

本発明の医薬組成物は、本発明の抗hTROP-2抗体及び／又は抗体－薬剤複合体を有効成分として含み、さらに薬学的に許容される担体を含む医薬組成物の形態で提供することが好ましい。また、本発明の医薬組成物は、公知の抗腫瘍剤との併用もすることができる。併用によりさらに高い抗腫瘍効果が得られる。

本発明の医薬組成物の適用の対象となる疾患（腫瘍）としては、hTROP-2の発現が確認されている前述した公知の各種ヒト腫瘍が挙げられる。なかでも特に、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌などの各種ヒト腫瘍のうちの１種又は２種以上が好ましく挙げられる。

これらの疾患は単独であってもよく、２つ以上が併発してもよい。また、対象となる腫瘍は、再発癌や転移癌であってもよく、本発明の医薬組成物（ひいては、本発明の抗hTROP-2抗体及び／又は抗体－薬剤複合体）は、再発癌や転移癌の治療剤及び診断剤としても有効に用いることができる。

「薬学的に許容され得る担体」とは、賦形剤、希釈剤、増量剤、崩壊剤、安定剤、保存剤、緩衝剤、乳化剤、芳香剤、着色剤、甘味剤、粘稠剤、矯味剤、溶解補助剤あるいはその他の添加剤等が挙げられる。そのような担体の１種以上を用いることにより、注射剤、液剤、カプセル剤、懸濁剤、乳剤あるいはシロップ剤等の形態の医薬組成物を調製することができる。これらの医薬組成物は、経口あるいは非経口的に投与することができる。非経口投与のためのその他の形態としては、１つ以上の活性物質を含み、常法により処方される注射剤などが含まれる。注射剤の場合には、生理食塩水又は市販の注射用蒸留水等の薬学的に許容される担体中に溶解または懸濁することにより製造することができる。

特に、本発明の抗hTROP-2抗体由来の抗体断片（中でも低分子のもの）を生体内に投与する場合は、上記に加えて、コロイド分散系を用いることができる。コロイド分散系は化合物（抗体断片）の生体内の安定性を高める効果や、特定の臓器、組織又は細胞へ化合物を効率的に輸送する効果が期待される。コロイド分散系は、通常用いられるものであればよく限定はされないが、ポリエチレングリコール、高分子複合体、高分子凝集体、ナノカプセル、ミクロスフェア、ビーズ、及び水中油系の乳化剤、ミセル、混合ミセル及びリポソームを包含する脂質をベースとする分散系を挙げることができ、好ましくは、特定の臓器、組織又は細胞へ化合物を効率的に輸送する効果のある、複数のリポソーム、人工膜の小胞である（Mannino et al., Biotechniques, 1988, 6, 682; Blume and Cevc, Biochem.et Biophys. Acta, 1990, 1029, 91; Lappalainen et al., Antiviral Res., 1994, 23, 119; Chonn and Cullis, Current Op. Biotech., 1995, 6, 698）。

本発明の医薬組成物の投与量は、患者の年齢、性別、体重及び症状、治療効果、投与方法、処理時間、あるいは医薬組成物に含有される本発明の抗hTROP-2抗体及び抗体－薬剤複合体の種類などにより異なる。通常、成人一人あたり、一回

につき600μgから6000mgの範囲で投与することができるが、この範囲に限定されるものではない。

例えば注射剤により投与する場合は、ヒト患者に対し、１回の投与において１kg体重あたり、100μg～100mgの量を、１日平均あたり１回～数回投与してもよく、好ましくは３日、１週間、１０日又は２週間に１回投与したり、あるいは単回（合計投与回数が１回）投与する態様も採ることができる。投与の形態としては、静脈内注射、皮下注射、皮内注射、筋肉内注射あるいは腹腔内注射などが挙げられるが、好ましくは静脈内注射である。また、注射剤は、場合により、非水性の希釈剤（例えばポリエチレングリコール、オリーブ油等の植物油、エタノール等のアルコール類など）、懸濁剤あるいは乳濁剤として調製することもできる。そのような注射剤の無菌化は、フィルターによる濾過滅菌、殺菌剤の配合等により行うことができる。注射剤は、用時調製の形態として製造することができる。すなわち、凍結乾燥法などによって無菌の固体組成物とし、使用前に無菌の注射用蒸留水または他の溶媒に溶解して使用することができる。

なお、本発明は、腫瘍を治療及び／又は診断する医薬（薬剤）を製造するための、前記本発明の抗hTROP-2抗体及び／又は抗体－薬剤複合体の使用を提供するものでもある。また、本発明は、腫瘍を治療用及び／又は診断用の、前記本発明の抗hTROP-2抗体及び／又は抗体－薬剤複合体を提供するものでもある。

さらに、本発明は、前記本発明の抗hTROP-2抗体及び／又は抗体－薬剤複合体を用いる（すなわち患者に投与する）ことを特徴とする腫瘍の治療及び／又は診断方法を提供するものであり、また、腫瘍を治療及び／又は診断するための、前記本発明の抗hTROP-2抗体及び／又は抗体－薬剤複合体の使用を提供するものでもある。

６．腫瘍の検出方法

本発明の腫瘍の検出方法は、前記本発明の抗hTROP-2抗体と、生体から採取された試料（以下、生体試料）とを反応させ、反応した抗体のシグナルを検出することを特徴とする方法である。

前述したように、hTROP-2は各種腫瘍細胞において特異的に発現することが

確認されているため、hTROP-2、特に遊離hTROP-2（hTROP-2の細胞外領域部分）は、各種腫瘍マーカーとして利用することが可能である。なかでも、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌及びヒト乳癌のマーカーとして利用することが好ましい。

そこで、本発明の抗hTROP-2抗体を生体試料と反応させ、反応した抗体のシグナルを検出することにより、腫瘍を検出することができる。得られた抗体のシグナルは、生体試料中の抗原量（すなわちhTROP-2量又は遊離hTROP-2量）の指標となる。本発明の抗体を用いた腫瘍の検出は、まず、検体として被験者から採取した生体試料、例えば検査対象とする組織片又は血液等と、本発明の抗体とを、抗原抗体反応によって結合させる。次いで、結合した抗体量の測定結果に基づいて、生体試料中の目的とする抗原量を測定することにより行う。当該測定は、公知の免疫学的測定法に従って行えばよく、例えば、免疫沈降法、免疫凝集法、標識免疫測定法、免疫比濁法、ウエスタンブロット法、フローサイトメトリー法などを用いることができる。標識免疫測定法では、抗体のシグナルは、標識抗体を用いて直接検出した標識量で表すほか、既知濃度あるいは既知抗体価の抗体を標準液として相対的に表してもよい。すなわち、標準液と検体を測定計により測定し、標準液の値を基準にして生体試料中の抗体シグナルを相対的に表すことができる。標識免疫測定法としては、例えばELISA法、EI法、RIA法、蛍光免疫測定(FIA)法、化学発光免疫測定法(Luminescence immunoassay)等が挙げられる。なかでも特に、ELISA法が、簡便かつ高感度という点で好ましい。

本発明においては、上記検出方法により得られた検出結果を指標として腫瘍の状態を評価又は診断することができる。例えば、検出結果が所定の基準値を超えるものを腫瘍陽性、所定の基準値以下のものを腫瘍陰性とし、陽性の場合には、いずれかの腫瘍を発症している可能性があると判断し、腫瘍の状態を評価することができる。ここで、腫瘍の状態とは、腫瘍の罹患の有無又はその進行度を意味し、腫瘍の発症の有無、進行度、悪性度、転移の有無及び再発の有無等が挙げられる。

上記評価にあたり、腫瘍の状態としては１つを選択してもよいし、複数個を組み合わせて選択してもよい。腫瘍の有無の評価は、得られた検出結果に基づいて、

所定の基準値を境界として腫瘍に罹患しているか否かを判断することにより行うことができる。腫瘍の悪性度は、癌がどの程度進行しているのかを示す指標となるものであり、検出結果に基づいて、病期(Stage)を分類して評価したり、あるいは早期癌や進行癌を分類して評価したりすることも可能である。例えば、検出結果を指標として早期癌又は進行癌であると評価することもできる。腫瘍の転移は、検出結果を指標として、原発巣の位置から離れた部位に新生物が出現しているか否かにより評価することができる。再発は、間欠期又は寛解の後に検出結果が再び所定の基準値を超えたか否かにより評価することができる。

7．腫瘍の検出用又は診断用キット

本発明の抗hTROP-2抗体は、腫瘍の検出用又は診断用キットの形態で提供することができる。本発明のキットは抗体を含むが、その他に、標識物質、あるいは抗体又はその標識物を固定した固相化試薬などを含めることができる。抗体の標識物質とは、酵素、放射性同位体、蛍光化合物及び化学発光化合物等によって標識されたものを意味する。本発明のキットは、上記の構成要素のほか、本発明の検出を実施するための他の試薬、例えば標識物が酵素標識物の場合は、酵素基質（発色性基質等）、酵素基質溶解液、酵素反応停止液、あるいは検体用希釈液等を含んでいてもよい。また、各種バッファー、滅菌水、各種細胞培養容器、各種反応容器（エッペンドルフチューブ等）、ブロッキング剤（Bovine Serum Albumin (BSA), Skim milk, Goat血清等の血清成分）、洗浄剤、界面活性剤、各種プレート、アジ化ナトリウム等の防腐剤、及び実験操作マニュアル（説明書）等を含んでいてもよい。

本発明のキットは、上記本発明の検出方法を行うために有効に用いることができ、極めて有用性が高いものである

以下に、実施例を挙げて本発明をより具体的に説明するが、本発明はこれらに限定されるものではない。

〔実施例１〕

[hTROP-2 遺伝子のクローニング]

hTROP-2 遺伝子全長の単離は、ヒト胎児肝臓（胎生 10 週）から RT-PCR 法により行った。まず hTROP-2 遺伝子（Genbank accession No. NM_002353）の配列をもとに以下に示す PCR プライマーを設計した。

フォワード側プライマー： 5'-ttcctccgccccaccatggc-3'（配列番号３）
リバース側プライマー： 5'-ctcgagcaagctcggttcctttctc-3'（配列番号４）

このとき、リバース側プライマーにはストップコドンを除き、Xho I による制限酵素消化配列を付加した。これらのプライマーとヒト胎児肝臓（胎生 10 週）より調製した全 RNA (TAKARA)から合成した cDNA を鋳型として PCR 反応を行った。その後、アガロースゲル電気泳動による展開、目的のバンドの抽出を行い、pCRII ベクター(Invitrogen)にクローニングした(pCRII-hTROP2)。クローニングした hTROP-2 の cDNA はシークエンスにより確認した。

発現ベクターの構築は、pCRII-hTROP2 から hTROP-2 遺伝子を含む *Eco* RI/*Xho* I 断片を切り出し、pcDNA4/*myc*-His© A ベクター（Invitrogen）の *Eco*RI/*Xho* I 部位に挿入して行った（pcDNA4-hTROP2-*myc*/His）。また pcDNA4-hTROP2-*myc*/His から hTROP-2 遺伝子を含む *Hind* III/*Pme* I 断片を切り出し（*Hind* III 切断部位は平滑末端化した）、pcDNA3.1(+)ベクター（Invitrogen）の *Pme* I 部位へ挿入することでネオマイシン耐性遺伝子を持つ発現ベクターを構築した（pcDNA3.1-hTROP2-*myc*/His）。

〔実施例２〕

[hTROP-2 遺伝子安定細胞株の構築]

上記の方法で作製した hTROP-2 の全長 cDNA をコードした発現ベクター(pcDNA3.1-hTROP2-myc/His)を、HEK293 細胞（RIKEN）、HuH-7 細胞（HSRRB）、7E2-C 細胞（WO 2005/052156 に記載）、CHO-K1 細胞（HSRRB）にリポフェクタミン 2000 試薬(Invitrogen)を用いて遺伝子導入し、抗生物質 G418（geneticin, GIBCO BRL）による選択を行った後、hTROP-2 を安定して

発現している細胞株を樹立することにより得た。

〔実施例３〕

[hTROP-2 細胞外領域のリコンビナントタンパク質作製]

hTROP-2 細胞外領域の一部（具体的には、配列番号２で示されるアミノ酸配列のうちの第 1 番目～第 263 番目のアミノ酸からなる領域）をコードする遺伝子断片は PCR 法により増幅した。増幅に使用したプライマーは以下の通りである。

フォワード側プライマー：5'-ttcctccgccccaccatggc-3' （配列番号３）
リバース側プライマー：5'-ctcgagctcgtccaggtaatagatgagcg-3' （配列番号５）

このとき、リバース側プライマーに Xho I による制限酵素消化部位を付加した。PCR 法により増幅した DNA 断片はアガロースゲル電気泳動により展開し、QIAquick（登録商標） Gel Extraction Kit（QIAGEN）を用いて精製した。精製した DNA 断片は pCR Blunt ベクター（Invitrogen）にサブクローニングし（pCRB-hTROP-2 EC）、遺伝子配列を確認した。次に pCRB-hTROP-2 EC から hTROP-2 の細胞外領域をコードする遺伝子断片を含む *Eco* RI/*Xho* I 断片を切り出し、pcDNA4/*myc*-His© A ベクター（Invitrogen）の *Eco* RI/*Xho* I 部位に挿入した（pcDNA4mH-hTROP-2 EC）。さらに pcDNA4mH-hTROP-2 EC の *Bam* HI/*Eco* RI 部位に *Nru* I 制限酵素切断部位を作製するため、以下に示すオリゴヌクレオチドを会合させ挿入した。

オリゴヌクレオチド１：5'-gatccactagtcgcgagtggtgg-3' （配列番号６）
オリゴヌクレオチド２：5'-aattccaccactcgcgactagtg-3' （配列番号７）

同様に pcDNA4mH-hTROP-2 EC の *Pme* I 部位に pBgl II リンカー（TAKARA）を挿入した（pcDNA4mH-NB-hTROP-2 EC）。バキュロウイルスを用いたリコンビナントタンパク質作製のため、pcDNA4mH-NB-hTROP-2

EC から hTROP-2 の細胞外領域をコードする遺伝子断片を含む *Nru* I/*Bgl* II 断片を切り出し、pPSC8 ベクター（日本農産工業）の *Nru* I/*Bgl* II 部位に挿入した（pPSC8-hTROP2 EC）。バキュロウイルスを用いた hTROP-2 細胞外領域のリコンビナントタンパク質作製は日本農産工業に委託した。

hTROP-2細胞外領域のリコンビナントタンパク質の精製は以下のように行った。リコンビナントタンパク質を含む培養上清にNi Sepharose 6 Fast Flow（GE Healthcare）を加え、4℃、2時間結合させた。その後、エコノカラム（BIO RAD）を用いて20mMイミダゾールを含むリン酸緩衝液で洗浄、300mMイミダゾールを含むリン酸緩衝液で溶出を行い精製した。

〔実施例４〕

[ヒト EpCAM cDNA の単離と発現ベクターの構築]

ヒト EpCAM 遺伝子全長の単離は、ヒト胎児肝臓（胎生 10 週）から RT-PCR 法により行った。まずヒト EpCAM 遺伝子（Genbank accession No. NM 002354）の配列をもとに以下に示す PCR プライマーを設計した。

フォワード側プライマー： 5'-tcctcgtgtcccactcccgg-3'（配列番号８）
リバース側プライマー： 5'-ctcgagtgcattgagttccctatgc-3'（配列番号９）

このとき、リバース側プライマーにはストップコドンを除き、*Xho* I による制限酵素消化配列を付加した。これらのプライマーとヒト胎児肝臓（胎生 10 週）由来の全 RNA (TAKARA)から合成した cDNA を鋳型として PCR 反応を行った。その後、アガロースゲル電気泳動による展開、目的のバンドの抽出を行い、pCRII ベクター(Invitrogen)にクローニングした(pCRII-hEpCAM)。クローニングしたヒト EpCAM の cDNA はシークエンスにより確認した。

発現ベクターの構築は、pCRII-hEpCAMからヒトEpCAM遺伝子を含む*Eco* RI/*Xho* I断片を切り出し、pcDNA4/*myc*-His© Aベクター（Invitrogen）の *Eco*RI/*Xho* I部位に挿入して行った（pcDNA4-hEpCAM-*myc*/His）。また pcDNA4-hEpCAM-*myc*/HisからヒトEpCAM遺伝子を含む*Hind* III/*Pme* I断片

を切り出し（*Hind* III切断部位は平滑末端化した）、pcDNA3.1(+)ベクター（Invitrogen）の*Pme* I部位へ挿入することでネオマイシン耐性遺伝子を持つ発現ベクターを構築した（pcDNA3.1-hEpCAM-*myc*His）。

〔実施例５〕

[抗 hTROP-2 モノクローナル抗体の作製]

免疫原としては、hTROP-2 発現安定細胞株（HEK293-hTROP-2 細胞、CHO-K1-hTROP-2 細胞、7E2-C-hTROP-2 細胞）、内在的に hTROP-2 タンパク質を細胞表面に発現している、ヒト膵臓癌細胞株（PK-59, RCB1901; RIKEN cell bank より購入）、または上記の方法で作製した、hTROP-2 の細胞外領域のリコンビナントタンパク質を用いた。

hTROP-2発現安定細胞株の場合は1×10^7細胞、リコンビナントhTROP-2タンパク質の場合は、20μgのタンパク質を、それぞれ免疫補助剤TiterMax Gold（フナコシ株式会社)と、１：１で混合してエマルジョンを調製し、マウス（C57/BL6, Balb/c）の両足蹠、または腹腔内に注射した（初回免疫）。両足蹠への短期免疫の場合は、初回免疫から３日後及び10日後に追加免疫を行い、最終免疫の翌日に、両膝膏リンパ節を採取し、リンパ球を調製した。腹腔内への長期免疫の場合は、初回免疫から１週間に１回の割合で、追加免疫を行い（１～２ヵ月間実施）、常法に従い、脾臓よりＢ細胞を単離した。追加免疫は、細胞免疫では5×10^6 細胞のPBSによる細胞懸濁液を用い、タンパク質抗原の場合は、5μgのPBS溶液を用いた。

調製したリンパ球とマウスミエローマ細胞株（P3-X63-Ag8.653）とを、３：１の割合で混合し、ポリエチレングリコール法で細胞融合を行い、HAT (ヒポキサンチン、アミノプテリン、チミジン）を含むメチルセルロース培地（商品名：ClonaCell-HY Cloning Medium D; Stem Cell）で7～28日間培養し、増殖してきたハイブリドーマの単一コロニーを、１つ１つを96穴平底プレートにピックアップして、HATを含む液体選択培地を用い、5% CO_2インキュベーターで培養した。増殖してきた単一コロニー由来のハイブリドーマの培養上清を、Cell ELISA（後述）による一次スクリーニング、HuH-7-hTROP-2細胞, PK-59を用

いたFACS解析で２次スクリーニングを行うことで、生細胞の細胞表面に発現しているhTROP-2タンパク質を認識する抗hTROP-2 モノクローナル抗体を産生するハイブリドーマを300種類樹立した。

〔実施例６〕

[Cell ELISA を用いた一次スクリーニング]

96 穴培養プレート（BD ファルコン）に、CHO-K1 細胞（hTROP-2 陰性コントロール：ヒューマンサイエンス振興事業団より購入）と CHO-K1-hTROP-2 細胞（または、HuH-7 細胞（hTROP-2 陰性コントロール：ヒューマンサイエンス振興事業団より購入）と HuH-7-hTROP-2 細胞）を 3×10^4 細胞/ウェルで交互に播種し、5% CO_2、37℃で１～２日間培養した。デカンテーションで細胞培養液を除去し、次いで、氷冷 PBS で洗浄後、4%パラホルムアルデヒド-PBS で５分間処理することにより細胞を固定し、氷冷 PBS で洗浄後 ELISA プレートとした。以後、常法に従い、ELISA 法を行った。具体的には以下のとおりである。

まず、2%スキムミルク-PBS溶液によるブロッキングを室温で30分～1時間行った。次に、ハイブリドーマ培養上清を加え、室温で１時間反応させた後、0.1% Tween20-PBS溶液で３回洗浄した。二次抗体として、ブロッキング溶液で1000倍希釈した、Horseradish peroxidase (HRP)標識抗マウスIgG (GEヘルスケアバイオサイエンス)を加え、室温で１時間反応させた後、0.1% Tween20-PBS溶液で３回洗浄した。TMB(3,3',5,5'-tetramethylbenzidine: SIGMA)基質溶液を添加して発色反応を行い、１M硫酸を加えて反応を停止させた。Microplate reader Model 550 (バイオ・ラッド)を用いて、吸光度（405nm）を測定した。陰性コントロールに対して、高い吸光度の値を示したハイブリドーマ培養上清に対応するハイブリドーマを24穴平底プレートに拡大培養を行い、FACS解析を用いた二次スクリーニング（後述）に移行した。

〔実施例７〕

[FACS 解析を用いた二次スクリーニング]

上記の Cell ELISA を用いた一次スクリーニングで陽性であったハイブリドーマについて、FACS 解析を用いた二次スクリーニングを行った。細胞は、hTROP-2 を発現していないヒト肝癌細胞株である HuH-7 細胞を陰性コントロールとして、hTROP-2 を発現させた安定細胞株である HuH-7-hTROP-2 細胞に対する反応性を指標に評価し、次に内在的に hTROP-2 タンパク質を細胞表面に発現しているヒト膵臓癌細胞株である PK-59 細胞（RCB1901; RIKEN cell bank より購入）との反応性で評価した。

細胞はトリプシン処理によって培養皿から剥がし、細胞懸濁液（細胞密度 2×10^6cells/mL）を調製した。Cell ELISA を用いた一次スクリーニングで陽性反応を示したハイブリドーマ培養上清と細胞懸濁液 100μL とを、4℃で 20 分間反応させた。PBS で洗浄後、PE 標識抗マウス IgG (BD ファーミンゲン)（0.1μg）と反応(4℃、30 分)させた後、FACSCalibur (ベクトンディッキンソン)により解析した。

最終的に、生細胞の細胞表面に発現しているhTROP-2タンパク質を認識する抗hTROP-2 モノクローナル抗体を産生するハイブリドーマを約300種類樹立した。

〔実施例８〕

[アイソタイプの同定]

作製した抗hTROP-2モノクローナル抗体のアイソタイプは、MOUSE MONOCLONAL ANTIBODY ISOTYPING TEST KIT (Serotec社)を用いて、キット添付の方法に従って行った。

〔実施例９〕

[TROP-2 抗体の腹水化及び抗体精製]

上記方法で作製したハイブリドーマのクローンを、予め（７日前）2,6,10,14-テトラメチルペンタデカン（プリスタン）を投与されたBALB/cヌードマウスの腹腔内に3×10^6個投与し、２週間後の腹水を採取した。さらに、この腹水から、カプリル酸沈澱、プロテインGカラム（HiTrap protein G；GEヘルスケアバイ

オサイエンス）、またはプロテインAカラム（HiTrap protein A；GEヘルスケアバイオサイエンス）を用いてアフィニティー精製を行うことで、各ハイブリドーマクローンが産生する抗hTROP-2モノクローナル抗体を得た。

〔実施例１０〕

[抗原親和性の測定(Kd 値の測定)]

作製した抗hTROP-2モノクローナル抗体の抗原親和性 (Kd値)は、ELISA を用いた方法で算出した（Djavadi-Ohaniance L. et al (1996), In Antibody Engineering, Chapter 4, pp77-97. IRL Press, Oxford）。

具体的には、96穴培養プレート(コーニング)に、精製したリコンビナントhTROP-2タンパク質（0.1μg/mL）を添加して抗原を固相化した（室温，１時間、または4℃，一晩）。次に、PBSで３回洗浄し、2% スキムミルク（PBS溶液）を加えてブロッキングを行った（室温、１時間）。PBSで２回洗浄後、予め抗原溶液（精製hTROP-2タンパク質；50, 25, 12.5, 6.25, 3.125 nM）と抗hTROP-2モノクローナル抗体の各クローン（0.5nM）とを混合し平衡化させておいた抗原－抗体複合体を、上記ELISAプレートに添加して反応させた（室温、1時間）。PBSで３回洗浄した後、ブロッキング液で希釈したHRP標識抗マウスIgG (最終濃度1μg/mL)（GEヘルスケアバイオサイエンス）と反応させた（室温、１時間）。次いで、0.1%Tween20-PBS溶液で３回洗浄した後に、TMB(3,3',5,5'-tetramethylbenzidine: SIGMA)基質溶液を添加して発色反応を行い、１M硫酸を加えて反応を停止させた。Microplate reader Model 550 (バイオ・ラッド)を用いて、吸光度を測定した。

解離定数(Kd)の計算式は以下の通りとした。

抗原抗体反応は質量作用の法則により、

$$Ag\ (Antigen) + Ab\ (Antibody) \underset{k2}{\overset{k1}{\Leftrightarrow}} Ag\text{-}Ab\ (Antigen\text{-}Antibody\ complex) \quad \cdots\cdots (1)$$

$$Kd = k2/k1 = Agf \times Abf \,/\, Ag\text{-}Ab = Agf \times Abf \,/\, x \quad \cdots\cdots (2)$$

(2)式で、Agf は遊離抗原の濃度、Abf は遊離抗体の濃度、Ag-Ab は抗原-抗体複合体の濃度を表し、Ag-Ab = x とおくと、遊離抗体濃度は、

$$Abf = Abt - x \quad \cdots\cdots\cdots (3)$$

(2)式は、

$$Kd = Agf \times (Abt - x) \,/\, x \quad \cdots\cdots\cdots (4)$$

(4)式の両項に x / Kd × Abt をかけると、

$$x \,/\, Abt = Agf \times (1 - x/Abt) \times 1/Kd$$

$$x/Abt \times 1/Agf = (1 - x/Abt) \times 1/Kd \quad \cdots\cdots\cdots (5)$$

(5)式において、X＝x/Abt, Y＝x/Abt × Agf とおくと、

$$Y = (1-X) \times 1/Kd \quad \cdots\cdots\cdots (6)$$

(6)式から、Kd 値を算出した。

上記の方法で、作製した抗 hTROP-2 モノクローナル抗体 300 クローンの Kd 値を測定した結果、1×10^{-10} (M)以下の Kd 値を示すクローンが 133 クローン、1×10^{-11} (M)以下の Kd 値を示すクローンが 59 クローン、1×10^{-12} (M)以下の

Kd 値を示すクローンが２クローンであった。

*in vivo*で腫瘍成長阻害活性を示した、抗hTROP-2モノクローナル抗体のうち、K5-70 (マウスIgG2a)、T6-16 (マウスIgG2a)、K5-107 (マウスIgG1)、K5-116-2-1 (マウスIgG2a)、及びT5-86 (マウスIgG1)のKd値は、それぞれ順に、6.8×10^{-12} (M)、4.3×10^{-12} (M)、4.7×10^{-12} (M)、2.69×10^{-11} (M)、及び8.49×10^{-11}(M)であった（図１、表１）。

表１

抗hTROP-2モノクローナル抗体のKd値

Clone No.	K5-70	T6-16	K5-107	K5-116-2-1	T5-86
Kd ($\times10^{-12}$ M)	6.8	4.3	4.7	26.9	84.9

〔実施例１１〕

[抗 hTROP-2 モノクローナル抗体のヒト癌細胞株との反応性]

ヒト癌細胞株（ヒト腫瘍細胞株）は、ヒューマンサイエンス振興事業団(HSRRB)、RIKEN cell bank (RIKEN), ATCC (American Type Culture Collection)、ECACC (European Collection of Cell Cultures)、DSMZ (German Collection of Microorganisms and Cell Cultures)より入手したものを使用した。具体的には、以下に列挙した通りである。

huH-1 (HSRRB), HUH-6 (HSRRB), HuH-7 (HSRRB), JHH-5 (HSRRB), JHH-6 (HSRRB), JHH-7(HSRRB), HLE (HSRRB), HLF (HSRRB), HepG2 (HSRRB), Alexander (HSRRB), KP-1N (HSRRB), KP-1NL (HSRRB), KP-2 (HSRRB), KP-3 (HSRRB), KP-3L (HSRRB), PK-1 (RIKEN), PANC-1 (RIKEN), MIA PaCa-2 (HSRRB), PK-59 (RIKEN), PK-45H (RIKEN), PK-45P (RIKEN), BxPC-3 (ATCC), SUIT-2 (HSRRB), TCC-PAN2 (HSRRB), SW480 (ATCC), DLD-1 (HSRRB), LoVo (HSRRB), COLO-320 (RIKEN), CACO-2 (RIKEN), CW-2 (RIKEN), HCT 116 (ATCC), HCC-56 (HSRRB), MCF-7 (HSRRB),

JIMT-1 (DSMZ), HCC1143 (ATCC), A549 (HSRRB), DU145 (RIKEN), PC-3 (HSRRB)

癌細胞はトリプシン処理によって培養皿から剥がし、細胞懸濁液（細胞密度 2 ×10^6cells/mL）を調製した。細胞懸濁液 100μL に抗 hTROP-2 モノクローナル抗体を加え（0.1μg）、4℃で 20 分間反応させた。PBS で洗浄後、PE 標識抗マウス IgG (BD ファーミンゲン)（0.1μg）と反応(4℃、30 分)させた後、FACSCalibur (ベクトンディッキンソン)により解析した。

作製した抗 hTROP-2 抗体は、いずれも内在的に hTROP-2 を発現していないヒト肝癌細胞株 HuH-7 とは結合せず、一方 hTROP-2 遺伝子を発現させた安定細胞株である HuH-7-hTROP-2 細胞には結合を示した（図２）。次に、作製した抗 hTROP-2 モノクローナル抗体のヒト癌細胞株（内在的に細胞表面に発現している hTROP-2 タンパク質）との反応性を FACS 解析により検討した結果、作製した 300 種類の抗 hTROP-2 モノクローナル抗体は、全て、ヒト膵臓癌細胞株（PK-59、BxPC-3）との結合を示し、特に、*in vivo* で腫瘍成長阻害活性を示した、K5-70, T6-16, K5-107, K5-116-2-1, T5-86 抗体は、PE 標識抗マウス IgG (BD ファーミンジェン)のみと反応させた場合と比較して、PK-59 細胞では、平均蛍光強度で K5-70 (44 倍)、T6-16 (59 倍)、K5-107 (89 倍)、K5-116-2-1 (122 倍)、T5-86 (15 倍)（図３）、BxPC-3 細胞では、K5-70 (45 倍)、T6-16 (25 倍)、K5-107 (90 倍)、K5-116-2-1 (121 倍)、T5-86 (10 倍) と、いずれの抗体も高い結合を示した（図４）。

PK-59とBxPC-3以外のヒト癌細胞株では、膵臓癌細胞株12種類のうち、KP-2, KP-3L, PK-1, PK-45H、SUIT-2、TCC-PAN2に結合し、KP-1N, KP-1NL, KP-3, PANC-1, MIA-PaCa2には結合しなかった（図５）。ヒト大腸癌細胞株では、CACO-2, SW480, DLD-1, HCT 116に結合し、COLO-320, CW-2には結合しなかった（図６）。その他、JIMT-1, HCC1143（いずれもヒト乳癌細胞株）、PC-3, DU145 (いずれもヒト前立腺癌細胞株)と結合し、多くのヒト癌細胞株の細胞表面に内在的に発現しているhTROP-2タンパク質を認識した（図６）。

〔実施例１２〕

[マウス TROP-2 タンパク質、ヒト TROP-1/EpCAM タンパク質との交差反応性]

作製した抗 hTROP-2 モノクローナル抗体の特異性を調べる目的で、hTROP-2 タンパク質とアミノ酸配列で 80％の相同性を示すマウス TROP-2 タンパク質、hTROP-2 タンパク質とアミノ酸配列で 50％の相同性を示すヒト TROP-1/EpCAM との反応性を FACS 解析にて行った。

具体的には、マウス TROP-2 遺伝子（GenBank accession No. NM_020047、Y08830）の全長 cDNA を含む発現ベクター（マウス TROP-2-pcDNA3.1(+)、東京大学・分子細胞生物学研究所より供与）、およびヒト TROP-1/EpCAM 遺伝子（GenBank accession No. NM_002354）の全長 cDNA を含む発現ベクター（pcDNA3.1-hEpCAM-*myc*/His）を、それぞれ Lipofectamine2000 試薬（Invitrogen）を用いて、一過性に CHO-K1 細胞に遺伝子導入を行い、24～48 時間後に細胞をトリプシン処理で培養皿から剥がして細胞懸濁液を調整し、作製した抗 hTROP-2 モノクローナル抗体（0.1μg）、PE 標識抗マウス IgG と順次反応させた後、FACSCalibur により解析した。

マウス TROP-2 遺伝子を一過性に発現させた CHO-K1 細胞において、陽性対照として用いたマウス TROP-2 と交差反応を示す T2-102 抗体（マウス IgG1）は強い結合を示し、一方、K5-70, T6-16, K5-107, K5-116-2-1, T5-86 抗体はマウス TROP-2 との交差反応は示さなかった（図７）。

同様に、ヒト EpCAM/TROP-1 遺伝子を一過性に発現させた CHO-K1 細胞において、陽性対照として用いた抗ヒト EpCAM モノクローナル抗体（BD ファーミンジェン）は強い結合を示し、一方、K5-70, T6-16, K5-107, K5-116-2-1, T5-86 抗体はヒト EpCAM/TROP-1 との交差反応は示さなかった（図８）。

以上の結果は、作製した抗hTROP-2モノクローナル抗体、特に*in vivo*で抗腫瘍活性を示したK5-70, T6-16, K5-107, K5-116-2-1, T5-86抗体は、hTROP-2に特異的に結合することが示された。

〔実施例１３〕

[細胞増殖阻害活性の測定]

抗 hTROP-2 モノクローナル抗体の hTROP-2 の機能阻害を調べる方法の１つとして、内在的に hTROP-2 を細胞表面に発現しているヒト癌細胞の細胞増殖に対する影響を、テトラカラーワン（生化学工業）を用いた生細胞数の測定に基づいて評価した。具体的には、PK-59 細胞を 0.5%のウシ胎児血清（BioWest 社製）を含む RPMI1640 培地に 2×10^5 細胞/mL の細胞濃度となるように細胞懸濁液を調製し、96 穴培養プレートに 100μL ずつ播種した。次いで、マウス IgG (陰性コントロール)、抗 hTROP-2 モノクローナル抗体(最終濃度 0.1, 1μg/mL)を添加し、37℃、5% CO_2 インキュベーターで 72 時間培養した。比較対照として、市販の抗 hTROP-2 モノクローナル抗体(クローン YY01, Santa Cruz)を使用した。細胞にテトラカラーワン（生化学工業）を添加し、5% CO_2 インキュベーターで 1～2 時間反応させた。反応後の 96 穴プレートを、そのままマイクロプレートリーダーを用いて波長 490nm（対照波長：655nm）の吸光度を測定した。各群 3 ウェルで実施し、Student's t 検定（Student's t-test）で有意差検定を行い、$P < 0.05$ を統計的に有意と判定した。

これまでに自社で作製した抗hTROP-2モノクローナル抗体のうち、約160クローンについて、上記の方法でPK-59細胞の細胞増殖に与える効果を調べたところ、*in vivo*で腫瘍成長阻害活性を示した、T6-16, T5-86, K5-70, K5-107は、マウス IgG (陰性コントロール)と比較して、20%～40%の細胞増殖阻害活性が確認され、これら抗hTROP-2抗体は、ヒト癌細胞表面に発現するhTROP-2タンパク質に結合し、hTROP-2タンパク質の機能を中和し、癌細胞の増殖を阻害する活性を有することが明らかとなった（図９）。

〔実施例１４〕

[スクラッチアッセイ]

抗 hTROP-2 モノクローナル抗体のヒト癌細胞の移動能をスクラッチアッセイで評価した。PK-59 細胞を 10%のウシ胎児血清を含む RPMI1640 培地に 3×10^5 cell/mL の細胞濃度となるように細胞懸濁液を調製し、96 穴培養プレートに 100μL ずつ播種した。細胞がコンフルエントに達した段階で、チップの先端

で縦に線を引くようにして単層培養した細胞の一部を剥離した。抗 hTROP-2 モノクローナル抗体、および陰性コントロールとしてマウス IgG を最終濃度が 0.1、および 1μg/mL となるように培地に添加し、24 時間培養を行い、抗体添加前(Day 0)と 24 時間培養後（Day 1）の剥離した領域の写真を撮影し、細胞間距離を測定した。また、剥離領域の面積を Scion Image ソフトウェアで定量化した。各群 8 ウェルで実施し、Student's t-test で有意差検定を行い、$P < 0.05$ を統計的に有意と判定した。

スクラッチ領域に浸潤する細胞の運動能に対するhTROP-2抗体の効果を調べた。細胞増殖阻害アッセイと同様に薬効のあった抗体を評価した。評価方法として、Day0(抗体添加時)およびDay1(24時間経過後)の細胞を撮影し、移動距離(μm)および、スクラッチ領域の面積を画像解析した。結果、図１０に示すように、細胞の運動能に明らかな違いが見られた。本試験に使用したT6-16およびK5-70はコントロールと比較し、どちらも有意な増殖阻害が見られ、再現性試験においても同様の傾向が確認できた。特にT6-16についてはP < 0.01 (by Student's t-test)であり、*in vivo*の試験との相関性が認められた。

〔実施例１５〕

[担癌マウスでの抗 hTROP-2 モノクローナル抗体の薬効評価]

prevention モデル

hTROP-2を発現する膵癌細胞株（PK-59, BxPC-3）をトリプシン処理で剥がし、PBSで1×10^8 cells/mLの細胞懸濁液を調製し、氷上にて等量のマトリゲル（BDファーミンゲン）と混合した。6 週齢の雌のヌードマウス（Balb/c, nu/nu）の右脇腹の皮下に26 Gのシリンジを用いて100μL（5×10^6 cell）注射した。癌細胞移植当日(Day 1)に、マウスを群分し、抗体の投与（1, 5, または10 mg/kg 体重、腹腔内投与）を開始した。以降は、3日に1回の間隔で同様の投与を行った。抗腫瘍活性は、腫瘍形成頻度及び腫瘍体積により評価した。腫瘍体積の計測は、以下の計算式を用いた。

$$腫瘍体積(mm^3) = (短径)^2 \times (長径) \times \pi/6$$

treatment モデル

hTROP-2を発現する膵癌細胞株（PK-59, BxPC-3）をトリプシン処理で剥がし、PBSで1×10^8 cells/mLの細胞懸濁液を調製し、氷上にて等量のマトリゲル（BDファーミンゲン）と混合した。6週齢の雌のヌードマウス（Balb/c, nu/nu）の右脇腹の皮下に26 Gのシリンジを用いて100μL（5×10^6 cells）注射した。癌細胞移植から5～6日後、腫瘍体積が50～150 mm^3 (平均値で約 100 mm^3)に成長したマウスを群分し、群分した当日を1日目（Day 1）として、抗体投与を開始した。抗体は3日に1回の間隔で腹腔内投与を行った（10 mg/kg 体重）。抗腫瘍活性は、腫瘍体積を計測することによって評価した。有意差検定は、Student's t-testで行い、$P<0.05$を統計的に有意と判定した。

〔実施例１６〕

[ヒト膵癌細胞 xenograft モデルにおける抗 hTROP-2 モノクローナル抗体の *in vivo* における抗腫瘍活性の検討]

hTROP-2 を標的とした癌治療用抗体は、xenograft treatment モデルにおいて、hTROP-2 を発現する腫瘍組織を特異的に死滅させるか、腫瘍の成長を阻害する活性を示すことが必須である。

本発明において新たに作製した抗 hTROP-2 モノクローナル抗体(約 160 クローン)を、膵癌細胞株 PK-59 細胞の xenograft treatment モデルで評価した。PK-59 細胞は、膵臓癌幹細胞マーカーである EpCAM を発現し（図 11A）(Chenwei Li, et al. Cancer Res 2007; 67: (3). 1030-1037), 薬剤耐性に関わる ABC トランスポーターである P-glycoprotein/MDR1（図 11B）, ABCG2/CDw338（図 11C)を細胞表面に発現している（Chen,C.J. et al. Cell 47 (3), 381-389 (1986), Allikmets,R., et al. Hum. Mol. Genet. 5 (10), 1649-1655 (1996)。また、膵臓癌幹細胞の特徴である CD24 と CD44 共陽性の細胞画分（8.93%）（図 11D)を含み、悪性度の高いヒト膵臓癌細胞株であると推測される(Chenwei Li, et al. Cancer Res 2007; 67: (3). 1030-1037、Jane E. Visvader and Geoffrey J. Lindeman.Nat Rev Cancer. Vol.8(10):755-68, 2008)。

新規に作製した約 160 クローンのうち、大部分のクローンは PK-59 細胞 xenograft treatment モデルでは薬効を示さなかったが、その中でも有意な腫瘍成長阻害活性を示す以下のクローン、すなわちクローン K5-70, T6-16, K5-107, T5-86 及び K5-116-2-1 が得られた。

クローン K5-70 (マウス IgG2a) 投与群では、腫瘍成長速度が統計的に有意に阻害され、投与開始後 21 日目(Day 21)において、コントロール群 (N=14) の腫瘍体積が 1200.8±377.3 mm^3 に対し、クローン K5-70 投与群では 748.7±175.0 mm^3 (P < 0.01 by Student's t-test) であった (図 12A)。抗体投与開始時点での腫瘍体積を 1.0 としたときの、21 日目(Day 21)における腫瘍体積は、コントロール群が 12.5 であるのに対し、クローン K5-70 投与群では 7.8 であった (図 12A)。摘出した腫瘍重量も、コントロール群が 0.73±0.26 g であったのに対し、クローン K5-70 投与群では 0.43±0.14 g (P < 0.01 by Student's t-test) であり、約 60%の阻害を示した (図 12B)。

同様に、クローン K5-107(マウス IgG1) 投与群(N=8)、クローン T6-16 (マウス IgG2a) 投与群(N=8)、クローン T5-86(マウス IgG1) 及びクローン K5-116-2-1 (マウス IgG2a) 投与群(N=8)でも、腫瘍成長速度が統計的に有意に阻害された。クローン K5-107 投与群 (N=8) 並びにクローン T6-16 投与群 (N=8) では、投与開始後 17 日目(Day 17)において、コントロール群 (N=8) の腫瘍体積が 1039.3±271.6 mm^3 に対し、それぞれ 698.2±175.9 mm^3 (P < 0.05 by Student's t-test)、707.2±254.5 mm^3 (P < 0.05 by Student's t-test) であった。同様に、クローン K5-116-2-1 投与群 (N=8) では、投与開始後 16 日目(Day 16)において、コントロール群 (N=8) の腫瘍体積が 797.0±172.9 mm^3 に対し、508.5±225.2 mm^3 (P < 0.05 by Student's t-test) であった (図 13)。

一方、クローン T5-86 については、投与開始後 15 日目(Day 15)において、コントロール群 (N=8) の腫瘍体積が 1033.2±319.4 mm^3 に対し、クローン T5-86 投与群 (N=8) では 744.1±289.1 mm^3 であり、腫瘍体積の比較では有意差はつかなかったものの、同日における腫瘍重量の比較では、コントロール群が 0.62±0.14 g であったのに対し、クローン T5-86 投与群では 0.44±0.13 g (P < 0.05 by Student's t-test) であり有意な阻害が示された。

また、下記表２に、各クローン抗体投与群の実験最終日におけるコントロール群との比率(T/C)を、腫瘍体積および腫瘍重量のそれぞれについて示した。表２に示した通り、各クローン抗体投与群において、それぞれ T/C=62～72%の有意な阻害が得られた。

表２

群	N(個体数)	腫瘍体積 T/C (%)	腫瘍重量 T/C (%)
K5-70	14	62.3**	58.8**
K5-107	8	67.2*	65.0*
T6-16	8	68.0*	64.7*
T5-86	8	72.0	70.5*
K5-116-2-1	8	63.8*	60.5*

* P < 0.05, ** P < 0.01 (by Student's t-test)

また、クローン K5-70、クローン T6-16、クローン K5-116-2-1 について、それぞれ膵癌細胞株 PK-59 細胞の xenograft prevention モデルでの抗腫瘍活性を検討した。それぞれのクローンにおいて、抗体投与後全ての個体で（N=8）腫瘍成長が阻害され、クローン K5-70 投与群（10 mg/kg 体重）では、投与開始後１８日目(Day 18)において、コントロール群（N=8）の腫瘍体積が 880.8±206.4 mm^3 に対し、62.4±80.4 mm^3（P < 0.01 by Student's t-test）と 92.9%の腫瘍成長阻害活性を示し、クローン T6-16 投与群（10 mg/kg 体重）では、投与開始後 28 日目(Day 28)において、コントロール群（N=8）の腫瘍体積が 992.3±250.8 mm^3 に対し、152.14±122.3 mm^3（P < 0.01 by Student's t-test）と 84.6%の腫瘍成長阻害活性、クローン K5-116-2-1 投与群（10 mg/kg 体重）では、クローン K5-116-2-1 投与群（10 mg/kg 体重）では、投与開始後 20 日目(Day20)において、コントロール群（N=8）の腫瘍体積が 1159.4±413.3 mm^3 に対し、207.7±319.2 mm^3（P < 0.01 by Student's t-test）と 82.1%の腫瘍成長阻害活性を示した（図 14, 表 3）。また、すべての実験において、試験期間を

通じて、コントロール群と抗 hTROP-2 抗体投与群の間に、平均体重の変化に有意な差はなかった。

下記表３に、各クローン抗体投与群の、実験最終日におけるコントロール群との比率(T/C)を、腫瘍体積および腫瘍重量それぞれについて示した。表３に示したとおり、各クローン抗体投与群において、有意な腫瘍成長阻害が見られ、特にクローン K5-70 投与群では、T/C=10%以下の顕著な効果が確認された。

表３

群	N(個体数)	腫瘍体積 T/C (%)	腫瘍重量 T/C (%)
K5-70	8	7.1**	5.8**
T6-16	8	15.3**	10.5**
K5-116-2-1	8	23.2**	21.5**

** P < 0.01 (by Student's t-test)

公知の抗 TROP-2 抗体 AR47A6.4.2（米国特許第 7420041 号）は、様々なヒト癌細胞株を用いた 20 mg/kg の投与量で、xenograft prevention モデルで腫瘍成長阻害効果を示しているが、ヒト膵臓癌細胞株 PL45 細胞では、ほぼ 100%の腫瘍成長を阻害しているものの、膵臓癌細胞株 BxPC-3 では約 50%、前立腺癌細胞株 PC-3 では約 40%, 乳癌細胞株である MCF-7 では約 60%, 大腸癌細胞株 Colo205 で約 40%の腫瘍成長阻害効果であったことに対して、本願発明の抗 hTROP-2 抗体は、半分の投与量(10 mg/kg 体重)で、より強い腫瘍成長阻害効果を発揮した。

〔実施例１７〕

[ヒト膵癌細胞株 BxPC-3 細胞の xenograft モデル（prevention モデルおよび treatment モデル）における抗腫瘍活性の検討]

上記のヒト膵癌細胞株 PK-59 細胞の xenograft treatment モデルを用いた場合と同様に、ヒト膵癌細胞株 BxPC-3 細胞の xenograft prevention モデル及び xenograft treatment モデルでの抗腫瘍活性を、クローン K5-70 について検討し

た。

クローン K5-70 投与群(N=8)では、コントロール群（N=8）と比較して、腫瘍成長が有意に阻害され、 52 日目(Day 52)において、コントロール群（N=8）の腫瘍体積が 616.3±266.8 mm^3 であったのに対し、クローン K5-70 投与群（N=8）では 236.0±136.4 mm^3 と 61.7%の腫瘍成長阻害効果を示した（$P < 0.01$ by Student's t-test）（図 15）。

以上の結果より、抗hTROP-2モノクローナル抗体は、少なくとも2つ以上の複数の癌細胞種に対して、*in vivo*での有意な腫瘍成長阻害活性を示すことが明らかとなった。

〔実施例１８〕

[抗 hTROP2 抗体（クローン K5-70）の、hTROP2 発現膵癌細胞株（PK-59 細胞）xenograft prevention モデルにおける用量依存的な抗腫瘍活性]

抗hTROP-2抗体の*in vivo*における腫瘍成長阻害活性を、より詳細に検討する目的で用量依存性試験を行った。図16に示したとおり、PK-59細胞の腫瘍の成長はK5-70抗体の投与によって用量依存的に阻害され、抗体投与後21日目(Day 21)において、コントロール群（N=8の腫瘍体積が937.8±295.3 mm^3であったのに対し、K5-70抗体（1 mg/kg 体重）投与群（N=8）が493.5±305.1 mm^3と50%の阻害率を示し、K5-70抗体（5mg/kg 体重）投与群（N= 8）が124.7±89.0 mm^3と90%の阻害効果を示し、公知の抗TROP-2抗体AR47A6.4.2（米国特許第7420041号）と比較して、２０分の１の投与量でほぼ同等の*in vivo*における腫瘍成長阻害効果を示し、４分の１の投与量では、より高い90%の阻害効果を示すことが明らかになった。

〔実施例１９〕

[エピトープアッセイ]

ヒト／マウスキメラ TROP-2 タンパク質の作製

ヒト／マウスキメラ TROP-2 遺伝子は PCR 法を用いて作製した。ヒト TROP-2 遺伝子配列およびマウス TROP-2 遺伝子配列（Genbank accession No.

NM_020047）をもとに以下に示すPCRプライマーを設計した。

ヒト／マウスTROP-2-Cプライマー

Y606（フォワード側）：5'-cctgagcctacgctgcgacgaagtggtgcg-3'（配列番号１０）

Y607（リバース側）：5'-cgcaccacttcgtcgcagcgtaggctcagg-3'（配列番号１１）

ヒト／マウスTROP-2-Aプライマー

Y612（フォワード側）：5'-gactgctccacgctgacttccaagtgcctg-3'（配列番号１２）

Y613（リバース側）：5'-caggcacttggaagtcagcgtggagcagtc-3'（配列番号１３）

ヒト／マウスTROP-2-Bプライマー

Y614（フォワード側）：5'-ctcgtggacaacgatggcctctacgacccg-3'（配列番号１４）

Y615（リバース側）：5'-cgggtcgtagaggccatcgttgtccacgag-3'（配列番号１５）

マウス／ヒトTROP-2-Dプライマー

Y608（フォワード側）：5'-ccaaagcctgcgctgcgatgagctggtgcgc-3'（配列番号１６）

Y609（リバース側）：5'-gcgcaccagctcatcgcagcgcaggctttgg-3'（配列番号１７）

マウス／ヒトTROP-2-Eプライマー

Y616（フォワード側）：5'-agcttcctatccgcggtgcactacgagcag-3'（配列番号１８）

Y617（リバース側）：5'-ctgctcgtagtgcaccgcggataggaagct-3'（配列番号１９）

マウス／ヒトTROP-2-Fプライマー

Y618（フォワード側）：5'-gacattaaaggcgagtctctattccagggc-3'（配列番号２０）

Y619（リバース側）：5'-gccctggaatagagactcgcctttaatgtc-3'（配列番号２１）

マウスTROP-2プライマー

フォワード側： 5-ctactccaccccaccctggcg-3'（配列番号２２）

リバース側： 5'-ctcgagcaagctaggttcgcttctc-3'（配列番号２３）

マウス TROP-2 リバース側プライマーにはストップコドンを除き、*Xho* I による制限酵素消化配列を付加した。作製したヒト／マウスキメラ TROP-2 タンパク質の模式図を図 17 に示す。

hmTROP-2-A キメラタンパク質は、hTROP-2 タンパク質の N 末端から 69 番目までのポリペプチドとマウス TROP-2 タンパク質の 64 番目から C 末端までのポリペプチドから成るキメラタンパク質である。hmTROP2-B キメラタンパク質は、hTROP-2 タンパク質の N 末端から 101 番目までのポリペプチドとマウス TROP-2 タンパク質の 96 番目から C 末端までのポリペプチドから成るキメラタンパク質である。hmTROP2-C キメラタンパク質は、hTROP-2 タンパク質の N 末端から 145 番目までのポリペプチドとマウス TROP-2 タンパク質の 140 番目から C 末端までのポリペプチドから成るキメラタンパク質である。mhTROP-2-D キメラタンパク質は、マウス TROP-2 タンパク質の N 末端から 139 番目までのポリペプチドと hTROP-2 タンパク質の 146 番目から C 末端までのポリペプチドから成るキメラタンパク質である。mhTROP-2-E キメラタンパク質は、マウス TROP-2 タンパク質の N 末端から 187 番目までのポリペプチドと hTROP-2 タンパク質の 194 番目から C 末端までのポリペプチドから成るキメラタンパク質である。mhTROP-2-F キメラタンパク質は、マウス TROP-2 タンパク質の N 末端から 227 番目までのポリペプチドと hTROP-2 タンパク質の 234 番目から C 末端までのポリペプチドから成るキメラタンパク質である。

上記キメラタンパク質を作製するための発現ベクターは、具体的には以下の方法で構築した。hmTROP-2-A キメラ遺伝子を作製するため hTROP-2 遺伝子を鋳型にして、hTROP-2 フォワード側プライマー及びヒト／マウス TROP-2-A プライマーY613 を用いて PCR を行った。同様にマウス TROP-2 遺伝子を鋳型にして、ヒト／マウス TROP-2-A プライマーY612 及びマウス TROP-2 リバース側プライマーを用いて PCR を行った。PCR により増幅した DNA 断片はアクリルアミドゲルを用いて展開、目的バンドの抽出により回収した。次に抽出した 2 種類の DNA 断片を混合して鋳型とし、hTROP-2 フォワード側プライマー及びマウス TROP-2 リバース側プライマーを用いて PCR を行った。PCR 産物はア

ガロースゲル電気泳動により展開し、目的の DNA 断片を抽出した。抽出した DNA 断片は pCR（登録商標）-Blunt ベクター（Invitrogen）にクローニングし（pCRB-hmTROP-2-A）、遺伝子配列を確認した。動物細胞用発現ベクターは、pcDNA3.1-hTROP-2-*myc*/His から Eco RI/Xho I 消化により hTROP-2 遺伝子を除き、pCRB-hmTROP-2-A から調製した hmTROP-2-A キメラ遺伝子を含む *Eco* RI/*Xho* I 断片を挿入することで作製した（pcDNA3.1-hmTROP-2-A-*myc*/His）。その他 hmTROP-2-B（ヒト TROP-2 フォワード側プライマー、ヒト／マウス TROP-2-B プライマーY615、ヒト／マウス TROP-2-B プライマーY614 及びマウス TROP-2 リバース側プライマーを使用）、hmTROP-2-C（ヒト TROP-2 フォワード側プライマー、ヒト／マウス TROP-2-C プライマーY607 及びヒト／マウス TROP-2-C プライマーY606、マウス TROP-2 リバース側プライマーを使用）、mhTROP-2-D（マウス TROP-2 フォワード側プライマー、マウス／ヒト TROP-2-D プライマーY609 及びマウス／ヒト TROP-2-D プライマーY608、ヒト TROP-2 リバース側プライマーを使用）、mhTROP-2-E（マウス TROP-2 フォワード側プライマー、マウス／ヒト TROP-2-E プライマーY617 及びマウス／ヒト TROP-2-E プライマーY616、ヒト TROP-2 リバース側プライマーを使用）、mhTROP-2-F（マウス TROP-2 フォワード側プライマー、マウス／ヒト TROP-2-F プライマーY619 及びマウス／ヒト TROP-2-F プライマーY618、ヒト TROP-2 リバース側プライマーを使用）についても同様の方法でキメラ遺伝子を作製し、発現ベクターを構築した（pcDNA3.1-hmTROP-2-B-*myc*/His、pcDNA3.1-hmTROP-2-C-*myc*/His、pcDNA3.1-mhTROP-2-D-*myc*/His、pcDNA3.1-mhTROP-2-E-*myc*/His、pcDNA3.1-mhTROP-2-F-*myc*/His）。

<u>hTROP-2、ヒト／マウスキメラ TROP-2-C、及びマウス／ヒトキメラ TROP-2-D タンパク質を恒常的に発現する HEK293 細胞株の樹立</u>

HEK293 細胞株に上記発現ベクター pcDNA3.1-hTROP-2-*myc*/His、pcDNA3.1-hmTROP-2-C-*myc*/His 及び pcDNA3.1-mhTROP-2-D-*myc*/His をそれぞれ遺伝子導入した。抗生物質 G418（Calbiochem）による選択を行い、

hTROP-2 タンパク質、hmTROP-2-C キメラタンパク質および mhTROP-2-D キメラタンパク質を恒常的に発現する HEK293 細胞株を樹立した。

膵臓癌細胞株 PK-59 を用いた xenograft treatment モデルにおいて、薬効を示した抗 hTROP-2 モノクローナル抗体 K5-70、T5-86、K5-107、T6-4、T6-16 および K5-116-2-1 について結合領域の同定を行った。まず hmTROP-2-C、mhTROP-2-D、それぞれのキメラタンパク質を恒常的に発現する HEK293 細胞を用いて、薬効を示した抗 hTROP-2 モノクローナル抗体の反応性を FACS 解析により検討した（図 18）。その結果、K5-70、K5-107、T5-86 および K5-116-2-1 抗体は hmTROP-2-C と反応したが mhTROP-2-D とは反応しなかった。一方、T6-4 および T6-16 抗体は mhTROP-2-D と反応したが hmTROP-2-C とは反応しなかった。これらのことから K5-70、K5-107、T5-84 および K5-116-2-1 抗体の結合領域は hTROP-2 の N 末端から 145 番目のアミノ酸までの領域、T6-4 および T6-16 抗体の結合領域は hTROP-2 の 146 番目のアミノ酸から C 末端までの領域に限定された（図 18）。

さらに詳細に結合領域の解析を行うため、hmTROP-2-A、hmTROP-2-B、mhTROP-2-E及びmhTROP-2-Fキメラタンパク質発現用ベクターを作製し、薬効を示す抗hTROP-2モノクローナル抗体との反応性を検討した（図19）。新たに作製したキメラタンパク発現用ベクターをHEK293細胞に遺伝子導入し、一過性に発現させた細胞を用いてFACS解析を行った。K5-70、K5-107、T5-86およびK5-116-2-1抗体はhmTROP-2-Aとは反応したがmhTROP-2-Bとは反応しなかった。調べた6種類のモノクローナル抗体はすべてhTROP-2とは反応を示した。このことからK5-70、K5-107、T5-86およびK5-116-2-1抗体の結合領域はhTROP-2のN末端から69番目のアミノ酸までの領域に存在することが明らかとなった。またT6-4およびT6-16抗体はmhTROP-2-E、mhTROP-2-F共に反応しなかったことから、hTROP-2の146番目から193番目のアミノ酸までの領域を認識することが示唆された。

〔実施例２０〕

[免疫組織化学]

＜材料・方法＞

免疫組織染色に使用した正常および癌組織アレイは以下の通りである。

ヒト正常組織アレイ：

Human, Normal organs in duplicates (Catalog No.: AB1, Super Bio Chips)

Normal tissues more than single spots (Catalog No.: A103(VI), ISU ABXIS)

肺癌組織アレイ：

Human Lung cancer-metastasis-normal (Catalog No.: CCA3, Super Bio Chips)

Human lung carcinoma tissue with margin tissue, 2 location cores (Catalog No.: OD-CT-RsLug03-002, Shanghai Outdo Biotech)

膵臓癌組織アレイ：

Human Pancreas carcinoma tissue with mono-pathological type from 60 cases, 2 location cores (Catalog No.: OD-CT-DgPan03-001, Shanghai Outdo Biotech)

肝臓癌組織アレイ：

Hepatocellular carcinoma, grade I～III with normal tissue controls, 63 cases tissue arrays (Catalog No.: CS03-01-002U, Cybrdi)

Human liver carcinoma tissue with mono-pathological type from 30 cases, 2 location cores (Catalog No.: OD-CT-DgLiv02-002, Shanghai Outdo Biotech)

大腸癌組織アレイ：

Human, Colorectal cancer (Catalog No.: CD3, Super Bio Chips)

Human colon carcinoma with margin tissue, 2 location cores (Catalog No.: OD-CT-DgCol03-002, Shanghai Outdo Biotech)

大腸癌リンパ節転移、肝転移組織アレイ：

Colorectal (colon and rectum) cancer with matched lymph node metastasis tissue array, 44 cases/99 cores, trial slide (Catalog No.: CO991t, Biomax us)

Colorectal (colon and rectum) cancer with matched lymph node metastasis and normal adjacent tissue array, 43 cases/99 cores (Catalog No.: CO992,

Biomax us)

Colon cancer tissues_liver metastasis (Catalog No.: A203(IV), ISU ABXIS)

乳癌組織アレイ：

Human, Breast cancer-metastasis-normal (Catalog No.: CBA3, Super Bio Chips)

Human breast carcinoma with margin tissue, 2 location cores (Catalog No.: OD-CT-RpBre03-002, Shanghai Outdo Biotech)

胃癌組織アレイ：

Human, Stomach cancer (Catalog No.: CQ1, Super Bio Chips)

Human gastric carcinoma with margin tissue, 2 location cores (Catalog No.: OD-CT-DgStm03-002, Shanghai Outdo Biotech)

食道癌組織アレイ：

Human, Esophagus cancer (Catalog No.: CR1, Super Bio Chips)

Human esophagus carcinoma with margin tissue, 2 location cores (Catalog No.: OD-CT-DgEso03-002, Shanghai Outdo Biotech)

卵巣癌組織アレイ：

Human, Ovary cancer (Catalog No.: CJ1, Super Bio Chips

前立腺癌組織アレイ：

Human, Prostate cancer-normal (Catalog No.: CA3, Super Bio Chips)

膀胱癌組織アレイ：

Bladder carcinoma / transitional cell carcinoma, grade I～III with normal tissue arrays (Catalog No.: CC12-01-001U, Cybrdi)

上記組織アレイの患者情報および臨床情報は添付のデータシートおよび各社のホームページから入手した。

免疫組織染色法

ヒト正常組織および癌組織の組織アレイパラフィン切片は、脱パラフィン処理後、37℃で5分間ペプシンによるプロテアーゼ処理を行い、抗hTROP-2モノク

ローナル抗体を用いた免疫染色に使用した。DAB(3,3'-ジアミノベンチジン)を基質とて発色反応を行った後、対比染色としてヘマトキシリンによる核染色を行った。

これらの操作はより具体的には次のようにして行なった。パラフィン包埋された切片を脱パラフィン処理した後、37℃で 5 分間ペプシン（DAKO）によるプロテアーゼ処理を行った。抗原賦活化後、メタノールに終濃度 0.3%となるように過酸化水素水を加えた溶液により、室温で 20 分間処理し内因性のペルオキシダーゼ活性を除いた。PBS で室温 5 分間の洗浄を 2 回行った後、1.5%の正常ウマ血清（DAKO）を含む PBS 溶液を用いて室温 30 分間のブロッキングを行い、組織中の非特異的結合部位をふさぐ操作を行った。次に 1.5%の正常ウマ血清を含む PBS 溶液により希釈した抗 hTROP-2 モノクローナル抗体 clone K5-63-17（終濃度 10μg/ml）を室温で 1 時間反応させた後、PBS で室温 5 分間の洗浄を 3 回行い、続いて 1.5%の正常ウマ血清を含む PBS 溶液により 200 倍に希釈したビオチン化抗マウス IgG 抗体（Vector）を室温で 30 分間反応させた。PBS による室温 5 分間の洗浄を 3 回行った後、Vectastain ABC キット（Vector）の試薬を説明書通りに混合し ABC コンプレックスを作り、これを室温で 30 分間反応させた。PBS で室温 5 分間の洗浄を 3 回行った後、ヒストファインペルオキシダーゼ基質シンプルステイン DAB 溶液（ニチレイバイオサイエンス）によって発色を行った。発色を確認した後、脱イオン水で 5 分間洗浄し、マイヤーヘマトキシリン溶液（和光純薬）によって核を染色し、その後アルコールで脱水、キシレンで透徹してエンテランニュー（メルク・ジャパン株式会社）で封入した。

＜結果＞

ヒト正常組織における hTROP-2 の発現

ヒト正常組織における hTROP-2 の発現パターンについて、抗 hTROP-2 モノクローナル抗体 クローン K5-63-17 を用いて解析した。ヒト正常組織組織アレイ（Catalog No.: AB1, Super Bio Chips）を脱パラフィン、親水化処理を行った後、タンパク質分解酵素ペプシンによる抗原の賦活化を行い、抗 hTROP-2 モノクローナル抗体 クローン K5-63-17 で免疫染色を行った（図 20）。その結果、

皮膚、食道、腎臓（皮質及び髄質）、膵臓、前立腺、膀胱、扁桃腺で染色が見られた。ほとんどの染色像は細胞膜への局在を示していたが（図 20 A, B, C, D, F, G. H）、一部細胞質にも発現が見られた（図 20. E, H）。一方、心臓、肝臓、胃、小腸、大腸、骨格筋、肺、脾臓、胸腺などでは染色されなかった（図 20. I, J）。

ヒト癌組織における hTROP-2 の発現

ヒト癌組織における hTROP-2 の発現（hTROP-2 の陽性率）を調べるため、抗 hTROP-2 モノクローナル抗体 クローン K5-63-17 を用いて、ヒトの各癌種の癌組織アレイの免疫染色を行った。癌細胞の 10%以上が染色される組織切片を hTROP-2 陽性とした。この染色結果を表４に示した。

表４

癌組織	TROP2陽性数/症例数	TROP2陽性率(%)
乳癌	32/80	40
肺癌	53/81	65.4
食道癌	69/90	76.7
胃癌	25/90	27.8
大腸癌	29/178	16.3
膵臓癌	26/62	41.9
肝臓癌	7/92	7.61
膀胱癌	42/59	71.2
前立腺癌	35/38	92.1
卵巣癌	14/58	24.1

また、代表的な染色像を図 21 に示した。hTROP-2 の発現を検討した癌種では、前立腺癌で最も陽性率が高く（92.1%）、肺癌（65.4%）、食道癌（76.7%）、膀胱癌（71.2%）などで高い陽性率であった。最も陽性率の低かったのは肝臓癌で 7.61%であった。癌細胞でも正常細胞と同様に、hTROP-2 は細胞膜に強く局在している染色像が見られた（図 21. A～F, H, I）。また一部は細胞質にも発現していた（図 21. A, B, E, G）。

膵臓癌での hTROP-2 の陽性率は 41.9%であった。このときの hTROP-2 陽性

率と膵臓癌のグレード（分化度）との関係について検討した。その結果、グレードが高い、すなわち低分化膵臓癌で高頻度に発現していた（表５）。

表５

膵臓癌 hTROP2陽性スポット 26/62 (41.94%)

Grade	-	+	陽性率	
I	8	0	0%	
I-II	5	0	0%	
II	19	21	52.5%	
II-III	4	5	55.6%	p<0.01
total	36	26		

〔実施例２１〕

[K5-70 抗体の単回投与による、ヒト膵癌細胞株 PK-59 xenograft prevention モデルでの抗腫瘍効果]

クローン K5-70（マウス IgG2a）の *in vivo* における強い抗腫瘍活性は、ヒト膵癌細胞株 PK-59 を用いた xenograft prevention モデルにおいて、10 mg/kg 体重の投与量で 1 回投与した場合においても示された。コントロール群（マウス IgG 10mg/kg 体重, N=3）では、全ての個体において腫瘍形成が観察され、細胞移植後 28 日目（Day 28）における腫瘍体積は 781.7±74.5 mm^3 であったのに対して、癌細胞移植日(Day 1)に 1 回 K5-70 抗体を投与した群では（10 mg/kg 体重、N=3）、Day 28 における腫瘍体積は 144.4±176.9 mm^3 ($P < 0.05$ by Student's t-test)と 81.5%の腫瘍成長阻害活性を示した（図 22A）。腫瘍重量においては、Day 28 においてコントロール群が 0.59±0.06 g であったのに対し、クローン K5-70 抗体投与群では 0.07±0.10 g ($P < 0.01$ by by Student's t-test)) と 88%の阻害活性を示した（図 22B）。腫瘍体積、腫瘍重量のいずれにおいても、K5-70 抗体投与群では 3 個体中 2 個体では、1 回の 10 mg/kg 体重の投与で、完全に腫瘍形成が阻害された（図 22C）。

〔実施例２２〕

[ヒト大腸癌細胞株 SW480 細胞 xenograft treatment モデルにおける抗ヒ

hTROP-2 モノクローナル抗体の抗腫瘍活性]

抗 hTROP-2 モノクローナル抗体（クローン K5-70, K5-116-2-1 および T6-16）の抗腫瘍活性を、ヒト大腸癌細胞株 SW480 を用いた xenograft treatment モデルで検討した。5×10^6 cells の SW480 細胞を 6 週齢雌の NOD-scid マウスの右脇腹皮下に移植（Day 1）し、平均の腫瘍体積が 100 mm^3 に達した段階で群分けを行い（Day 7 又は Day 10）, Day 7 又は Day 10 から 3 日に 1 回の投与間隔で、抗体の腹腔内投与を開始した。クローン K5-70 抗体の抗腫瘍活性評価と、クローン K5-116-2-1 抗体およびクローン T6-16 抗体の抗腫瘍活性評価は、それぞれ独立した試験で評価した。K5-70 抗体の抗腫瘍活性評価試験においては、癌細胞移植から 44 日目（Day 44）におけるコントロール群（マウス IgG（10 mg/kg 体重）, N=8）の腫瘍体積が 365.4±214.6 mm^3 であったのに対して、K5-70 抗体(10 mg/kg 体重)投与群では、27.4±29.4 mm^3 ($P < 0.01$ by Student's t-test)と著しく腫瘍形成が阻害された（阻害率 92.5 %）（図 23A）。腫瘍重量においては、コントロール群が 0.11±0.07 g であったのに対して、K5-70 抗体投与群では 0.005±0.007 (g) ($P < 0.01$ by Student's t-test)と 95.5 %の阻害率であった（図 23B）。特に、K5-70 抗体投与群では 8 個体中 2 個体では、完全に腫瘍形成が阻害され腫瘍の確認が出来なかった。

別に行った K5-116-2-1 抗体および T6-16 抗体の抗腫瘍活性評価試験においては、Day 42 におけるコントロール群の腫瘍体積が 713.8±354.5 mm^3 (N=8)であったのに対して、K5-116-2-1 抗体 投与群 (10 mg/kg 体重)では、188.9±97.4 mm^3 (N=8, $P < 0.01$ by Student's t-test) (図 24A), T6-16 抗体投与群(10 mg/kg 体重)では、292.8±199.7 mm^3 (N=8, $P < 0.05$ by Student's t-test) (図 25A)と、それぞれ 73.5 %, 59.0%の阻害率であった。腫瘍重量についても同様に、コントロール群では 0.39±0.19 g であったのに対して、K5-116-2-1 抗体投与群、T6-16 抗体投与群では、0.10±0.07 g ($P < 0.01$ by Student's t-test), 0.17±0.14 g ($P < 0.05$ by Student's t-test)と、それぞれ 72.2 %, 56.4 %の阻害率を示した（図 24B, 図 25B）。

〔実施例２３〕

[ヒト大腸癌細胞株 SW480 細胞 xenograft treatment モデルにおけるクローン K5-70 の用量依存的な抗腫瘍活性]

次に、K5-70 抗体の用量依存的な抗腫瘍活性を、ヒト大腸癌細胞株 SW480 を用いた xenograft treatment モデルにおいて検討した。5×10^6 cells の SW480 細胞を 6 週齢雌の NOD-scid マウスの右脇腹皮下に移植し、移植から 10 日後（Day 10）に平均の腫瘍体積が 100 mm^3 に達した段階でコントロール群（マウス IgG 10 mg/kg 体重投与群、N=8, 104.4±17.6 mm^3）, 1 mg/kg 体重の K5-70 抗体投与群 (N=8, 104.3±16.1 mm^3), 5mg/kg 体重の K5-70 抗体投与群（N=8, 104.6±15.9 mm^3）, 10 mg/kg 体重の K5-70 抗体投与群（N=8, 104.8±14.9 mm^3）に群分を行い、3 日に 1 回の腹腔内投与を行った。Day 42 におけるコントロール群の腫瘍体積が 713.8 ± 354.5 mm^3 であったのに対して、K5-70 抗体投与群では用量依存的な腫瘍形成阻害活性が観察され、1 mg/kg 体重投与群では、485.0±207.3 mm^3 (阻害率 32.1%), 5 mg/kg 体重投与群では、339.5 ± 253.2 mm^3 (阻害率 52.4%), 10 mg/kg 体重投与群では、355.4±202.8 mm^3 (阻害率 50.2 %, $P < 0.05$ by Student's t-test)であった（図 26A）。同様に、Day 42 における腫瘍重量については、コントロール群が 0.39±0.19 g であったのに対して、K5-70 抗体の 1 mg/kg 体重　投与群では, 0.24±0.11 g (阻害率 37.8%), 5 mg/kg 体重投与群では、0.17±0.14 g (阻害率 55.8%, $P < 0.05$ by Student's t-test), 10 mg/kg 体重投与群では、0.20±0.13 g (阻害率 47.1 %) となり、用量依存的な抗腫瘍活性が確認された（図 26B）。

〔実施例２４〕

[ヒト大腸癌細胞株 SW480 細胞 xenograft treatment モデルにおける K5-70 抗体の投与間隔の検討]

さらに、K5-70 抗体の至適投与間隔を検討するために、週に 1 回（7 日に 1 回）投与を行った際の抗腫瘍活性を、ヒト大腸癌細胞株 SW480 を用いた xenograft treatment モデルにおいて検討した。5×10^6 cells の SW480 細胞を 6 週齢雌の NOD-scid マウスの右脇腹皮下に移植し、移植から 10 日後（Day 10）に平均の腫瘍体積が 100 mm^3 に達した段階でコントロール群（マウス IgG, 10

mg/kg 体重、N=8, 104.42±15.1 mm^3）, K5-70 抗体投与群 （10 mg/kg 体重、N=8, 104.3±16.1 mm^3)に群分を行い、7 日に 1 回の腹腔内投与を行った。Day 42 におけるコントロール群の腫瘍体積が 713.8±354.5 mm^3 であったのに対して、K5-70 抗体投与群(週 1 回投与)では、332.3±239.9 mm^3 (阻害率 55%, $P < 0.05$ by Student's t-test) となった（図 27A）。更に、投与間隔を空け 10 日に 1 回および 2 週間に 1 回投与した場合、Day 39 におけるコントロール群の腫瘍体積が 956.9±367.8 mm^3 であったのに対して、10 日に 1 回の K5-70 抗体投与群では、525.4±180.6 mm^3 (阻害率 45.1%, $P < 0.01$ by Student's t-test)2 週間に 1 回の K5-70 抗体投与群では 459.4±217.6 mm^3 (阻害率 52.0%, $P < 0.01$ by Student's t-test)となった（図 27B）。先行技術（US 7420040, US 7420041）では、膵臓癌細胞株（BxPC-3）を用いた xenograft treatment モデルにおいて、20 mg/kg 体重、週に 3 回の抗体投与（投与間隔 2 日間）で、阻害率 50-60%の抗腫瘍活性を示しているのに対し、K5-70 抗体は 1 回の投与量が半分（10 mg/kg 体重）、かつ 2 週間に 1 回の投与（投与間隔 13 日間）で、先行技術に匹敵する抗腫瘍活性を示した。従って K50-70 抗体は少なくとも先行技術の 12 分の 1 の総投与量で顕著な抗腫瘍活性を示すことが明らかとなった。

〔実施例２５〕

[ヒト大腸癌細胞株 SW480 細胞 xenograft treatment モデルにおける T6-16 抗体の用量依存的な抗腫瘍活性]

次に、T6-16 抗体の用量依存的な抗腫瘍活性を、ヒト大腸癌細胞株 SW480 を用いた xenograft treatment モデルにおいて検討した。5×10^6 cells の SW480 細胞を 6 週齢雌の NOD-scid マウスの右脇腹皮下に移植し、移植から 10 日後（Day 10）に平均の腫瘍体積が 100 mm^3 に達した段階でコントロール群（マウス IgG 10 mg/kg 体重、N=8, 105.8±9.9mm^3）, 1 mg/kg 体重の T6-16 抗体投与群 (N=8, 104.4±13.3 mm^3), 5mg/kg 体重の T6-16 抗体投与群（N=8, 104.7±13.0 mm^3）, 10 mg/kg 体重の T6-16 抗体投与群（N=8, 104.8±12.4 mm^3）に群分を行い、3 日に 1 回の腹腔内投与を行った。Day 43 におけるコントロール群の腫瘍体積が 473.5±137.0 mm^3 であったのに対して、T6-16 抗体

投与群では用量依存的な腫瘍形成阻害活性が観察され、1 mg/kg 体重投与群では、397.9±97.5 mm^3 (阻害率 16.0 %), 5 mg/kg 体重投与群では、195.9±89.7 mm^3 (阻害率 58.7 %, $P < 0.01$ by Student's t-test), 10 mg/kg 体重投与群では、190.2±56.5 mm^3 (阻害率 59.8 %, $P < 0.01$ by Student's t-test)であった（図 28A）。同様に、Day 43 における腫瘍重量は、コントロール群が 0.19±0.07 g であったのに対して、1 mg/kg 体重の T6-16 抗体投与群では, 0.20±0.08 g, 5 mg/kg 体重の投与群では、0.08±0.04 g (阻害率 57.9 %, $P < 0.01$ by Student's t-test), 10 mg/kg 体重の投与群では、0.09±0.04 g (阻害率 52.6 %, $P < 0.01$ by Student's t-test) となり、用量依存的な抗腫瘍活性が確認された（図 28B）。

〔実施例２６〕

[ヒト大腸癌細胞株 SW480 細胞 xenograft treatment モデルにおける T6-16 抗体の投与間隔の検討]

さらに、T6-16 抗体の至適投与間隔を検討するために、週に 1 回（7 日に 1 回）および 10 日に 1 回の投与間隔での抗腫瘍活性を、ヒト大腸癌細胞株 SW480 を用いた xenograft treatment モデルにおいて検討した。5×10^6 cells の SW480 細胞を 6 週齢雌の NOD-scid マウスの右脇腹皮下に移植し、移植から 10 日後（Day 10）に平均の腫瘍体積が 100 mm^3 に達した段階でコントロール群（マウス IgG, 10 mg/kg 体重、N=8, 105.8±9.9mm^3），週 1 回の T6-16 抗体投与群(10 mg/kg 体重、N=8, 105.0±11.6 mm^3), 10 日に 1 回の T6-16 抗体投与群（10 mg/kg 体重、N=5, 130.8±2.4 mm^3)に群分けを行い、投与を開始した。Day 43 におけるコントロール群の腫瘍体積が 473.5±137.0 mm^3 であったのに対して、週 1 回の T6-16 抗体投与群では、243.7±65.3 mm^3 (阻害率 48.5 %, $P < 0.01$ by Student's t-test), 10 日に 1 回の T6-16 抗体投与群では、297.8±54.4 mm^3 (阻害率 37.1%, $P < 0.05$ by Student's t-test)となった（図 29）。先行技術（US 7420040, US 7420041）が膵臓癌細胞株（BxPC-3）を用いた xenograft treatment モデルにおいて、20 mg/kg 体重の投与量で、週に 3 回投与（投与間隔 2 日間）で、阻害率 50-60%の抗腫瘍活性を示しているのに対し、T6-16 抗体は、1 回の投与量が、先行技術の半分の用量かつ、10 日に 1 回の投与（投与間

隔9日間）で、顕著な抗腫瘍活性を示した。従って、T6-16抗体は少なくとも先行技術の8分の1の総投与量で顕著な抗腫瘍活性を示すことが明らかとなった。

〔実施例２７〕

[ヒト前立腺癌細胞株DU-145 xenograft preventionモデルにおける抗腫瘍活性の検討]

クローン K5-70 のヒト前立腺癌に対する抗腫瘍活性を、DU-145 細胞（RIKEN Cell Bank, RCB2143）を用いたxenograft preventionモデルで評価した。5×10^6 細胞の DU-145 細胞を6週齢の雌のヌードマウス（Balb/c, nu/nu）の皮下に移植し、移植当日を Day 1 とし、コントロール群（マウスIgG）（N=8）とK5-70抗体投与群（N=8）に群分けを行い、Day 1から3日に1回の頻度で 10 mg/kg 体重の投与量で腹腔内投与した。Day 40 において、コントロール群における腫瘍体積が 368.2 ± 307.8 mm^3 であったのに対し、K5-70抗体投与群では 30.6 ± 29.6 mm^3 ($P < 0.05$ by Student's t-test)と約90%の腫瘍形成阻害活性を示した（図 30A）。腫瘍重量においては、更に顕著な抗腫瘍活性が認めらた。Day 40におけるコントロール群の腫瘍重量が 0.18 ± 0.18 g であったのに対して、K5-70 抗体投与群では 8 個体の全てにおいて腫瘍が消失しており、完全に腫瘍形成が阻害された（図 30B）。以上の結果から、抗ヒトTROP-2 モノクローナル抗体クローン K5-70 は、ヒト前立腺癌細胞に対しても強い抗腫瘍活性を示すことが明らかとなった。

〔実施例２８〕

[ヒト膵癌細胞株 PK-59 細胞 の肝転移モデルを用いた K5-70 抗体の転移抑制活性]

消化器癌の治療において、癌の転移は臨床上の予後を左右する重要な因子であり、転移をコントロールすることは治療上重要な意味を有する。 TROP-2 を標的とした癌治療用抗体投与により、腫瘍形成の抑制のみならず、癌細胞の他臓器への転移を抑制することが可能であれば、高い臨床上の有用性が期待されるため、癌治療抗体として望ましい特性である。

TROP-2 は多くの癌腫における発現が確認されているが、特に転移巣において高発現していることが示されている（Br. J. Cancer (2008); 99: 1290-1295, Clin. Cancer Res. (2006); 12: 3057-3063, Mod. Pathol. (2008); 21: 186-191）。さらに、Trop-2 遺伝子を導入した癌細胞を経脾的あるいは経膵的にヌードマウスに移植した場合に、肝転移の発症率が増加すること（WO 2010/089782、Molecular Cancer (2010); 9: 253）が報告され、TROP-2 の癌転移過程における重要性が示唆されている。しかしながら、TROP-2 を標的とした抗体を用いて*in vivo*で転移抑制作用を具体的に示した報告はこれまでに認められない。

本発明により見出された抗hTROP-2 マウスモノクローナル抗体K5-70 は、ヒト膵臓癌細胞を皮下に移植したゼノグラフトモデルにおいて高い治療効果が示されているが、癌細胞の増殖抑制効果に加え、ヒト膵癌細胞株PK-59 の遊走能を抑制することが、*in vitro*のスクラッチアッセイで示されており、癌の転移を*in vivo*においても抑制する可能性が考えられた。そこで、PK-59 細胞をヌードマウスの脾臓から注入し、肝転移を発生させるモデルを用いて抗hTROP-2 マウスモノクローナル抗体の転移抑制効果を検証した。

癌細胞移植の前日に、マウスを群分し、抗hTROP-2 モノクローナル抗体K5-70 あるいは対照抗体（精製マウスIgG）を 10 mg/kg 体重にて腹腔内投与した。翌日、hTROP-2 を内在的に発現するヒト膵癌細胞株（PK-59）をトリプシン処理で剥がし、PBSで 2×10^7 cells/mLの細胞懸濁液を調製した。細胞懸濁液は移植時まで氷上に保管した。6～7 週齢の雌のヌードマウス（Balb/c, nu/nu）をペントバルビタールの腹腔内投与により麻酔し、麻酔下で左側腹部を 10～15mm切開し、脾臓を腹腔外に引き出し、27 Gのシリンジを用いて 50μLの細胞懸濁液（1 x 10^6 cell）を脾臓内に注入した。細胞注入 4 分後に脾門部を 5-0silkにて結紮し、脾臓を摘出した。切開した腹膜を 4-0silkで閉創し、Wound Clips (AUTOCLIP 9mm, Becton Dickinson)を用いて閉腹した。癌細胞移植 7 日後に、さらにK5-70 抗体および対照抗体を 10mg/kg 体重で投与した。癌細胞移植から 4～6 週間後に頸椎脱臼によりマウスを安楽死させ、肝臓を摘出し、転移巣の有無を確認した。

コントロールとして用いたマウスIgGを投与した群においては、PK-59 細胞を

移植した6匹のマウス中、4匹において、移植後4から6週間後に肝葉辺縁部への明らかな転移巣（2～7 個）が認められた（図 31A、転移発生率 67%, 表 6）。これに対し、K5-70 抗体投与群では、PK-59 細胞を移植した4匹のマウスにおいて、いずれの個体でもまったく肝臓への転移巣が認められず、転移発生率は0%であった（図 31B, 表 6）。

表6

PK-59 細胞をヌードマウスに経脾的に移植した肝転移モデルにおける
クローンK5-70 の転移抑制効果

投与群	移植後週	個体番号	転移巣数	転移判定	
Control群	4W	C-1	0	−	
	4W	C-2	5	++	
	6W	C-3	7	++	
	6W	C-4	7	++	
	6W	C-5	2	+	
	6W	C-6	0	−	
		平均転移巣数	3.5	転移発生率	67%
K5-70投与群	4W	K-1	0	−	
	6W	K-2	0	−	
	6W	K-3	0	−	
	6W	K-4	0	−	
		平均転移巣数	0	転移発生率	0%

これらの結果から、抗hTROP-2 抗体K5-70 は、ヒト膵癌細胞株PK-59 の肝転移に対して極めて強い抑制作用を有することが明らかとなった。

〔実施例２９〕

[ヒト大腸癌細胞株 SW480 xenograft モデルにおける塩酸イリノテカン投与後の癌再発モデルにおける K5-70 抗体の抗腫瘍活性]

癌治療剤として近年、癌細胞の増殖を抑制する化学療法剤が多く開発され、一定の治療成績を上げているが、癌細胞以外の正常細胞に対する増殖抑制作用に伴う副作用と、治療中止後の癌の再発が大きな問題となっている。したがって、TROP-2 を標的とした癌治療用抗体投与により、化学療法剤による治療後の腫瘍再発を抑制することが可能であれば、高い臨床上の有用性が期待されるため、癌治療抗体として望ましい特性である。

大腸癌の治療剤としては、5-FU, 白金製剤に加え、トポイソメラーゼ阻害効果を有する塩酸イリノテカン（トポテシン、第一三共株式会社）が近年臨床適応され、動物モデルにおいても、大腸癌をはじめとした種々のヒト腫瘍細胞を移植したマウスモデルにおける抗腫瘍効果が報告されている（Cancer Chemother Pharmacol. (1991); 28(3):192-8）。そこで、塩酸イリノテカン投与後の腫瘍再発に対する抗 hTROP-2 抗体クローン K5-70（マウス IgG2a）の再発予防効果について、ヒト大腸癌細胞株 SW480 を用いた xenograft モデルにおいて検討した。5×10^6 cells の SW480 細胞を 8 週齢雌の NOD-scid マウスの右脇腹皮下に移植し、移植から 11 日後（Day 11）に平均の腫瘍体積が 100 mm^3 以上に達した段階で無処置群（生理食塩水投与群、N=8, 130.7±16.2 mm^3），塩酸イリノテカン（CPT-11, トポテシン、第一三共株式会社）投与群（N=16, 123.0±21.4 mm^3）に群分けを行い、塩酸イリノテカンを 40mg/kg 体重で 3 日に 1 回、合計 3 回（Day 11, 14, 17）腹腔内投与した。塩酸イリノテカンの最終投与から 3 日目(Day 20)に、無処置群の腫瘍体積は 232.1±21.1 mm^3 に達したのに対し、塩酸イリノテカン投与群は 126.6.±26.6mm^3 ($P < 0.01$ by Student's t-test)であり、明らかな腫瘍抑制効果が認められた。この段階で腫瘍サイズに基づき塩酸イリノテカン投与群を 2 群に分け、一方は K5-70 抗体投与群 （10 mg/kg 体重, N=8, Day 20 の腫瘍体積 126.0±28.0 mm^3)とし、もう一方はマウス IgG 投与群 (10mg/kg 体重、N=8, Day 20 の腫瘍体積 127.2±27.0 mm^3）とした。それぞれ 3 日に 1 回の抗体の腹腔内投与と腫瘍体積の測定を行い腫瘍の再発を評価した（図 32）。マウス IgG 投与群では、塩酸イリノテカンの最終投与から 18 日目(Day 35)より腫瘍体積が 300 mm^3 を超える明らかな再発腫瘍を有する個体が複数認められ、塩酸イリノテカンの最終投与から 30 日目(Day 47)においては、8 例中 5 例で 300 mm^3 を超える腫瘍が認められた（腫瘍平均体積 401.7±172.7 mm^3）。これに対し、K5-70 抗体投与群では顕著に腫瘍再発が抑制され、腫瘍平均体積は、180.5±142.1 mm^3, ($P < 0.05$ by Student's t-test)であった（図 32）。特に、K5-70 抗体投与群では、Day 47 における腫瘍体積が群分け時（126.0±28.0 mm^3）よりも退縮し、100 mm^3 未満となった個体が 8 例中 4 例認められた。これらの結果から、抗 hTROP-2 抗体 K5-70 は、塩酸イリノテカ

ン投与後の腫瘍再発に対しても極めて強い抑制作用を有することが明らかとなった。

〔実施例３０〕

[CLIPS 技術を用いたエピトープマッピング]

＜材料と方法＞

ペプチド合成

本実験で使用した直鎖型 15-mer 及び 30-mer の TROP-2 細胞外領域に由来するペプチドは、Fmoc (9-Fluorenylmethoxycarbonyl)法による固相合成により取得した。また不連続エピトープ解析のため、両端にシステイン残基を付加した TROP-2 細胞外領域に由来する 17-mer のペプチドを合成し、CLIPS 技術（Chemically Linked Peptides on Scaffolds technology）によりループ構造を一つまたは二つ有する立体構造を再構築した。付加したシステイン残基近傍に別のシステイン残基が存在する場合、アラニンに置換した。

エピトープスクリーニング ELISA

5034 種類の合成したペプチドを PEPSCAN カード（455 ペプチド/カード）へ共有結合させ、ELISA 法により合成ペプチドと抗体の結合を検証した。PEPSCAN カードはブロッキングバッファー（4%ウマ血清、5%卵白アルブミン、1% Tween を含むリン酸緩衝液）で 1 μg/mL に希釈した抗ヒト TROP-2 モノクローナル抗体（K5-70、K5-107、K5-116-2-1、T5-86、T6-16）と反応させた。洗浄後、1000 倍希釈したペルオキシダーゼ二次抗体複合体と 25℃、1 時間反応させた。洗浄後、基質溶液（2,2'-azino-di-3-ethylbenzthiazoline sulfonate(ABTS)と 2μL の 3%過酸化水素水を含む溶液）を加え、1 時間発色反応を行った。抗体結合活性は CCD カメラで撮影し、画像解析を行い定量した。

＜結果＞

薬効を示した抗 hTROP-2 モノクローナル抗体 K5-70、K5-107、K5-116-2-1、T5-86、T6-16 について CLIPS（Chemically Linked Peptides on Scaffolds）技術を利用しエピトープ解析を行った。なお、以下、本実施例において「アミノ酸

番号」とは、配列番号２に示されるアミノ酸配列（hTROP-2 タンパク質（323 アミノ酸残基））中のアミノ酸番号を意味する。

K5-70 抗体についての解析結果を下記表 7 に示す。その結果、33 ペプチドが K5-70 抗体と強い結合を示すことが明らかとなった。これら 33 ペプチドの中で、VCSPDGPGGRCQCRALGSGMAVD（アミノ酸番号 43-65）を含む配列（表 7 中に記載のペプチド No. 1-7, 9）、HHILIDLRHRPTAG（アミノ酸番号 152-165）を含む配列（表 7 中に記載のペプチド No. 14, 22-24, 28）、VHYEQPTIQIELRQ（アミノ酸番号 193-206）を含む配列（表 7 中に記載のペプチド No. 8, 10, 12, 13, 18, 20, 21, 23, 26, 28, 30, 32）、DLDAELRRLFRER（アミノ酸番号 171-183）を含む配列（表 7 中に記載のペプチド No. 11, 16, 18, 19, 21, 22, 29, 31）は、繰り返し出現した。K5-70 抗体は、特に VCSPDGPGGRCQCRALGSGMAVD を含む配列に強く結合した。これらの結果から、hTROP-2 タンパク質中、上記 4 種類のペプチド配列領域が K5-70 抗体のエピトープである可能性が示唆された。

表 7

ヒト TROP-2 細胞外領域に由来する CLIPS ペプチドに対する

K5-70 抗体の結合

number	peptide	binding of KS-70	SEQ ID No.
1	NKMTVCSPDGPGGRCQCRALGSGMAVDCST	2742	101
2	TVCSPDGPGGRCQCRALGSGMAVDCSTLTS	2604	102
3	TNKMTVCSPDGPGGRCQCRALGSGMAVDCS	2562	103
4	MTVCSPDGPGGRCQCRALGSGMAVDCSTLT	2402	104
5	KMTVCSPDGPGGRCQCRALGSGMAVDCSTL	1770	105
6	PTNKMTVCSPDGPGGRCQCRALGSGMAVDC	1391	106
7	VCSPDGPGGRCQCRALGSGMAVDCSTLTSK	932	107
8	CAAVHYEQPTIQIELRCAAVHYEQPTIQIELRC	876	108
9	CPTNKMTVCSPDGPGGRCQCRALGSGMAVD	839	109
10	CVHYEQPTIQIELRQNCVHYEQPTIQIELRQNC	825	110
11	HSDLDAELRRLFRERCHSDLDAELRRLFRERC	725	111
12	RLFRERYRLHPKFVAAVHYEQPTIQIELRQ	687	112
13	AVHYEQPTIQIELRQ	642	113
14	CAGAFNHSDLDAELRRCHHILIDLRHRPTAGAC	624	114
15	CPKFVAAVHYEQPTIQCGLDLRVRGEPLQVERC	S79	115
16	CHSDLDAELRRLFRERCGLDLRV	S38	116
17	FQGRGGLDLRVRGEP	S38	117
18	CVHYEQPTIQIELRQNCDLDAELRRLFRERYRC	S24	118
19	CHSDLDAELRRLFRERCRGEPLQ	S19	119
20	CTIQIELRQNTSQKAACVHYEQPTIQIELRQNC	S13	120
21	CVHYEQPTIQIELRQNCHSDLDAELRRLFRERC	S11	121
22	CHHILIDLRHRPTAGACHSDLDAELRRLFRERC	489	122
23	CHHILIDLRHRPTAGACVHYEQPTIQIELRQNC	489	123
24	CHHILIDLRHRPTAGACGLDLRVRGEPLQVERC	488	124
25	CDAELRRLFRERYRLHCDELVRTHHILIDLRHC	483	125
26	CVHYEQPTIQIELRQNC	483	126
27	CAFNHSDLDAELRRLFCVHYEQPTIQIELRQNC	478	127
28	CVHYEQPTIQIELRQNCHHILIDLRHRPTAGAC	473	128
29	CDLDAELRRLFRERYRCDELVRTHHILIDLRHC	472	129
30	VHYEQPTIQIELRQNCGLDLRVRGEPLQVERC	470	130
31	CDELVRTHHILIDLRHCDLDAELRRLFRERYRC	469	131
32	AVHYEQPTIQIELRQCAVHYEQPTIQIELRQC	468	132
33	CHSDLDAELRRLFRERCDELVRTHHILIDLRHC	466	133

K5-107 についての解析結果を下記表 8 に示す。その結果、VCSPDGPGGRCQCRALGSGMAVD（アミノ酸番号 43-65）を含む配列が 20 ペプチド中 10 ペプチド（表 8 中に記載のペプチド No. 1-6, 8, 9, 14, 17）に含まれていた（表 8）。

従って、hTROP-2 タンパク質中、上記 VCSPDGPGGRCQCRALGSGMAVD のペプチド配列領域が K5-107 抗体のエピトープであることが示唆された。

表 8

ヒト TROP-2 細胞外領域に由来する CLIPS ペプチドに対する

K5-107 抗体の結合

number	peptide	binding of K5-107	SEQ ID No.
1	TNKMTVCSPDGPGGRCQCRALGSGMAVDCS	2763	134
2	NKMTVCSPDGPGGRCQCRALGSGMAVDCST	2761	135
3	KMTVCSPDGPGGRCQCRALGSGMAVDCSTL	2752	136
4	MTVCSPDGPGGRCQCRALGSGMAVDCSTLT	2726	137
5	CPTNKMTVCSPDGPGGRCQCRALGSGMAVD	2723	138
6	TVCSPDGPGGRCQCRALGSGMAVDCSTLTS	2720	139
7	TCPTNKMTVCSPDGPGGRCQCRALGSGMAV	2716	140
8	PTNKMTVCSPDGPGGRCQCRALGSGMAVDC	2689	141
9	VCSPDGPGGRCQCRALGSGMAVDCSTLTSK	2655	142
10	CSPDGPGGRCQCRALGSGMAVDCSTLTSKC	2655	143
11	CTCPTNKMTVCSPDGPGGRCQCRALGSGMA	2207	144
12	NCTCPTNKMTVCSPDGPGGRCQCRALGSGM	1816	145
13	DNCTCPTNKMTVCSPDGPGGRCQCRALGSG	1525	146
14	CTVASPDGPGGRAQARCLGSGMAVDASTLTSKC	1118	147
15	QDNCTCPTNKMTVCSPDGPGGRCQCRALGS	874	148
16	SPDGPGGRCQCRALGSGMAVDCSTLTSKCL	561	149
17	CTNKMTVCSPDGPGGRCQCRALGSGMAVDASTC	380	150
18	TVCSPDGPGGRCQCR	312	151
19	CAPKNARTLVRPSEHACARTLVRPSEHALVDNC	284	152
20	HSDLDAELRRLFRERCHSDLDAELRRLFRERC	274	153

K5-116-2-1 抗体についての解析結果を下記表 9 に示す。当該解析では、VCSPDGPGGRCQCRALGSGMAVD（アミノ酸番号 43-65）を含む配列（表 9 中に記載ののペプチド No. 1-7, 15, 25）、HHILIDLRHRPTAG（アミノ酸番号 152-165）を含む配列（表 9 中のペプチド No. 8-11, 16, 17, 19, 20, 22-24, 27-29）、DLDAELRRLFRER（アミノ酸番号 171-183）を含む配列（表 9 中に記載のペプチド No. 11-14, 17, 19, 21, 23, 29）の 3 種類のペプチド配列が複数回出現した（表 9）。従って、hTROP-2 タンパク質中、これら 3 種類のペプチド配列領域が K5-116-2-1 抗体のエピトープであることが示唆された。

表 9

ヒト TROP-2 細胞外領域に由来する CLIPS ペプチドに対する

K5-116-2-1 抗体の結合

number	peptide	binding of K5-116-2-1	SEQ ID No.
1	TVCSPDGPGGRCQCRALGSGMAVDC5TLT5	2672	154
2	NKMTVCSPDGPGGRCQCRALGSGMAVDCST	2613	155
3	TNKMTVCSPDGPGGRCQCRALGSGMAVDCS	2482	156
4	MTVCSPDGPGGRCQCRALGSGMAVDCSTLT	2440	157
5	KMTVCSPDGPGGRCQCRALGSGMAVDCSTL	2423	158
6	CPTNKMTVCSPDGPGGRCQCRALGSGMAVD	2136	159
7	PTNKMTVCSPDGPGGRCQCRALGSGMAVDC	1723	160
8	CAGAFNH5DLDAELRRCHHILIDLRHRPTAGAC	1643	161
9	CTHHILIDLRHRPTAGC	1586	162
10	CVHYEQPTIQIELRQNCHHILIDLRHRPTAGAC	1504	163
11	CHHILIDLRHRPTAGACH5DLDAELRRLFRERC	1475	164
12	H5DLDAELRRLFRERCH5DLDAELRRLFRERC	1467	165
13	CDAELRRLFRERYRLHCH5DLDAELRRLFRERC	1462	166
14	CDAELRRLFRERYRLHCPK	1442	167
15	VCSPDGPGGRCQCRALG5GMAVDC5TLT5K	1432	168
16	DL5LRCDELVRTHHILIDLRHRPTAGAFNH	1421	169
17	CDELVRTHHILIDLRHCDLDAELRRLFRERYRC	1392	170
18	CFQGRGGLDLRVRGEPC	1376	171
19	CDAELRRLFRERYRLHCDELVRTHHILIDLRHC	1366	172
20	CGLDLRVRGEPLQVERCHHILIDLRHRPTAGAC	1342	173
21	CH5DLDAELRRLFRERCH5DLDAELRRLFRERC	1331	174
22	CDELVRTHHILIDLRHCHHILIDLRHRPTAGAC	1323	175
23	CDAELRRLFRERYRLHCDELVRTHHILIDLRHC	1266	176
24	CHHILIDLRHRPTAGACRGEPLQVERTLIYYLC	1229	177
25	CSPDGPGGRCQCRAL	1227	178
26	CTVASPDGPGGRAQARCVHYEQPTIQIELRQNC	1223	179
27	CHHILIDLRHRPTAGACVHYEQPTIQIELRQNC	1222	180
28	L5LRCDELVRTHHILIDLRHRPTAGAFNH5	1220	181
29	CDELVRTHHILIDLRHCH5DLDAELRRLFRERC	1205	182

T5-86 及び T6-16 抗体についての解析結果を下記表 10 及び表 11 に示す。当該解析では、DPEGRFKARQCN（アミノ酸番号 109-120）の配列を含むペプチドと強く結合した。T5-86 抗体に対しては結合する 26 ペプチド中 22 ペプチド（表 10 中に記載のペプチド No. 1-4, 6-8, 10-13, 15-19, 21-26）に、T6-16 抗体に対しては結合する 26 ペプチド中 4 ペプチド（表 11 中に記載のペプチド No. 1, 2, 9, 13）に、上記ペプチド配列が含まれていた（表 10 及び表 11）。また T5-86 抗体についての解析では、DPEGRFKARQCN（アミノ酸番号 109-120）を含む配列以外にも VCSPDGPGGRCQCRA（アミノ酸番号 43-57）を含む配列（表 10 中に記載ののペプチド No. 5, 14, 20）が複数回出現した。さらに、T6-16 抗体についての解析でも、他の配列として HHILIDLRHRPTAG（アミノ酸番号 152-165）を含む配列（表 11 中に記載のペプチド No. 4-8, 10-12, 19, 21,

23, 25, 26）が複数回確認された。従って、T5-86 抗体のエピトープとしては、hTROP-2 タンパク質中、DPEGRFKARQCN（アミノ酸番号 109-120）、VCSPDGPGGRCQCRA（アミノ酸番号 43-57）の 2 種類のペプチド配列領域が示唆され、T6-16 抗体のエピトープとしては、hTROP-2 タンパク質中、DPEGRFKARQCN（アミノ酸番号 109-120）、HHILIDLRHRPTAG（アミノ酸番号 152-165）の 2 種類のペプチド配列領域が示唆された。

表１０

ヒト TROP-2 細胞外領域に由来する CLIPS ペプチドに対する T5-86 抗体の結合

number	peptide	binding of T5-86	SEQ ID No.
1	CYDPDADPEGRFKARQCADPEGRFKARQANQTC	2306	183
2	PDCDPEGRFKARQCN	2292	184
3	CADPEGRFKARQANCPDADPEGRFKARQANC	2287	185
4	CPDADPEGRFKARQANCPDADPEGRFKARQANC	2263	186
5	VCSPDGPGGRCQCRA	2260	187
6	CYDPDADPEGRFKARQCPDADPEGRFKARQANC	2240	188
7	CADPEGRFKARQANQTCYDPDADPEGRFKARQC	2208	189
8	DCDPEGRFKARQCNQ	2150	190
9	CTVASPDGPGGRAQARCHSDLDAELRRLFRERC	2086	191
10	CDADPEGRFKARQANQCDADPEGRFKARQANQC	2035	192
11	CGRFKARQANQTSVAWCARTLVRPSEHALVDNC	2019	193
12	CADPEGRFKARQANQTCPDADPEGRFKARQANC	1980	194
13	CPDADPEGRFKARQANCPDADPEGRFKARQANC	1950	195
14	CSPDGPGGRCQCRAL	1946	196
15	CEGRFKARQANQTSVACEGRFKARQANQTSVAC	1895	197
16	CTVASPDGPGGRAQARCPDADPEGRFKARQANC	1890	198
17	CGLYDPDADPEGRFKACPDADPEGRFKARQANC	1857	199
18	DPDCDPEGRFKARQCNCQTSVCWCVNSVGVR	1850	200
19	CPEGRFKARQANQTSVCDELVRHHILIDLRHC	1841	201
20	CPDGPGGRAQARALGSCHSDLDAELRRLFRERC	1830	202
21	CTLVRPSEHALVDNDGCGRFKARQANQTSVAWC	1820	203
22	CPDADPEGRFKARQANCYDPDADPEGRFKARQC	1795	204
23	CGLYDPDADPEGRFKACPEGRFKARQANQT5VC	1793	205
24	YDPDCDPEGRFKARQ	1775	206
25	CPDADPEGRFKARQANCADPEGRFKARQANQTC	1773	207
26	CDPEGRFKARQCNQT	1772	208

表１１

ヒト TROP-2 細胞外領域に由来する CLIPS ペプチドに対する

T6-16 抗体の結合

number	peptide	binding of T6-16	SEQ ID No.
1	CVN5VGVRRTDKGDL5CYDPDADPEGRFKARQC	1072	209
2	C5VGVRRTDKGDL5LRCYDPDADPEGRFKARQC	786	210
3	H5DLDAELRRLFRERCH5DLDAELRRLFRERC	714	211
4	CDELVRTHHILIDLRHCDLDAELRRLFRERYRC	713	212
5	CVN5VGVRRTDKGDL5LRCDELVRTHHILI	688	213
6	VRRTDKGDL5LRCDELVRTHHILIDLRHRP	670	214
7	CVERTLIYYLDEIPPKCHHILIDLRHRPTAGAC	626	215
8	CHHILIDLRHRPTAGACH5DLDAELRRLFRERC	620	216
9	CVN5VGVRRTDKGDL5CPDADPEGRFKARQANC	611	217
10	CVHYEQPTIQIELRQNCHHILIDLRHRPTAGAC	602	218
11	VGVRRTDKGDL5LRCDELVRTHHILIDLRH	601	219
12	CAGAFNH5DLDAELRRCHHILIDLRHRPTAGAC	592	220
13	C5VGVRRTDKGDL5LRCPDADPEGRFKARQANC	585	221
14	CVRP5EHALVDNDGLYCSVGVRRTDKGDL5LRC	573	222
15	CDAELRRLFRERYRLHCH5DLDAELRRLFRERC	566	223
16	CSVGVRRTDKGDL5LRCNDGLYDPDADPEGRFC	559	224
17	CVN5VGVRRTDKGDL5CGLYDPDADPEGRFKAC	553	225
18	CDLDAELRRLFRERYRCH5DLDAELRRLFRERC	534	226
19	CDELVRTHHILIDLRHCHHILIDLRHRPTAGAC	534	227
20	CAGAFNH5DLDAELRRCDLDAELRRLFRERYRC	529	228
21	CDAELRRLFRERYRLHCDELVRTHHILIDLRHC	527	229
22	CVHYEQPTIQIELRQNCDLDAELRRLFRERYRC	526	230
23	CHHILIDLRHRPTAGACVHYEQPTIQIELRQNC	524	231
24	CGVRRTDKGDL5LRADCGVRRTDKGDL5LRADC	524	232
25	CGLDLRVRGEPLQVERCHHILIDLRHRPTAGAC	521	233
26	CDLDAELRRLFRERYRCDELVRTHHILIDLRHC	516	234

〔実施例３１〕

[マウス抗 hTROP-2 抗体（クローン K5-70, K5-107, K5-116-2-1 および T6-16）の抗体遺伝子の可変領域の配列決定]

3×10^6 個のマウス抗 TROP-2 モノクローナル抗体産生ハイブリドーマから、TRIzol 試薬（Invitrogen）を用いて全 RNA を抽出した。クローン K5-70, クローン K5-107 およびクローン K5-116-2-1 については、SMARTer™ RACE cDNA Amplification kit (Clontech)を用いて、キット添付の方法に従い、マウス IgG の H 鎖特異的プライマー (5'-TCCAKAGTTCCA-3' (配列番号２４))と、L 鎖特異的プライマー（5'-GCTGTCCTGATC-3' (配列番号２５)）を用いて cDNA を合成した。クローン T6-16 については、GeneRacer Kit（Invitrogen）を用いて、キット添付の方法に従い、オリゴ dT プライマーを用いて cDNA を合成した。

クローン K5-70 (マウス IgG2a), クローン K5-107 (マウス IgG1)、およびクローン K5-116-2-1 (マウス IgG2a)の H 鎖及び L 鎖の可変領域（VH, VL）をコードする遺伝子は、上記合成後の cDNA を鋳型として、PCR 法を用いてクローニングした。この際、5'-プライマーは SMARTer™ RACE cDNA Amplification kit に添付されている 10 × Universal Primer A Mix（UPM）を使用した。一方、3'-プライマーは、VH 増幅用 3'-プライマーとしてはマウス IgG H 鎖に特異的な配列を有するものを使用し、VL 増幅用 3'-プライマーとしてはマウス IgG L 鎖に特異的なな配列を有するものを使用した。

5'-プライマー（10 × Universal Primer A Mix（UPM））：

Long (0.4 μM)

5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT -3'（配列番号２６）

Short (2 μM)

5'- CTAATACGACTCACTATAGGGC-3'（配列番号２７）

3'-プライマー（R プライマー）：

VH： 5'-GGGAARTARCCCTTGACCAGGCA-3'（配列番号２８）

5'-GGGAARTAGCCTTTGACAAGGCA-3'（配列番号２９）

VL： 5'-CACTGCCATCAATVCTCCACTTGACA-3'（配列番号３０）

上記各プライマーを用いた PCR は、以下の反応液組成及び反応条件で行った。また VH の cDNA 増幅の R プライマーは上記の２配列を等モル数ずつ混合して使用した。

＜反応液組成＞

鋳型cDNA:	2.5μL
5×PrimeSTAR バッファー(Mg^{2+} plus):	10μL
2.5mM dNTP:	4μL
PrimeSTAR HS DNA ポリメラーゼ(2.5U/μL):	0.5μL
10 × Universal Primer A Mix (UPM):	5μL
Rプライマー (10μM):	1μL
滅菌水：	27μL
合計：	50μL

＜反応条件＞

94℃（10 sec）反応させた後、「熱変性･解離：98℃（10 sec）→アニーリング：60℃（5 sec）→合成･伸長：72℃（60 sec）」を1サイクルとして計 30 サイクル反応させた後、最後に 72℃（3 min）反応させた。

合成した VH 及び VL の cDNA を、pMD20-T vector (タカラバイオ)にサブクローニングして、塩基配列を決定した。複数の VH クローン及び VL クローンの塩基配列を解読し、マウス H 鎖及び L 鎖の可変領域に典型的な塩基配列を同定した。図 33 及び図 34 に、K5-70 の VH 及び VL のコンセンサス cDNA 塩基配列、ならびに推定アミノ酸配列を示した。図 35 及び図 36 に、K5-107 の VH 及び VL のコンセンサス cDNA 塩基配列、ならびに推定アミノ酸配列を示した。図 37 及び図 38 に、K5-116-2-1 の VH 及び VL のコンセンサス cDNA 塩基配列、ならびに推定アミノ酸配列を示した。

クローン T6-16 の H 鎖及び L 鎖の可変領域（VH, VL）をコードする遺伝子は、上記合成後の cDNA を鋳型として、PCR 法を用いてクローニングした。この際、この際、5'-プライマーは GeneRacer Kit に添付されているものを使用した。一方、3'-プライマーは、VH 増幅用 3'-プライマーとしてはマウス IgG H 鎖に特異的な配列を有するものを使用し、VL 増幅用 3'-プライマーとしてはマウス IgG L

鎖に特異的な配列を有するものを使用した。

5'-プライマー（F プライマー）：

5'-CGACTGGAGCACGAGGACACTGA-3'（配列番号３１）

3'-プライマー（R プライマー）：

VH: 5'- GCCAGTGGATAGACAGATGG -3'（配列番号３２）

VL: 5'- GATGGATACAGTTGGTGCAGC -3'（配列番号３３）

上記各プライマーを用いた PCR は、以下の反応液組成及び反応条件で行った。

＜反応液組成＞

鋳型cDNA：	1.0μL
5×PrimeSTAR バッファー(Mg^{2+} plus)：	10μL
2.5mM dNTP：	4μL
PrimeSTAR HS DNA ポリメラーゼ(2.5U/μL)：	0.5μL
Fプライマー (10μM)：	3μL
Rプライマー (10μM)：	1.0μL
滅菌水：	30.5μL
合計：	50μL

＜反応条件＞

「熱変性･解離：98℃（10 sec）→アニーリング：57℃（10 sec）→合成･伸長：72℃（60 sec）」を 1 サイクルとして計 35 サイクル。

合成した VH 及び VL の cDNA を、pCR4Blunt-TOPO vector (Invitrogen)にサブクローニングして、塩基配列を決定した。複数の VH クローン及び VL クローンの塩基配列を解読し、マウス H 鎖及び L 鎖の可変領域に典型的な塩基配列を同定した。図 39 及び図 40 に、T6-16 の VH 及び VL のコンセンサス cDNA 塩基配列、ならびに推定アミノ酸配列を示した。

〔実施例３２〕

[ヒト型化 K5-70 抗体のデザイン]

先の実施例で作製した K5-70 抗体について、その可変領域（VH, VL）のヒト型化を、Queen らの方法（Proc. Natl. Acad. Sci. USA 86: 10029-10033, 1989）に従って以下のように行った。まず、K5-70 抗体可変領域の立体構造の分子モデリングをコンピュータで行い、次いで、ヒト抗体遺伝子の可変領域配列とのホモロジー検索によって、K5-70 VH のヒト型化に必要なフレームワーク（FR）領域を提供するアクセプターとして GenBank accession number が DA980102 の cDNA 配列 (DA980192 VH)を選択した(Genome Res. 16:55-65, 2006)。同様に、K5-70 VL のヒト型化に必要なフレームワーク（FR）領域を提供するアクセプターとして GenBank accession number が L41174 の cDNA 配列(L41174 VL)を選択した(J. Biol. Chem. 270:12457-12465, 1995)。

K5-70 VH のヒト型化では、K5-70 VH の CDR 配列をアクセプターである DA980102 VH の対応する位置に置き換えて挿入した。コンピュータモデリングによる立体構造解析により、K5-70 VH の CDR に隣接し、その構造維持に重要な役割を果たしていると考えられるアミノ酸残基（48 番目のイソロイシン (I)、66 番目のリジン(K)、67 番目のアラニン(A)、71 番目のバリン(V)、93 番目のスレオニン(T)）については K5-70 VH のものを保持し、それ以外の FR 領域をアクセプター配列に置き換えた。VH および VL 内のアミノ酸残基位置番号は Kabat らの定義（Sequences of Proteins of Immunological Interests, Fifth edition, NIH Publication No. 91-3242, U.S. Department of Health and Human Services, 1991）に準拠した。

また、アクセプター配列 DA980102 VH の 82 番目のアミノ酸残基であるメチオニン（M）は、somatic hypermutation による特殊なアミノ酸残基である可能性が高いと考えられる。そこで潜在的抗原性を小さくするために、82 番目のアミノ酸残基として最も一般的なロイシン（L）に置き換えた。上述の通りデザインしたヒト型化 K5-70 VH（HuK5-70 VH）、K5-70 VH および DA980102 VH のアミノ酸配列アライメントを図 41 に示した。

ヒト型化 K5-70 VL のデザインついても同様に CDR 配列の移植を行い、CDR の構造維持に重要なアミノ酸残基（49 番目のリジン(K)）は K5-70 VL のものを保持し、それ以外の FR 領域をアクセプター配列に置き換えた（HuK5-70 VL）。HuK5-70 VL、K5-70 VL および L41174 VL のアミノ酸配列アライメントを図 42 に示した。

〔実施例３３〕

[ヒト型化 T6-16 抗体のデザイン]

先の実施例で作製した T6-16 抗体について、その可変領域（VH, VL）のヒト型化を、Queen らの方法（Proc. Natl. Acad. Sci. USA 86: 10029-10033, 1989）に従って以下のように行った。まず、T6-16 抗体可変領域の立体構造の分子モデリングをコンピュータで行い、次いで、ヒト抗体遺伝子の可変領域配列とのホモロジー検索によって、T6-16 VH のヒト型化に必要なフレームワーク（FR）領域を提供するアクセプターとして GenBank accession number が DA935238 の cDNA 配列（DA935238 VH）を選択した(Genome Res. 16:55-65, 2006) 。同様に、T6-16 VL のヒト型化に必要なフレームワーク（FR）領域を提供するアクセプターとして GenBank accession number が M99608 の cDNA 配列(M99608 VL)を選択した(J. Immunol. 149:2518-2529, 1992)。

T6-16 VH のヒト型化では、T6-16 VH の CDR 配列をアクセプターである DA935238 VH の対応する位置に置き換えて挿入した。コンピュータモデリングによる立体構造解析により、T6-16 VH の CDR に隣接し、その構造維持に重要な役割を果たしていると考えられるアミノ酸残基（48 番目のイソロイシン (I)、67 番目のアラニン (A)、73 番目のリジン (K)）については T6-16 VH のものを保持し、それ以外の FR 領域をアクセプター配列に置き換えた（HuT6-16 VH1）。また 73 番目のリジン残基（K）については、抗原結合部位の適切な形成に対する影響が少ない可能性があり、HuT6-16 VH1 のリジン残基をより寛容なアミノ酸残基であるスレオニン（T）に置き換えたアミノ酸配列を別途デザインした（HuT6-16 VH2）。デザインしたヒト型化 T6-16 VH（HuT6-16 VH1 および HuT6-16 VH2）、T6-16 VH および DA980102 VH のアミノ酸配列アライ

メントを図 43 に示した。

ヒト型化 T6-16 VL のデザインについても、同様に CDR 配列の移植を行った。CDR の構造維持に重要なアミノ酸残基はアクセプター配列でも保持されており、FR 配列はアクセプター配列と同じ配列を用いた（HuT6-16 VL）。HuT6-16 VL、T6-16 VL および L41174 VL のアミノ酸配列アライメントを図 44 に示した。

〔実施例３４〕

[ヒト型化 K5-70 VH および VL 遺伝子合成]

HuK5-70 VH および HuK5-70 VL をコードする遺伝子は以下のように作製した。上述のようにデザインした HuK5-70 VH および VL の N 末端側に、それぞれ K5-70 VH、VL 由来のシグナルペプチド配列を付加したアミノ酸配列をもとに遺伝子合成を行った（Operon 社）。その際、それぞれ合成した HuK5-70 VH および HuK5-70 VL の遺伝子配列の 5'末端側に Kozak 配列（ACC ACC）を付加し、また HuK5-70 VH の 5'末端には、制限酵素部位として EcoRI の部位（GAA TTC）を付加し、3'末端には NheI（GCT AGC）の部位を付加した。同様に、HuK5-70 VL の 5'末端には、制限酵素部位として Age I の部位（ACC GGT）を付加し、3'末端には BsiWI の部位（CGT ACG）を付加した。合成した HuK5-70 VH 遺伝子、および HuK5-70 VL 遺伝子は、pCR2.1 ベクター（Invitrogen）に TA クローニングにより組み込んだ。遺伝子合成により作製した HuK5-70 VH および VL の遺伝子配列をそれぞれ図 45 及び 46 に示した。

〔実施例３５〕

[ヒト型化 T6-16 VH1、T6-16 VH2 および T6-16 VL 遺伝子合成]

HuT6-16 VH1、HuT6-16 VH2 および HuT6-16 VL をコードする遺伝子は以下のように作製した。デザインした HuT6-16 VH1、HuT6-16 VH2 の N 末端側に T6-16 VH 由来のシグナルペプチド配列を、HuT6-16 VL の N 末端側に T6-16 VL 由来のシグナルペプチド配列をそれぞれ付加したアミノ酸配列をもとに遺伝子合成を行った（Operon 社）。その際、それぞれ合成した HuT6-16 VH1、

HuT6-16 VH2 および HuT6-16 VL の遺伝子配列の 5'末端側に Kozak 配列（ACC ACC）を付加し、また HuT6-16 VH1 および HuT6-16 VH2 の 5'末端には、制限酵素部位として EcoRI の部位（GAA TTC）を付加し、3'末端には NheI の部位（GCT AGC）を付加した。同様に、ヒト型化 T6-16 VL の 5'末端には、制限酵素部位として Age I の部位（ACC GGT）を付加し、3'末端には BsiWI の部位（CGT ACG）を付加した。合成した HuT6-16 VH1 遺伝子および HuT6-16 VH2 遺伝子ならびに HuT6-16 VL 遺伝子は、pCR2.1 ベクター（Invitrogen）に TA クローニングにより組み込んだ。

遺伝子合成により作製した HuT6-16 VH1、HuT6-16 VH2 および HuT6-16 VL の遺伝子配列をそれぞれ図 47～49 に示した。

〔実施例３６〕

[ヒト型化 K5-70 VH および VL 遺伝子発現ベクター構築]

pCR2.1 ベクター（Invitrogen）に組み込まれた HuK5-70 VH および VL 遺伝子は、それぞれ制限酵素 EcoRI 及び Nhe I、Age I 及び BsiWI によって消化し、遺伝子断片の回収を行った。次に、切り出した HuK5-70 VH 遺伝子はヒト IgG1 フォームの発現用の動物細胞発現ベクターである、pFUSE-CHIg-hG1 ベクター（InvivoGen）の EcoRI/Nhe I 部位に挿入し（pFUSE-CHIg-HuK5-70）、HuK5-70 VL 遺伝子はヒト Ig κフォーム発現用ベクターである pFUSE2-CLIg-hk ベクター（InvivoGen）の Age I/BsiWI 部位に挿入して（pFUSE2-CLIg-HuK5-70）、それぞれ、コンストラクトを完成させた。

〔実施例３７〕

[ヒト型化 T6-16 VH1、T6-16 VH2 および T6-16 VL 遺伝子発現ベクター構築]

pCR2.1 ベクター(Invitrogen)に組み込まれた T6-16 VH1、T6-16 VH2 遺伝子は制限酵素 EcoRI 及び Nhe I で消化し、T6-16 VL 遺伝子は Age I 及び BsiWI で消化し、遺伝子断片の回収を行った。次に、切り出した T6-16 VH1 遺伝子は pFUSE-CHIg-hG1 ベクター（InvivoGen）の EcoRI/Nhe I 部位に挿入し（pFUSE-CHIg-HuT6-16-1）、T6-16 VH2 遺伝子も同様に pFUSE-CHIg-hG1

ベクター（InvivoGen）の EcoRI/Nhe I 部位に挿入し（pFUSE-CHIg-HuT6-16-2）、T6-16 VL 遺伝子は pFUSE2-CLIg-hk ベクター（InvivoGen）の Age I/BsiWI 部位にに挿入して（pFUSE2-CLIg-HuT6-16）、それぞれ、コンストラクトを完成させた。

〔実施例３８〕

[HuK5-70 抗体、HuT6-16-1 抗体および HuT6-16-2 抗体を安定的に発現する 293F 細胞株の樹立]

293F 細胞（Invitrogen）は、FreeStyle 293 Expression Medium（Invitrogen）で維持、培養した。293F 細胞への遺伝子導入は 293 fectin 試薬（Invitrogen）を用い、添付のプロトコルに従い行った。pFUSE-CHIg-HuK5-70 および pFUSE2-CLIg-HuK5-70 を共に 293F 細胞へ導入し、Zeocin（InvivoGen）および Blasticidin（InvivoGen）による薬剤選択を行うことで HuK5-70 抗体を安定的に発現する細胞株を樹立した。同様に、pFUSE-CHIg-HuT6-16-1 および pFUSE2-CLIg-HuT6-16 を共に 293F 細胞へ導入し、上記の薬剤選択を行うことで HuT6-16-1 抗体を安定的に発現する細胞株を樹立した。また同様に、pFUSE-CHIg-HuT6-16-2 および pFUSE2-CLIg-HuT6-16 を共に 293F 細胞へ導入し、上記の薬剤選択を行うことで HuT6-16-2 抗体を安定的に発現する細胞株を樹立した。

〔実施例３９〕

[HuK5-70 抗体、HuT6-16-1 抗体および HuT6-16-2 抗体タンパク質の精製]

樹立した各抗体発現細胞株は 1～2.5 x 10^5 cells/ml で FreeStyle 293 Expression Medium（Invitrogen）に接種し、6～8 日間ローラーボトル培養を行った。培養上清を回収した後、rProtein A Sepharose Fast Flow（GE Healthcare）を用いて定法に従い各ヒト型化抗体の精製を行った。

図 50 に、HuK5-70 抗体、HuT6-16-1 抗体及び HuT6-16-2 抗体を発現させた 293F 細胞の培養上清中の、各ヒト型化抗体タンパク質の発現をウエスタンブロットで確認した図を示した。具体的には、SDS-PAGE を行った後、PVDF メン

ブレン (Immobilon-P, Millipore, IPVH00010)に転写した。メンブレンは5%スキムミルクを含むTBS (Tris-buffered saline)を用いて室温、30分間ブロッキングを行った。0.1%TBST (0.1% Tween 20含有TBS)で5分間、3回洗浄した後、1次抗体との反応を行った。

レーン1は遺伝子導入を行っていない293F細胞の培養上清（ネガティブコントロール）、レーン2はpFUSE-CHIg-HuK5-70およびpFUSE2-CLIg-HuK5-70を導入した293F細胞の培養上清を示す。HuK5-70抗体の重鎖および軽鎖タンパク質の検出はビオチン標識した抗ヒトIgG F(ab')^{2}抗体（Rockland）を用いた。レーン3は293F細胞にpFUSE-CHIg-HuT6-16-1およびpFUSE2-CLIg-HuT6-16を導入した293F細胞の上清、レーン4はpFUSE-CHIg-HuT6-16-2およびpFUSE2-CLIg-HuT6-16を導入した293F細胞の上清を示し、HuT6-16-1抗体およびHuT6-16-2抗体の重鎖タンパク質はビオチン標識した抗ヒトIgG Fc抗体（Rockland）で、HuT6-16-1抗体およびHuT6-16-2抗体の軽鎖タンパク質はビオチン標識した抗ヒトIgG F(ab')^{2}抗体（Rockland）でそれぞれ検出した。

その結果、HuK5-70抗体、HuT6-16-1抗体及びHuT6-16-2抗体のいずれにおいても、重鎖および軽鎖タンパク質の培養上清中での発現が確認された。

また、図51に、精製したHuK5-70抗体、HuT6-16-1抗体及びHuT6-16-2抗体を、SDS-PAGEで展開後、CBB染色した図を示した。還元条件下で、いずれも約50kDの重鎖と約25kDの軽鎖が検出され、上述したウエスタンブロットで検出された各重鎖および軽鎖と同じ位置にバンドが確認された。以上の結果から、HuK5-70抗体タンパク質、HuT6-16-1抗体タンパク質及びHuT6-16-2抗体タンパク質の産生が確認された。

〔実施例４０〕

[ヒト型化K5-70抗体（HuK5-70）及びヒト型化T6-16抗体（HuT6-16-1, HuT6-16-2）の抗原親和性]

精製したHuK5-70抗体、HuT6-16-1抗体及びHuT6-16-2抗体の抗原親和性をFACS、及びELISAを用いた方法で検討した。

FACSは、実施例5に記載したHEK293細胞にヒト全長TROP-2遺伝子を安定的に発現させたHEK293-hTROP-2細胞と内在的にヒトTROP-2タンパク質を細胞表面に発現している膵臓癌細胞株PK-59を用いて行った。トリプシン処理によって培養皿から剥がした細胞（HEK293-hTROP-2細胞、又はPK-59細胞）の懸濁液（5 x 10^5 cells）に対し、1次抗体として10% FCSを含む培地で1μg/mlに希釈した抗体溶液100μl加え、4℃で20分間incubationした。その後、1mlの10% FCSを含む培地で洗浄し、フィコエリスリン（PE）標識anti-mouse IgG抗体（BD Pharmingen）およびビオチン標識anti-human IgG Fc抗体（Rockland）をそれぞれ200倍、2000倍希釈した2次抗体溶液100μlを加えた。4℃で20分間incubationした後、再び1mlの10% FCSを含む培地で洗浄した。2次抗体としてビオチン標識anti-human IgG Fc抗体を用いた場合は、蛍光標識試薬としてストレプトアビジン標識PE（BD Pharmingen）を400倍希釈した標識溶液を100μl加え、4℃で20分間incubationした後、1mlの10% FCSを含む培地で洗浄した。その後、標識細胞を含むサンプルは1mlの1% FCS、2mM EDTA入りのPBSに懸濁し、FACSCalibur (Becton Dickinson)を用いて解析した。その結果、HEK293-hTROP-2細胞においても、PK-59細胞においても、HuK5-70抗体は、マウスK5-70抗体と同等の反応性を示した。同様にHuT6-16-1抗体およびHuT6-16-2抗体はT6-16抗体と同等の反応性を示した（図52）。

更にELISA法により抗原親和性について検証した。ELISAは、実施例3で示したhTROP-2細胞外領域のリコンビナントタンパク質を固相化したELISAプレートを使用して行った。具体的には、96 well plate（BD FALCON）をPBSで0.5μg/mlに希釈したhTROP-2細胞外領域のリコンビナントタンパク質を50μl/wellでコーティングした（4℃、一晩）。洗浄バッファー（0.05% Tween 20を含むPBS）で洗浄後、ブロッキングバッファー（2%スキムミルク、0.05% Tween 20を含むPBS）を200μl/wellで添加しブロッキングを行った（室温、1時間）。洗浄バッファーで洗浄した後、HuK5-70抗体、HuT6-16-1抗体、HuT6-16-2抗体、K5-70抗体およびT6-16抗体をELISAバッファー（1%スキムミルク、0.025% Tween 20を含むPBS）で3.05 x 10^{-4}～5μg/mlの

範囲で 2 倍希釈系列サンプルを調製し、各 50μl/well で添加した（室温、2 時間）。洗浄バッファーで洗浄した後、検出用抗体として ELISA バッファーで 2000 倍希釈した HRP 標識 goat 抗ヒトκ鎖抗体（SouthernBiotech）もしくは 2000 倍希釈した HRP 標識 sheep 抗マウス IgG 抗体（GE Healthcare）を 50μl/well で添加した（室温、1 時間）。洗浄バッファーで洗浄後、TMB（3,3',5,5'-tetramethylbenzidine、SIGMA）を基質溶液として 50μl/well で添加し、発色反応を行った。1M 硫酸を 25μl/well で加え反応を停止させた後、Microplate reader Model 550 （BioRad）を用いて、655nm の吸光度をリファレンスとして 450nm の吸光度を測定した。その結果、K5-70 および HuK5-70 の反応曲線はほぼ重なり、EC50 はぞれぞれ 27ng/ml、22ng/ml であった（図 53）。同様に、T6-16、HuT6-16-1 及び HuT6-16-2 の反応曲線もほぼ重なり、EC50 はそれぞれ 30ng/ml、27ng/ml、27ng/ml であった（図 54）。これらの結果から HuK5-70 抗体、HuT6-16-1 抗体および HuT6-16-2 抗体は、それぞれ、ヒト型化する前の親抗体である K5-70 又は T6-16 抗体と同等の抗原親和性を示すことが明らかとなった。

〔実施例４１〕

[ヒト型化抗 hTROP-2 抗体（HuK5-70）の *in vivo* における抗腫瘍活性]

次に、HuK5-70抗体の*in vivo*における抗腫瘍活性を、内在的にヒトTROP-2を細胞表面に発現するヒト大腸癌細胞株SW480を用いたxenograft treatmentモデルで検討した。5×10^6 cellsのSW480細胞を7週齢雌のNOD-scidマウスの右脇腹皮下に移植（Day 1）し、平均の腫瘍体積が100 mm^3 に達した段階で群分けを行い（Day 9），Day 9から3日に1回の投与間隔で、抗体の腹腔内投与を開始した。癌細胞移植から39日目（Day 39）におけるコントロール群（PBS投与, N=8）の腫瘍体積が824.3±188.8 mm^3であったのに対して、HuK5-70抗体投与群 (10 mg/kg 体重, N=8)では、455.5±208.6 mm^3 ($P < 0.01$ by Student's t-test)であり有意に腫瘍形成が阻害された（阻害率44.7 %）（図55A）。腫瘍重量においては、コントロール群が0.509±0.161 g であったのに対して、HuK5-70抗体投与群では0.272±0.162 g ($P < 0.05$ by Student's t-test)であり46.6 %の阻

害率であった（図55B）。

〔実施例４２〕

［ヒト型化抗 hTROP-2 抗体（HuK5-70 及び HuT6-16-2）のヒト大腸癌細胞株 SW480 細胞 xenograft treatment モデルにおける用量依存的な抗腫瘍活性］

HuK5-70 及び HuT6-16-2 抗体の用量依存的な抗腫瘍活性を、ヒト大腸癌細胞株 SW480 を用いた xenograft treatment モデルにおいて検討した。5 × 10^6 cells の SW480 細胞を 7 週齢雌の NOD-scid マウスの右脇腹皮下に移植（Day 1）し、癌細胞移植から 9 日目（Day 9）に平均の腫瘍体積が 100 mm^3 に達した段階でコントロール群（PBS 投与群、N=8, 101.65±8.35 mm^3），1 mg/kg 体重の HuK5-70 抗体投与群 (N=8, 103.18±9.86 mm^3), 5 mg/kg 体重の HuK5-70 抗体投与群（N=8, 101.34±8.94 mm^3），10 mg/kg 体重の HuK5-70 抗体投与群（N=8, 101.53±8.98 mm^3），1 mg/kg 体重の HuT6-16-2 抗体）投与群 (N=8, 103.18±9.86 mm^3), 5 mg/kg 体重の HuT6-16-2 抗体投与群（N=8, 101.34±8.94 mm^3），10 mg/kg 体重の HuT6-16-2 抗体投与群（N=8, 101.53±8.98 mm^3）に群分を行い、Day 9 より 3 日に 1 回の間隔で抗体の腹腔内投与を行った。癌細胞移植から 48 日目(Day 48)におけるコントロール群の腫瘍体積が 754.67±276.05 mm^3 であったのに対して、HuK5-70 抗体投与群では、1 mg/kg 体重投与群では、521.81±183.45 mm^3 (阻害率 30.9%), 5 mg/kg 体重投与群では、258.78±137.02 mm^3 (阻害率 65.7%, $P < 0.01$ by Student's t-test), 10 mg/kg 体重投与群では、314.60±152.89 mm^3 (阻害率 58.3 %, $P < 0.01$ by Student's t-test)であった（図 56A)。HuT6-16-2 抗体投与群では、1 mg/kg 体重投与群では、600.41±319.84 mm^3 (阻害率 20.4%), 5 mg/kg 体重投与群では、315.32±189.02 mm^3 (阻害率 58.2%, $P < 0.01$ by Student's t-test), 10 mg/kg 体重投与群では、270.79±266.71 mm^3 (阻害率 64.1 %, $P < 0.01$ by Student's t-test)であった（図 57A)。Day 48 における腫瘍重量については、コントロール群が 0.422±0.201 g であったのに対して、HuK5-70 抗体投与群では、 1 mg/kg 体重投与群で, 0.301±0.160 g (阻害率 28.7%), 5 mg/kg 体重投与群で、0.115±0.083 g (阻害率 72.7%, $P < 0.01$ by Student's t-test), 10 mg/kg 体重投与群で、

0.244± 0.181 g (阻害率 42.2%) となった（図 56B)。HuT6-16-2 抗体投与群では、1 mg/kg 体重投与群で, 0.422±0.255 g (阻害率 0%), 5 mg/kg 体重投与群で、0.247±0.151 g (阻害率 41.5%), 10 mg/kg 体重投与群で、0.190±0.190 g (阻害率 53.1%, P < 0.01 by Student's t-test) となった（図 57B)。これらの結果から、HuK5-70 及び HuT6-16-2 抗体の用量依存的な抗腫瘍活性が確認された。

〔実施例４３〕

［抗 hTROP-2 マウスモノクローナル抗体 K5-70 及び T6-16 のヒト卵巣癌細胞株 SK-OV-3 細胞 xenograft treatment モデルにおける抗腫瘍活性］

親抗体である K5-70 及び T6-16 抗体の *in vivo* における抗腫瘍活性を、内在的に hTROP-2 を細胞表面に発現するヒト卵巣癌細胞株 SK-OV-3 を用いた xenograft treatment モデルで検討した。5 × 10^6 cells の SK-OV-3 細胞を 7 週齢雌のヌードマウスの右脇腹皮下に移植（Day 1）し、癌細胞移植から 11 日目（Day 11)に、明らかな腫瘍形成が確認された個体（平均の腫瘍体積約 50 mm^3 ）について群分けを行い, Day 11 から週に 2 回の投与間隔で、抗体の腹腔内投与を行った。癌細胞移植から 56 日目（Day 56）におけるコントロール群（PBS 投与, N=8）の腫瘍体積が 652.6±349.1 mm^3 であったのに対して、K5-70 抗体投与群 (10 mg/kg 体重, N=8)では、253.7±137.3 mm^3 (P < 0.01 by Student's t-test)と有意に腫瘍形成が阻害され（阻害率 61.1 %）、T6-16 抗体投与群(10 mg/kg 体重, N=8)では、214.6±98.6 mm^3 (P < 0.01 by Student's t-test)と有意に腫瘍形成が阻害された（阻害率 67.1 %）（図 58A）。腫瘍重量においては、コントロール群が 0.413±0.218 g であったのに対して、K5-70 抗体投与群では 0.194±0.112 (g) (P < 0.05 by Student's t-test)と 53.0 %の阻害率、T6-16 抗体投与群では 0.183±0.093 (g) (P < 0.05 by Student's t-test)と 55.7 %の阻害率であった（図 58B）。

〔実施例４４〕

［抗 hTROP-2 マウスモノクローナル抗体 K5-70 及び T6-16 のヒト乳癌細胞株 MDA-MB-468 細胞 xenograft treatment モデルにおける抗腫瘍活性］

同様に、K5-70 ならびに T6-16 抗体の *in vivo* における抗腫瘍活性を、内在的に hTROP-2 を細胞表面に発現するヒト乳癌細胞株 MDA-MB-468 を用いた xenograft treatment モデルで検討した。5 × 10^6 cells の MDA-MB-468 細胞を 7 週齢雌のヌードマウスの右脇腹皮下に移植（Day 1）し、癌細胞移植から 12 日目(Day 12）に、明らかな腫瘍形成が確認された個体（平均の腫瘍体積約 50 mm^3 ）について群分けを行い, Day 12 から週に 2 回の投与間隔で、抗体の腹腔内投与を行った。癌細胞移植から 54 日目（Day 54）におけるコントロール群（PBS 投与, N=8）の腫瘍体積が 218.6±75.5 mm^3 であったのに対して、K5-70 抗体投与群(10 mg/kg 体重, N=8)では、70.2±37.4 mm^3 ($P < 0.01$ by Student's t-test)と有意に腫瘍形成が阻害され（阻害率 67.9 %）、T6-16 抗体投与群投与群(10 mg/kg 体重, N=8)では、88.3±42.9 mm^3 ($P < 0.01$ by Student's t-test)と有意に腫瘍形成が阻害された（阻害率 59.6 %）（図 59A）。Day 54 における腫瘍重量においては、コントロール群が 0.142±0.049 g であったのに対して、K5-70 抗体投与群では 0.050±0.033 (g) ($P < 0.01$ by Student's t-test)と 64.8 %の阻害率、T6-16 抗体投与群では 0.077±0.046 (g) ($P < 0.05$ by Student's t-test)と 45.8 %の阻害率であった（図 59B）。

〔実施例４５〕

［抗 hTROP-2 マウスモノクローナル抗体 K5-70 及び T6-16 のヒト肺癌細胞株 Calu-3 細胞 xenograft treatment モデルにおける抗腫瘍活性］

同様に、K5-70 抗体の *in vivo* における抗腫瘍活性を、内在的に hTROP-2 を細胞表面に発現するヒト肺癌細胞株 Calu-3 を用いた xenograft treatment モデルで検討した。5 × 10^6 cells の Calu-3 細胞を 7 週齢雌のヌードマウスの右脇腹皮下に移植（Day 1）し、癌細胞移植から 9 日目(Day 9)に、明らかな腫瘍形成が確認された個体（平均の腫瘍体積約 100 mm^3 ）について群分けを行い, Day 9 から週に 2 回の投与間隔で、抗体の腹腔内投与を行った。癌細胞移植から 41 日目（Day 41）におけるコントロール群（PBS 投与, N=8）の腫瘍体積が 395.7 ±221.2 mm^3 であったのに対して、K5-70 抗体投与群(10 mg/kg 体重, N=8)では、120.7±125.6 mm^3 ($P < 0.01$ by Student's t-test)と有意に腫瘍形成が阻害さ

れ（阻害率 69.5 %）、T6-16 抗体投与群投与群(10 mg/kg 体重, N=8)では、146.3 ± 128.4 mm^3 (P < 0.05 by Student's t-test) と有意に腫瘍形成が阻害された（阻害率 63.0%）（図 60A）。腫瘍重量においては、コントロール群が 0.301 ±0.189 g であったのに対して、K5-70 抗体投与群では 0.08±0.085 (g) (P < 0.01 by Student's t-test)と 73.5 %の阻害率、T6-16 抗体投与群では 0.106 ± 0.096 (g) (P < 0.05 by Student's t-test)と 64.9 %の阻害率であった（図 60B）。

〔実施例４６〕

［抗 hTROP-2 マウスモノクローナル抗体 K5-70 のヒト胆管癌細胞株 TFK-1 細胞 xenograft prevention モデルにおける抗腫瘍活性］

同様に、K5-70 抗体の *in vivo* における抗腫瘍活性を、内在的に hTROP-2 を細胞表面に発現するヒト胆管癌細胞株 TFK-1 を用いた xenograft preventiont モデルで検討した。5 × 10^6 cells の TFK-1 細胞を 7 週齢雌のヌードマウスの右脇腹皮下に移植（Day 1）し、同日より、週に 2 回の投与間隔で、抗体の腹腔内投与を開始した。癌細胞移植から 31 日目（Day 31）におけるコントロール群（PBS 投与, N=5）の腫瘍体積が 1000.4±268.9 mm^3 であったのに対して、K5-70 抗体投与群(10 mg/kg 体重, N=5)では、197.2±215.5 mm^3 (P < 0.01 by Student's t-test)と有意に腫瘍形成が阻害された（阻害率 80.3 %）（図 61A）。腫瘍重量においては、コントロール群が 0.443±0.070 g であったのに対して、K5-70 抗体投与群では 0.063±0.052 (g) (P < 0.01 by Student's t-test)と 85.8 %の阻害率であった（図 61B）。

〔実施例４７〕

［HuK5-70 および HuT6-16-2 抗体の avidity 解析］

HuK5-70 および HuT6-16-2 抗体の抗原結合活性について、低密度の抗原を固相化した 96 well plate を用いた ELISA 法（低密度抗原固相化 ELISA）により検討した。96 穴プレートに 0.1M 酢酸バッファー(pH5.3)で 0.1 μg/mL に調製したリコンビナント hTACSTD2-Fc-His タンパク(Creative BioMart)を 50 μL/ウェルで添加し、4℃で一晩コーティングした後、実施例４０と同様の解析をおこ

なった。被験抗体は ELISA バッファー（1%スキムミルク、0.025% Tween 20 を含む PBS）で 20μg/mL から 2 倍希釈系列（15 点）を作製し使用した。その結果、HuT6-16-2 抗体は T6-16 抗体とほぼ同等の結合活性であるのに対し（EC50 値はそれぞれ 49ng/mL と 41ng/mL）、HuK5-70 抗体の EC50 値は K5-70 抗体の約 20 倍となった（それぞれ 222ng/mL、12ng/mL、図 62）。

次に HuK5-70 および K5-70 抗体の抗原結合活性について、、一価の抗原一抗体反応を解析する ELISA により検討した。 96 穴プレートに 0.1M 酢酸バッファー(pH5.3)で 1μg/mL に希釈した Goat anti-human IgG (Fcγ specific) (Southern Biotech)および 3μg/mL に希釈した Goat anti-mouse IgG (γ chain specific) (Southern Biotech)を 50μL/ウェルで添加し、4℃で一晩コーティングした。洗浄バッファー（0.05% Tween 20 を含む PBS）で洗浄後、ブロッキングバッファー（2%スキムミルク、0.05% Tween 20 を含む PBS）を 200μL/ウェルで添加しブロッキングを行った（室温、1 時間）。洗浄バッファーで洗浄した後、ELISA バッファー（1%スキムミルク、0.025% Tween 20 を含む PBS）で 1μg/mL 希釈した試験抗体を 50μL/ウェルで添加した。この際、Goat anti-human IgG (Fcγ specific)をコートしたウェルには HuK5-70 抗体を添加し、Goat anti-mouse IgG (γ chain specific)をコートしたウェルには K5-70 抗体を添加した。室温で 1 時間静置した後、洗浄バッファーで洗浄し、実施例 3 で示した hTROP-2 細胞外領域のリコンビナントタンパク質 (hTROP-2 EC)を ELISA バッファーで 5μg/mL から 3 倍希釈系列（10 点）を作製し、各 50μL/ウェルで添加した。室温で 1 時間静置した後、洗浄バッファーで洗浄し、1 次抗体として ELISA バッファーで 2μg/mL に希釈した anti-His (G-18) (Santa Cruz) を 50μL/ウェルで添加した。室温で 1 時間静置した後、洗浄バッファーで洗浄し、2 次抗体として ELISA バッファーで 1000 倍希釈した HRP 標識 anti-rabbit IgG (GE Healthcare) を 50μL/ウェルで添加した。室温で 1 時間静置した後、洗浄バッファーで洗浄し、TMB（3,3',5,5'-tetramethylbenzidine, SIGMA）を基質溶液として 50μL/ウェルで添加し、発色反応を行った。1M 硫酸を 25μL/ウェルで加え反応を停止させた後、Microplate reader Model 550（Bio Rad）を用いて、655nm の吸光度をリファレンスとして 450nm の吸光度を測定した。

その結果、hTROP-2EC タンパク質の K5-70 および HuK5-70 抗体との結合カーブから算出される EC50 はそれぞれ 7ng/mL、6ng/mL となった（図 63）。このことから一価の抗原一抗体反応においては HuK5-70 抗体と K5-70 抗体は同等の抗原親和性（アフィニティー）であることが示された。

実施例４０で示したとおり、高密度の抗原(0.5μg/mL)を固相化した 96 well plate を用いた ELISA 法（抗原固相化 ELISA）では K5-70 抗体と HuK5-70 抗体の抗原親和性が同等であったことから（図 53）、低密度の抗原（0.1μg/mL）を固相化した ELISA 法で HuK5-70 抗体の抗原結合活性が K5-70 抗体と比べて相対的に低い原因として、HuK7-50 抗体では 2 つの抗原結合アームの運動能に関するフレキシビィリティー、いわゆる“アビディティー（Avidity）”が、K5-70 抗体と比べて相対的に低いことが考えられた。

〔実施例４８〕

［ヒト型化 K5-70 抗体の変異体の作製とキャラクラリゼーション］

HuK5-70 抗体の“アビディティー（Avidity）”を向上させることを目的として、以下の実験を行った。

前述のとおり HuK5-70 抗体の“アビディティー（Avidity）”が相対的に低いことが、VH と VL のどちらに起因するのかを以下の実験で検討した。まず、親抗体である K5-70 抗体の H 鎖可変領域 (K5-70 VH),および L 鎖可変領域（K5-70 VL）をコードする遺伝子を遺伝子合成により作製した（Operon 社）。その際、K5-70 VH および K5-70 VL の遺伝子配列の 5'末端側に Kozak 配列（ACC ACC）を付加した。さらに K5-70 VH の 5'末端には制限酵素部位として EcoRI の部位(GAA TTC)を、3'末端には NheI の部位(GCT AGC)を付加した。同様に、K5-70 VL の 5'末端には制限酵素部位として Age I の部位(ACC GGT)を、3'末端には BsiWI の部位(CGT ACG)を付加した。合成した K5-70 VH 遺伝子、および K5-70 VL 遺伝子は、pCR2.1 ベクター（Invitrogen）に組み込んだ。遺伝子合成により作製した K5-70 VH および VL の遺伝子配列をそれぞれ図 64 と図 65 に示した。pCR2.1 ベクターに組み込まれた K5-70 VH および K5-70 VL 遺伝子は、それぞれ制限酵素 EcoRI および Nhe I、Age I および BsiWI によって消化

し、遺伝子断片の回収を行った。次に、切り出した K5-70 VH 遺伝子は、ヒト IgG1 フォーム発現用ベクターである、pFUSE-CHIg-hG1 ベクター（InvivoGen）の EcoRI/Nhe I 部位に、K5-70 VL 遺伝子はヒト Ig κフォーム発現用ベクターである pFUSE2-CLIg-hk ベクター（InvivoGen）の Age I/BsiWI 部位にそれぞれ挿入し、マウス-ヒトキメラコンストラクトを完成させた（pFUSE-CHIg-MuK5-70、pFUSE2-CLIg-MuK5-70）。

上記で作製したコンストラクトと、実施例３６で作製した HuK5-70 H 鎖発現ベクター（pFUSE-CHIg-HuK5-70）および HuK5-70 L 鎖発現ベクター（pFUSE2-CLIg-HuK5-70）を、下記表の 1～4 のとおりの組み合わせで 293F 細胞（Invitrogen）に共発現させた。

	H鎖	L鎖	産生される抗体	備考
1	pFUSE-CHIg-HuK5-70	pFUSE2-CLIg-HuK5-70	HuK5-70 抗体	ヒト型化 K5-70 抗体
2	pFUSE-CHIg-HuK5-70	pFUSE2-CLIg-MuK5-70	HuVH/MuVL 抗体	ヒト型化 K5-70VH/ マウス K5-70VL
3	pFUSE-CHIg-MuK5-70	pFUSE2-CLIg-HuK5-70	MuVH/HuVL 抗体	マウス K5-70VH/ ヒト型化 K5-70VL
4	pFUSE-CHIg-MuK5-70	pFUSE2-CLIg-MuK5-70	ChK5-70 抗体	キメラ K5-70 抗体

293F 細胞(Invitrogen)へのトランスフェクションは、NeoFection 試薬（Astec）を用いて添付の方法に従った。トランスフェクション後、5 日間 FreeStyle 293 Expression Medium（Invitrogen）を用いて 37℃、CO_2 濃度 8% の CO_2 インキュベーターで培養を行った後培養上清を回収した。培養上清中の

抗体濃度は、サンドイッチELISA法で測定した。具体的には、96穴プレートをPBSで1μg/mLに希釈したGoat anti-human IgG (Fcγ specific) (Southern Biotech)を50μL/ウェルで添加し、4℃で一晩コーティングした。洗浄バッファー（0.05% Tween20を含むPBS）で洗浄した後、ブロッキングバッファー（2%スキムミルクと0.05% Tween20を含むPBS）を200μL/ウェルで添加し、1時間、室温にてブロッキングを行った。洗浄バッファーで洗浄した後、ELISAバッファー(1% スキムミルクと0.025% Tween 20を含むPBS)で適当な希釈倍率に希釈した培養上清を50μL/ウェルで添加し、室温で2時間反応させた。標準品としてHuK5-70抗体を用いた。洗浄バッファーで洗浄した後、検出用抗体としてELISAバッファーで1,000倍希釈したHRP標識Goat anti-human kappa (κ chain specific) (Southern Biotech)を50μL/ウェルで添加し、室温で1時間反応させた。洗浄バッファーで洗浄した後、TMB（3,3',5,5'-tetramethylbenzidine, SIGMA）基質溶液を50μL/ウェルで添加し、発色反応を行った。1M 硫酸を25μL/ウェルで加え反応を停止させた後、iMark Microplate reader（Bio Rad）を用いて655nmの吸光度をリファレンスとして450nmの吸光度を測定した。4種類の培養上清中の抗体とhTROP-2との結合は、前述した低密度抗原固相化ELISAで測定した。その結果、マウスK5-70 VHとHuK5-70 VLから構成されるMuVH/HuVL抗体のhTROP-2に対する結合はChK5-70抗体と同等であったが、HuK5-70 VHとマウスK5-70 VLから構成されるHuVH/MuVL抗体のhTROP-2に対する結合活性は、ChK5-70と比べて相対的に低かった（図66）。このことから、HuK5-70 VHがHuK5-70の“アビディティー”に関与していることが示唆された。そこで、HuK5-70抗体の“アビディティー”を向上させた改変抗体を作製するために、HuK5-70 VHのアミノ酸の置換を行った。図41に示したHuK5-70 VHとK5-70 VHのアミノ酸配列のアライメントから分かるとおり、アミノ酸番号が5, 7, 11, 12, 13, 20, 38, 40, 44, 73, 75, 81, 82c, 83, 87, 108および109番目の合計17個のアミノ酸が、HuK5-70 VHとK5-70 VHとの間で異なっている（アミノ酸番号はKabatらの定義(1991)に従った）。HuK5-70 VHのこれらのアミノ酸を対応するK5-70 VHのアミノ酸に1つずつ置換した変異体を遺伝子合成により作製し、発現

ベクター（pFUSE-CHIg-HuK5-70 変異体）を作製した。さらに、Landolfi らの報告(J. Immunol. 166:1748, 2001)によると、VH の 11 番目と 38 番目のアミノ酸がヒト型化抗体のアビディティーに関与しており、両者のアミノ酸を対応するマウス由来のアミノ酸に置換することにより、アビディティー、および生物活性が向上することが報告されている。そこで、11 番目と 38 番目のアミノ酸の 2 重変異体も併せて作製した。図 67 に作製した 18 種類の HuK5-70 VH 変異体の名称とアミノ酸配列を示した。

作製した 18 種類の HuK5-70 VH 変異体（pFUSE-CHIg-HuK5-70 変異体の発現ベクター）を、それぞれ HuK5-70 L 鎖発現ベクター（pFUSE2-CLIg-HuK5-70）と組み合わせて、HEK293 細胞にトランスフェクションし、その培養上清を用いて各アミノ酸置換抗体と hTROP-2 との結合活性を低密度抗原固相化 ELISA で検討した。その結果、18 種類の HuK5-70 VH 変異体のうち、HuK5-70 VH の４４番目の R(アルギニン)を G(グリシン)に置換した R44G 変異体（HuK5-70 R44G；以下 HuK5-70-2）において、明らかな抗原結合活性の向上が認められた（図 68）。HuK5-70 VH R44G (以下 HuK5-70 VH2)遺伝子の配列は図 69 に示した。

〔実施例４９〕

［HuK5-70-2 抗体の精製およびキャラクラリゼーション］

HuK5-70 R44G 変異型抗体である HuK5-70-2 抗体は、以下のように精製した。pFUSE-CHIg-HuK5-70 R44G と pFUSE2-CLIg-HuK5-70 を 293F 細胞にトランスフェクションし、5 日間培養を行った後、培養上清を回収した。回収した培養上清から rProtein A sepharose Fast Flow (GE Healthcare)を用いて、HuK5-70-2 抗体を精製した。精製した HuK5-70-2 抗体を還元条件下で SDS-PAGE により展開したところ、約 50kDa の H 鎖と約 25kDa の L 鎖が確認され、それぞれの抗体の純度は 95%以上であった（図 70）。

精製した HuK5-70-2 および HuK5-70 抗体の hTROP-2 との結合活性を高密度抗原固相化 ELISA および低密度抗原固相化 ELISA で検討した。高密度抗原

固相化 ELISA で（1μg/mL の hTROP-2 を固相化した 96 ウェルプレートを使用）、アフィニティーの検証を行った結果、HuK5-70 抗体と HuK5-70-2 抗体の結合曲線はほぼ重なり、両者のアフィニティーは同等であり、次に低密度抗原固相化 ELISA（0.1μg/mL の hTROP-2 を固相化した 96 ウェルプレートを使用）によりアビディティーの検証を行った結果、培養上清を用いた場合の結果と同様、HuK5-70-2 抗体の抗原結合活性は HuK5-70 抗体より明らかに高かった（図 71）。具体的には、EC_{50} 値は、K5-70 抗体が 11.4ng/mL、HuK5-70 抗体が 33.4ng/mL、HuK5-70-2 抗体が 11.4ng/mL であり、HuK5-70-2 抗体は、HuK5-70 抗体と比べて“アビディティー”が向上し、K5-70 抗体と同等の活性を有するものであることが示された。

〔実施例５０〕

［ヒト型化抗 hTROP-2 抗体の抗体依存性細胞傷害(ADCC)活性の測定］

(1) 標的細胞溶液の調整

標的細胞としては、内在的に hTROP-2 を発現するヒト大腸癌細胞株 SW480、ヒト膵臓癌細胞株 PK-59、ヒト前立腺癌細胞株 PC-3 を用いた。10 cm 細胞培養ディッシュで培養したターゲット細胞をトリプシン処理によってプレートから剥離し、アッセイ培地に懸濁した。ADCC アッセイ用の培地は,0.5% FBS を加えた Leivovitz L-15 培地 (SW480 細胞) または RPMI-1640 培地（PK-59、PC-3 細胞）を用いた。遠心後（1000 rpm, 3 分、室温）、ペレットを同じ培地で 2×10^5 cell/mL の細胞密度になるように調製し、標的細胞溶液とした。

(2) ヒト末梢血単核細胞の分離

健常人静脈血をヘパリン採血し、PBS で 2 倍に希釈後、Lymphoprep (第一化学薬品) 上に重層し遠心した（室温、750 rpm, 5 分、次に 2000 rpm, 20 分）。遠心後、中間層分画にある単核細胞（健常人末梢血単核球）を回収し、PBS で 3 回洗浄後、アッセイ培地で細胞懸濁液を調製し、エフェクター細胞として使用した。

(3) ヒト型化抗 hTROP-2 抗体（HuK5-70, HuK5-70-2 および HuT6-16-2 抗体）の ADCC 活性

96 穴平底プレート（FALCON 社製）に調製した標的細胞溶液の 100μL（2×10^4 cell/ウェル）を分注した。次いで、ヒト末梢血単核細胞（エフェクター細胞）を、エフェクター細胞：ターゲット細胞比が 40:1 となるように添加した。更に、被験抗体として、ヒト型化抗 hTROP-2 抗体（HuK5-70, HuK5-70-2 および HuT6-16-2 抗体）を最終濃度が 0.1～30μg/mL となるように加えて、全量を 200μL として CO_2 インキュベーター内（37℃、5%CO_2）で 6 時間培養を行った。培養後、Cytotoxicity Detection Kit (LDH) (Roche, Cat. No. 11 644 793 001）を用い、Kit 添付のプロトコールに従って、エフェクター細胞により傷害を受けたターゲット細胞の細胞質から培養上清へ放出される乳酸脱水素酵素（LDH）の活性を測定し、この測定結果を指標として ADCC 活性を評価した。

図 72A～72C に示したとおり、HuK5-70、および HuT6-16-2 抗体は hTROP-2 を細胞表面に発現するヒト大腸癌細胞株 SW480（図 72A）、ヒト膵癌細胞株 PK-59（図 72B）、ヒト前立腺癌細胞株 PC-3（図 72C）に対して、用量依存的に ADCC 活性を発揮することが明らかとなった。また図 72B および図 72C に示したとおり、HuK5-70-2 抗体はヒト膵癌細胞株 PK-59 およびヒト前立腺癌細胞株 PC-3 細胞に対して、HuK5-70 抗体よりも強い ADCC 活性を発揮することが明らかとなった。HuK5-70-2 抗体は、前述のとおり、HuK5-70 VH の４４番目の R(アルギニン)を G(グリシン)に置換することにより、hTROP-2 に対する結合能（アビディティー）が向上した抗体であり、HuK5-70 抗体に比べて向上したアビディティーが ADCC 活性に反映していることが示された。したがって、HuK5-70-2 抗体は、*in vivo* においても、HuK5-70 抗体と同様に優れた抗腫瘍活性を有する（好ましくは、HuK5-70 抗体より高い抗腫瘍活性を有し得る）ものと推認される。以上の結果から、HuK5-70、HuK5-70-2 及び HuT6-16-2 抗体は、hTROP-2 を細胞表面に発現する癌に対して有効な治療用抗体となることが示唆された。

産業上の利用可能性

本発明によれば、hTROP-2と特異的に反応し、*in vivo*において高い抗腫瘍活性を有する抗体、具体的には、低用量で*in vivo*での高い抗腫瘍活性を有するモノクローナル抗体、特に当該抗体のうちヒト型化抗体であるものを提供することができる。さらに本発明は、当該抗体を産生するハイブリドーマ、当該抗体の断片、当該抗体等と各種薬剤との複合体、腫瘍の診断用又は治療用医薬組成物、腫瘍の検出方法、腫瘍の検出用又は診断用キットを提供することができる。

配列表フリーテキスト

配列番号３：合成DNA

配列番号４：合成DNA

配列番号５：合成DNA

配列番号６：合成DNA

配列番号７：合成DNA

配列番号８：合成DNA

配列番号９：合成DNA

配列番号１０：合成DNA

配列番号１１：合成DNA

配列番号１２：合成DNA

配列番号１３：合成DNA

配列番号１４：合成DNA

配列番号１５：合成DNA

配列番号１６：合成DNA

配列番号１７：合成DNA

配列番号１８：合成DNA

配列番号１９：合成DNA

配列番号２０：合成DNA

配列番号２１：合成DNA

配列番号２２：合成DNA

配列番号２３：合成DNA
配列番号２４：合成DNA
配列番号２５：合成DNA
配列番号２６：合成DNA
配列番号２７：合成DNA
配列番号２８：合成DNA
配列番号２９：合成DNA
配列番号３０：合成DNA
配列番号３１：合成DNA
配列番号３２：合成DNA
配列番号３３：合成DNA
配列番号７４：組換えDNA
配列番号７５：合成コンストラクト（組換えタンパク質）
配列番号７６：組換えDNA
配列番号７７：合成コンストラクト（組換えタンパク質）
配列番号７８：組換えDNA
配列番号７９：合成コンストラクト（組換えタンパク質）
配列番号８０：組換えDNA
配列番号８１：合成コンストラクト（組換えタンパク質）
配列番号８２：組換えDNA
配列番号８３：合成コンストラクト（組換えタンパク質）
配列番号９２：組換えタンパク質
配列番号９３：組換えタンパク質
配列番号９４：組換えタンパク質
配列番号９５：組換えタンパク質
配列番号９６：組換えタンパク質
配列番号９７：組換えタンパク質
配列番号９８：組換えタンパク質
配列番号９９：組換えDNA

配列番号１００：組換えDNA

請 求 の 範 囲

１．Ｈ鎖Ｖ領域のアミノ酸配列が配列番号９２又は９８で示されるアミノ酸配列からなり、Ｌ鎖Ｖ領域のアミノ酸配列が配列番号９３で示されるアミノ酸配列からなるヒトTROP-2に対する抗体。

２．抗体のＨ鎖Ｖ領域のＣＤＲ１～３のアミノ酸配列が、それぞれ順に、配列番号３６～３８で示されるアミノ酸配列であり、及び／又は、抗体のＬ鎖Ｖ領域のＣＤＲ１～３のアミノ酸配列が、それぞれ順に、配列番号４１～４３で示されるアミノ酸配列である、請求項１記載の抗体。

３．Ｈ鎖Ｖ領域のアミノ酸配列が配列番号９４又は９５で示されるアミノ酸配列からなり、Ｌ鎖Ｖ領域のアミノ酸配列が配列番号９６で示されるアミノ酸配列からなる、ヒトTROP-2に対する抗体。

４．抗体のＨ鎖Ｖ領域のＣＤＲ１～３のアミノ酸配列が、それぞれ順に、配列番号６６～６８で示されるアミノ酸配列であり、及び／又は、抗体のＬ鎖Ｖ領域のＣＤＲ１～３のアミノ酸配列が、それぞれ順に、配列番号７１～７３で示されるアミノ酸配列である、請求項３記載の抗体。

５．抗体がヒト型化抗体である、請求項１～４のいずれか１項に記載の抗体。

６．抗体が*in vivo*で抗腫瘍活性を有するものである、請求項１～５のいずれか１項に記載の抗体。

７．5～20mg/kg体重の投与量で50％以上の腫瘍成長阻害活性を示すものである、請求項１～６のいずれか１項に記載の抗体。

８．前記腫瘍成長阻害活性を示すための投与回数が多くても１週間に１回である、請求項７記載の抗体。

９．10mg/kg体重の単回投与で50％以上の腫瘍成長阻害活性を示すものである、請求項１～６のいずれか１項に記載の抗体。

１０．2種以上のヒト腫瘍細胞株に対して抗腫瘍活性を有する、請求項１～９のいずれか１項に記載の抗体。

１１．解離定数（Kd値）が1.0×10^{-10} M以下である、請求項１～１０のいずれか１項に記載の抗体。

１２．抗体がモノクローナル抗体である、請求項１～１１のいずれか１項に記載の抗体。

１３．腫瘍が、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌からなる群より選ばれる少なくとも１種である、請求項６～１２のいずれか１項に記載の抗体。

１４．腫瘍がヒト膵癌、ヒト大腸癌、ヒト肺癌、ヒト乳癌及びヒト卵巣癌からなる群より選ばれる少なくとも１種である、請求項６～１２のいずれか１項に記載の抗体。

１５．腫瘍が再発癌又は転移癌である、請求項６～１４のいずれか１項に記載の抗体。

１６．腫瘍細胞株が、ヒト膵癌細胞株PK-59、ヒト膵癌細胞株BxPC-3、ヒト膵癌細胞株KP-3L、ヒト膵癌細胞株KP-2、ヒト膵癌細胞株PK-1、ヒト膵癌細胞株PK-45H、ヒト膵癌細胞株PK-45P、ヒト膵癌細胞株TCC-PAN2、ヒト膵癌細胞株SUIT-2、ヒト大腸癌細胞株CACO-2、ヒト大腸癌細胞株SW480、ヒト大腸癌細胞株DLD-1、ヒト大腸癌細胞株HCT 116、ヒト乳癌細胞株JIMT-1、ヒト乳癌細胞株HCC1143、ヒト乳癌細胞株MCF-7、ヒト乳癌細胞株MDA-MB-468、ヒト前立腺癌細胞株DU145、ヒト前立腺癌細胞株PC-3、ヒト卵巣癌細胞株SK-OV-3、ヒト肺癌細胞株Calu-3、ヒト胆管癌細胞株TFK-1からなる群より選ばれる少なくとも2種である、請求項１０～１５のいずれか１項に記載の抗体。

１７．腫瘍細胞株が、ヒト膵癌細胞株PK-59、ヒト膵癌細胞株BxPC-3、ヒト大腸癌細胞株SW480、ヒト肺癌細胞株Calu-3、ヒト乳癌細胞株MBA-MD-468及びヒト卵巣癌細胞株SK-OV-3からなる群より選ばれる少なくとも2種である、請求項１０～１５のいずれか１項に記載の抗体。

１８．請求項１～１７のいずれか１項に記載の抗体に由来する抗体断片。

１９．配列番号９２又は９８で示されるアミノ酸配列、及び／又は、配列番号９３で示されるアミノ酸配列を含むものである、請求項１８記載の抗体断片。

２０．配列番号９４又は９５で示されるアミノ酸配列、及び／又は、配列番号９６で示されるアミノ酸配列を含むものである、請求項１８記載の抗体断片。

２１．請求項１～１７のいずれか１項に記載の抗体と、抗腫瘍活性及び／又は殺細胞活性を有する物質とを含む、抗体－薬剤複合体。

２２．請求項１８～２０のいずれか１項に記載の抗体断片と、抗腫瘍活性及び／又は殺細胞活性を有する物質とを含む、抗体断片－薬剤複合体。

２３．腫瘍が、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌からなる群より選ばれる少なくとも１種である、請求項２１又は２２記載の複合体。

２４．腫瘍がヒト膵癌、ヒト大腸癌、ヒト肺癌、ヒト乳癌及びヒト卵巣癌からなる群より選ばれる少なくとも１種である、請求項２１又は２２記載の複合体。

２５．腫瘍が再発癌又は転移癌である、２１～２４のいずれか１項に記載の複合体。

２６．請求項１～１７のいずれか１項に記載の抗体、請求項１８～２０のいずれか１項に記載の抗体断片、及び請求項２１～２５のいずれか１項に記載の複合体からなる群より選ばれる少なくとも１種を含む、医薬組成物。

２７．腫瘍の治療に用いるものである、請求項２６記載の組成物。

２８．体重減少の副作用を有しないものである、請求項２７記載の組成物。

２９．腫瘍の診断に用いるものである、請求項２６記載の組成物。

３０．腫瘍が、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌からなる群より選ばれる少なくとも１種である、請求項２６～２９のいずれか１項に記載の組成物。

３１．腫瘍が再発癌又は転移癌である、請求項２６～３０のいずれか１項に記載の組成物。

３２．請求項１～１７のいずれか１項に記載の抗体、請求項１８～２０のいずれか１項に記載の抗体断片、及び請求項２１～２５のいずれか１項に記載の複合体からなる群より選ばれる少なくとも１種を含む、腫瘍治療剤。

３３．体重減少の副作用を有しないものである、請求項３２記載の治療剤。

３４．腫瘍が、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌からなる群より選ばれる少なくとも１種である、請求項３２又は３３記載の治療剤。

３５．請求項１～１７のいずれか１項に記載の抗体、請求項１８～２０のいずれか１項に記載の抗体断片、及び請求項２１～２５のいずれか１項に記載の複合体からなる群より選ばれる少なくとも１種を含む、腫瘍診断剤。

３６．腫瘍が、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌からなる群より選ばれる少なくとも１種である、請求項３５記載の診断剤。

３７．請求項１～１７のいずれか１項に記載の抗体、請求項１８～２０のいずれか１項に記載の抗体断片、及び請求項２１～２５のいずれか１項に記載の複合体からなる群より選ばれる少なくとも１種と、生体から採取された試料とを反応させ、反応した抗体及び／又は抗体断片のシグナルを検出することを特徴とする、腫瘍の検出方法。

３８．腫瘍が、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌からなる群より選ばれる少なくとも１種である、請求項３７記載の方法。

３９．請求項１～１７のいずれか１項に記載の抗体、請求項１８～２０のいずれか１項に記載の抗体断片、及び請求項２１～２５のいずれか１項に記載の複合体からなる群より選ばれる少なくとも１種を含む、腫瘍の治療用、診断用又は検出用キット。

４０．腫瘍が、ヒト膵癌、ヒト前立腺癌、ヒト大腸癌、ヒト乳癌、ヒト卵巣癌、ヒト肺癌及びヒト胆管癌からなる群より選ばれる少なくとも１種である、請求項３９記載のキット。

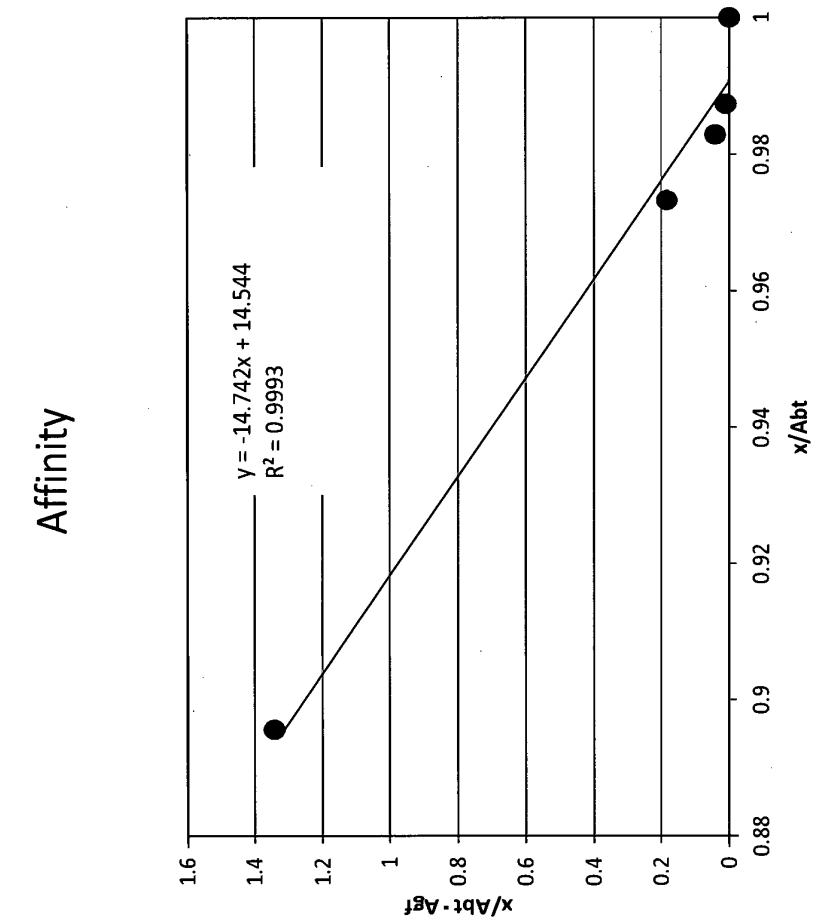
４１．請求項１～１７のいずれか１項に記載の抗体をコードするポリヌクレオチド。

４２．請求項１８～２０のいずれか１項に記載の抗体断片をコードするポリヌクレオチド。

４３．請求項４１又は４２記載のポリヌクレオチドを含む組換えベクター。

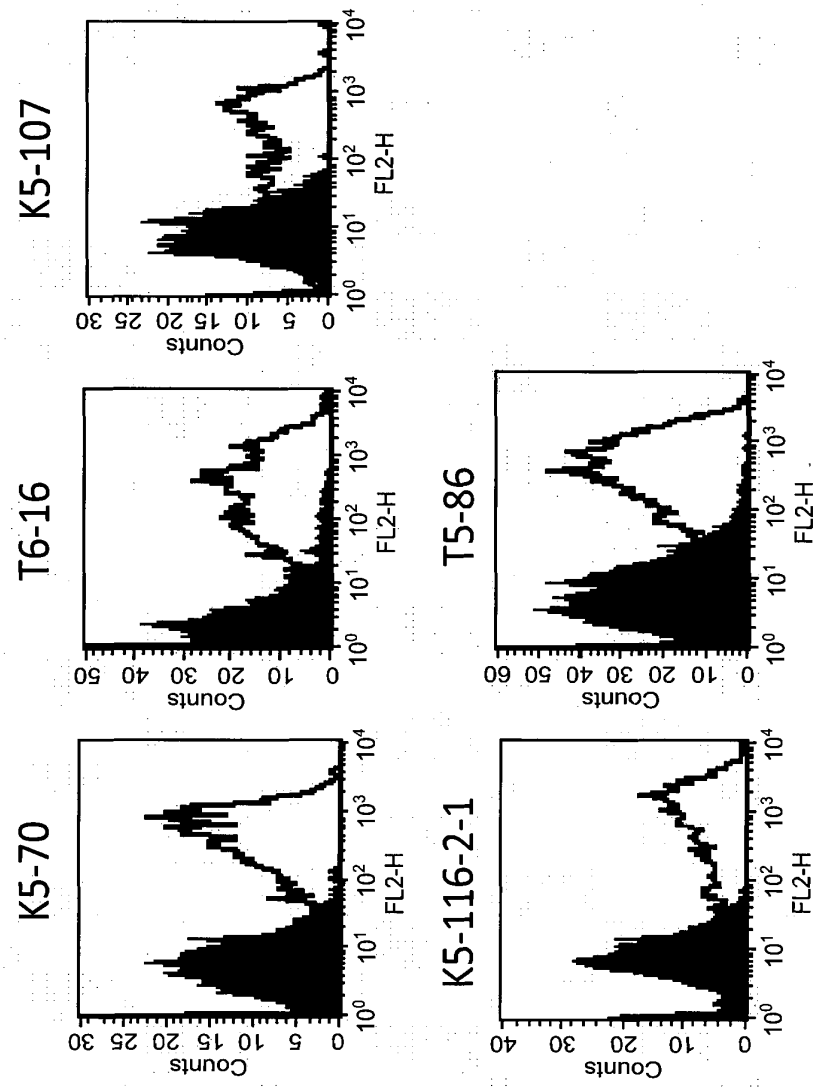
４４．請求項４３記載の組換えベクターを含む形質転換体。

図1


Affinity
y = -14.742x + 14.544
R² = 0.9993
x/Abt - Agf
x/Abt
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
0.98
0.96
0.94
0.92
0.9
0.88

OnCusp
Ex. 1002

図2



差替え用紙(規則26)

図3

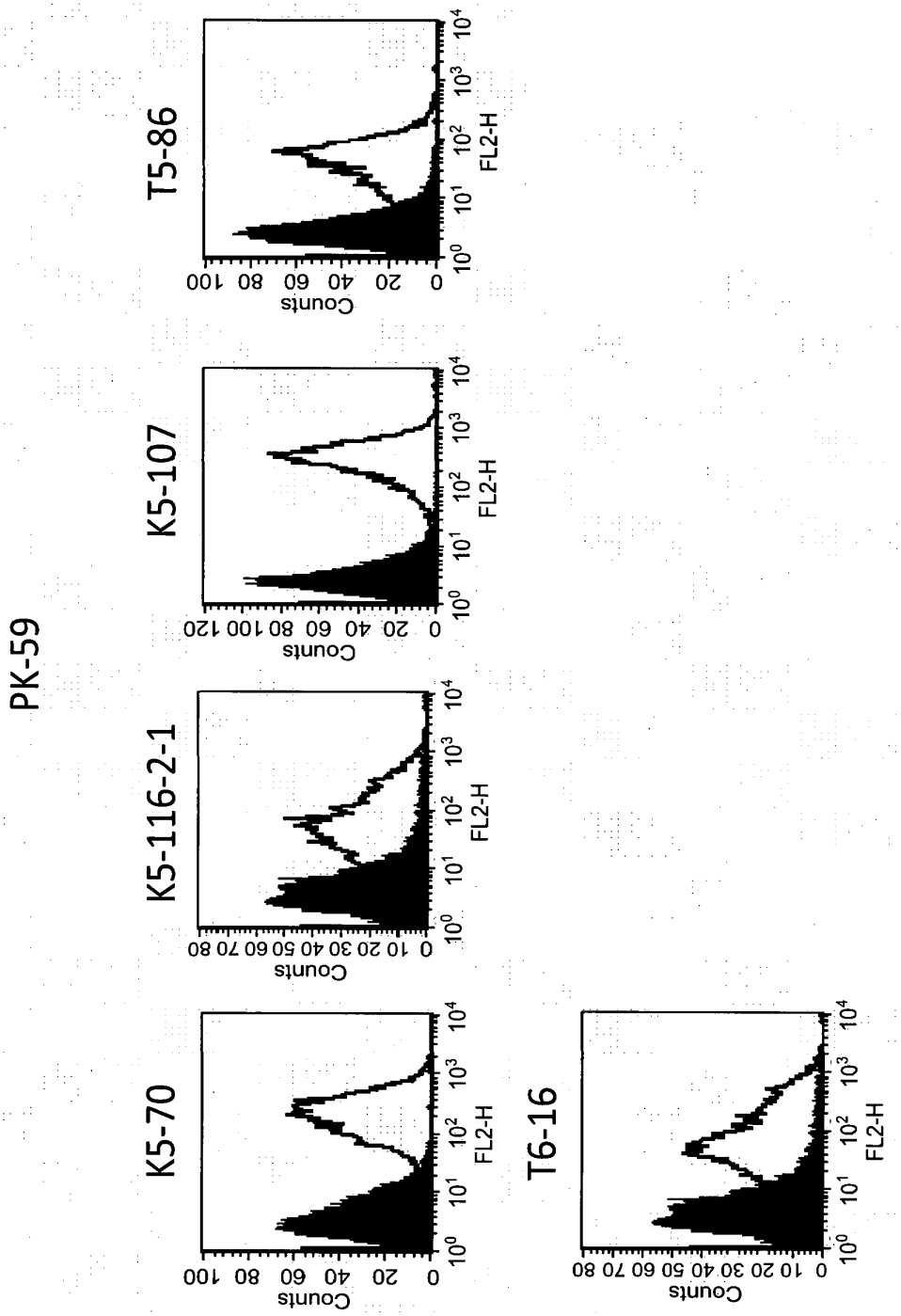
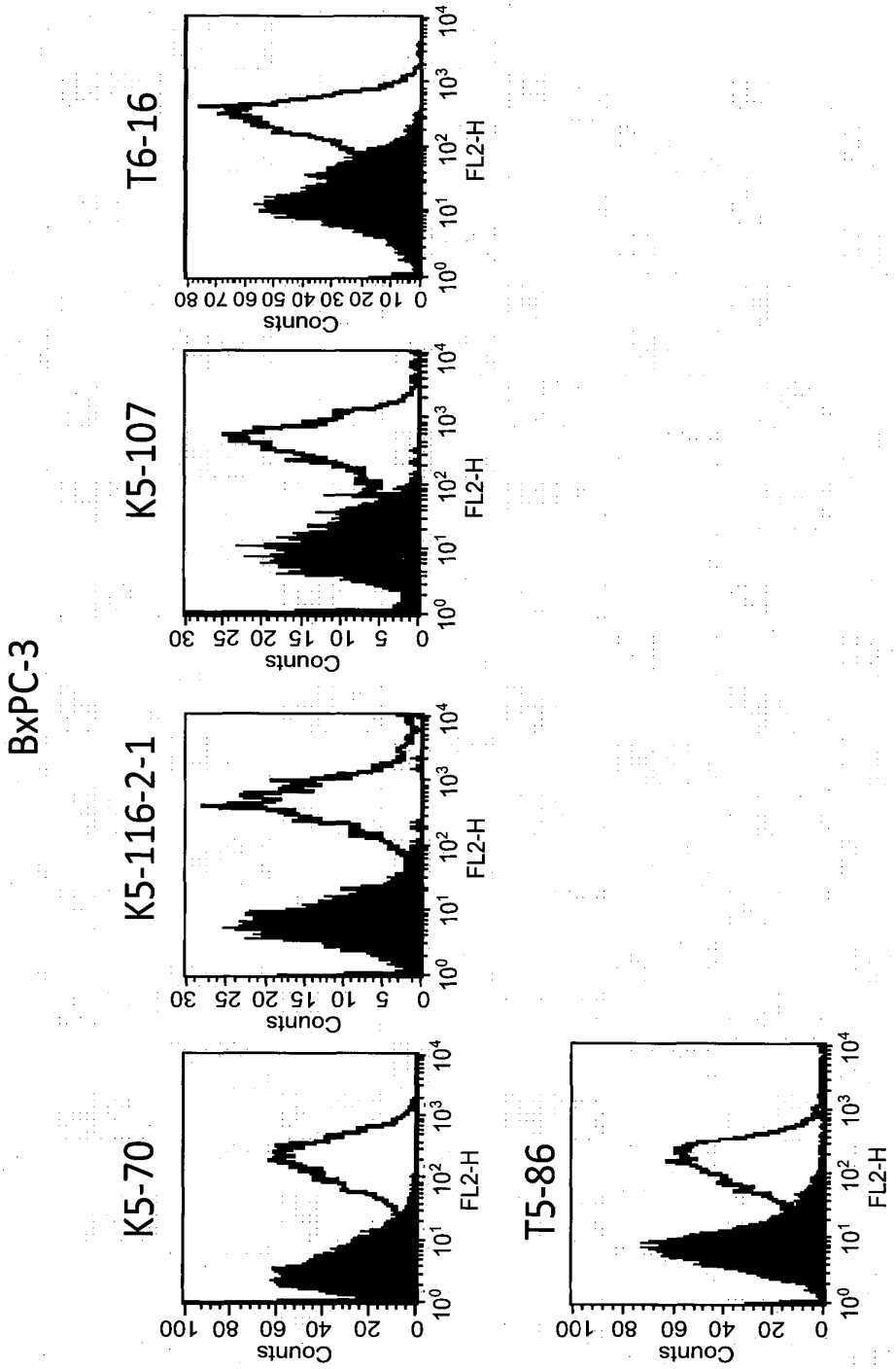


図4



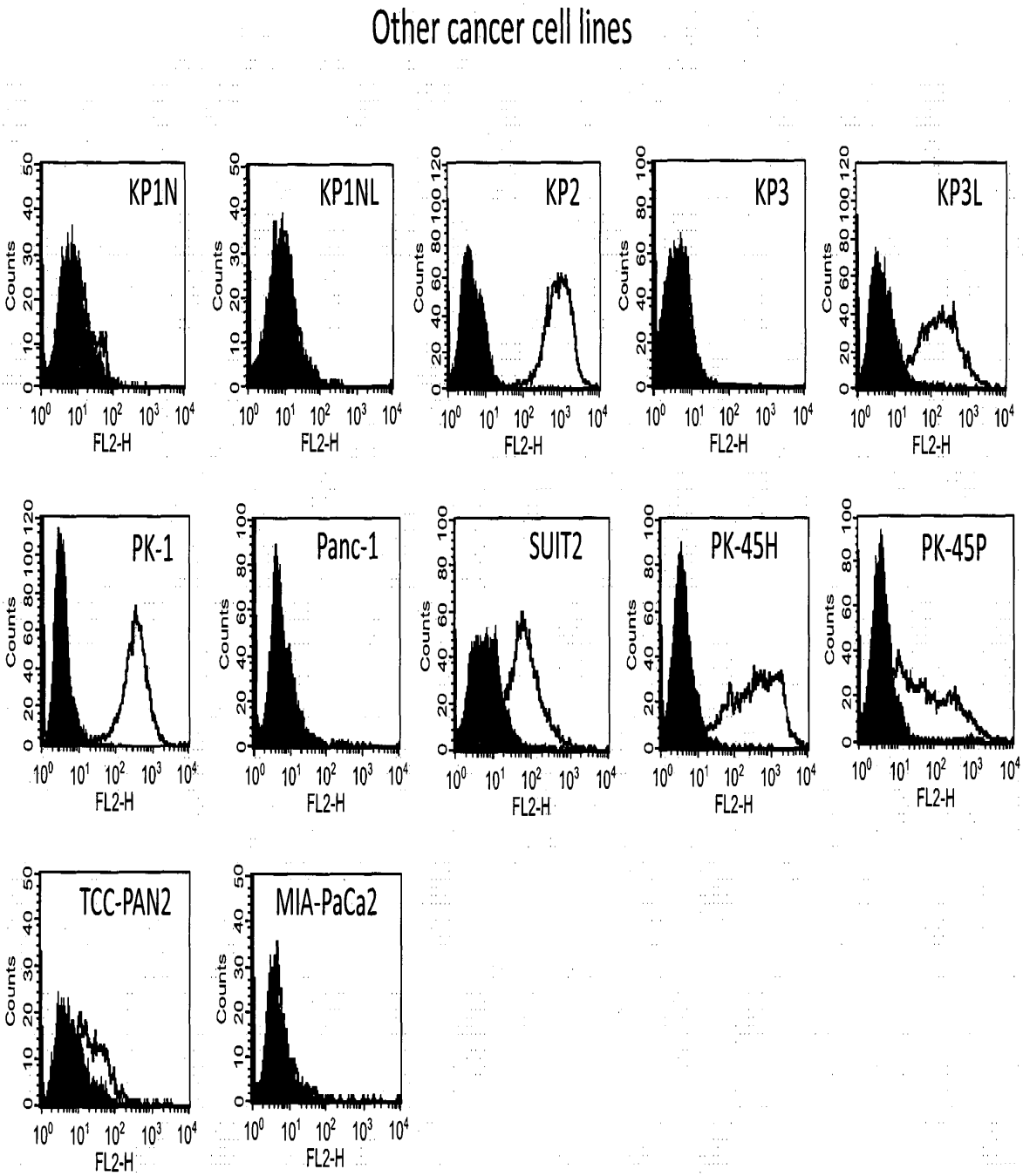
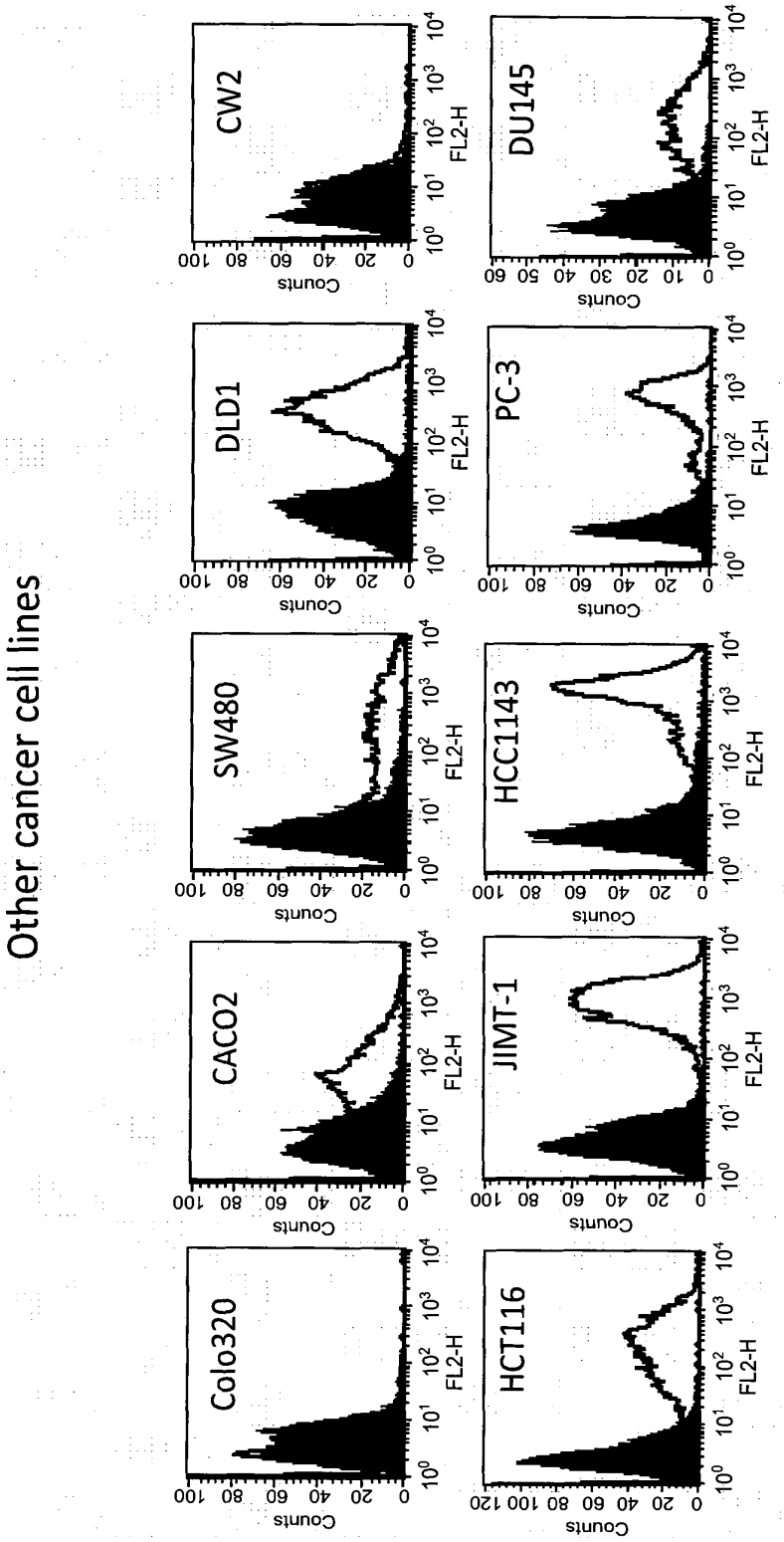

Other cancer cell lines
KP1N
KP1NL
KP2
KP3
KP3L
PK-1
Panc-1
SUIT2
PK-45H
PK-45P
TCC-PAN2
MIA-PaCa2
Counts
FL2-H

図5

図6


Other cancer cell lines
CW2
DU145
DLD1
PC-3
SW480
HCC1143
CACO2
JIMT-1
Colo320
HCT116
Counts
FL2-H

Reactivity with mouse TROP-2
T2-102
K5-70
K5-107
K5-116-2-1
T5-86
T6-16
Counts
FL2-H

図７

図8

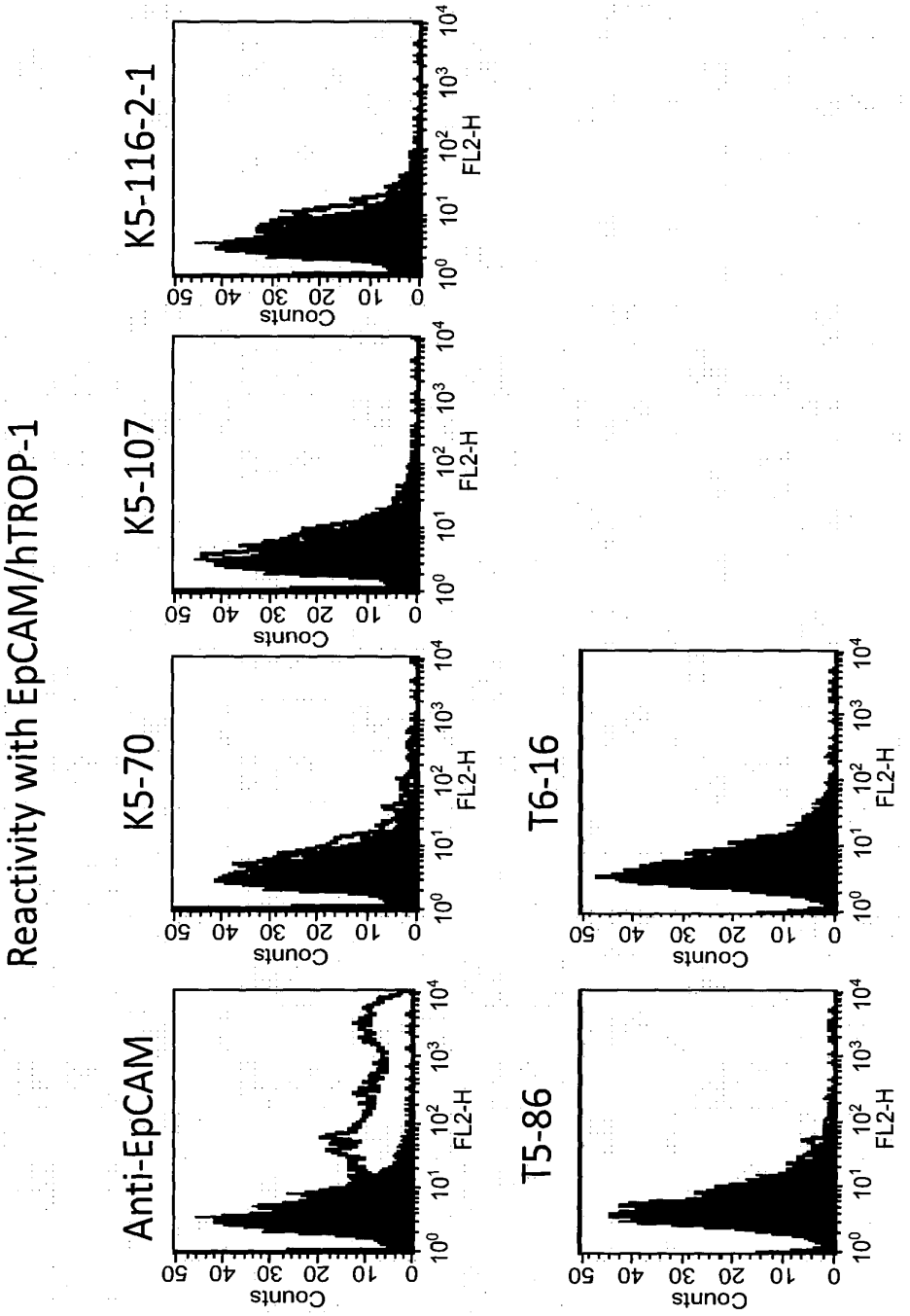

Reactivity with EpCAM/hTROP-1
K5-116-2-1
K5-107
K5-70
T6-16
Anti-EpCAM
T5-86
Counts
FL2-H

図9

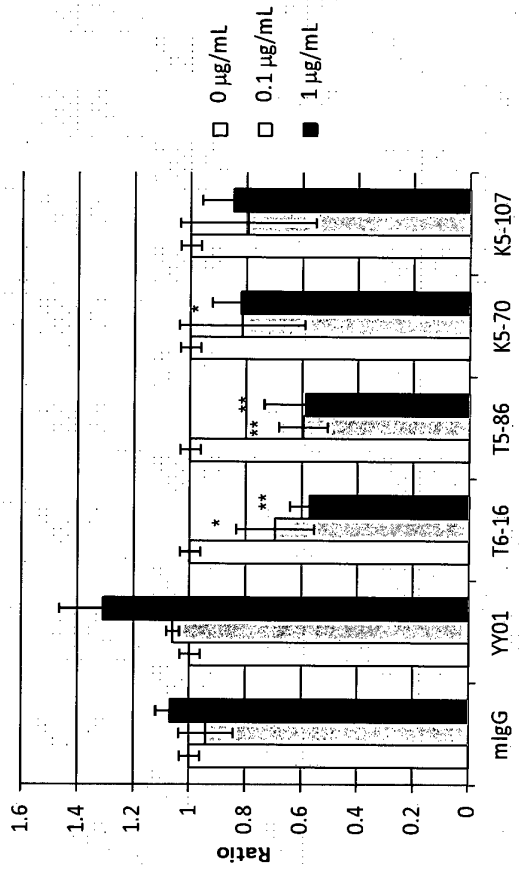
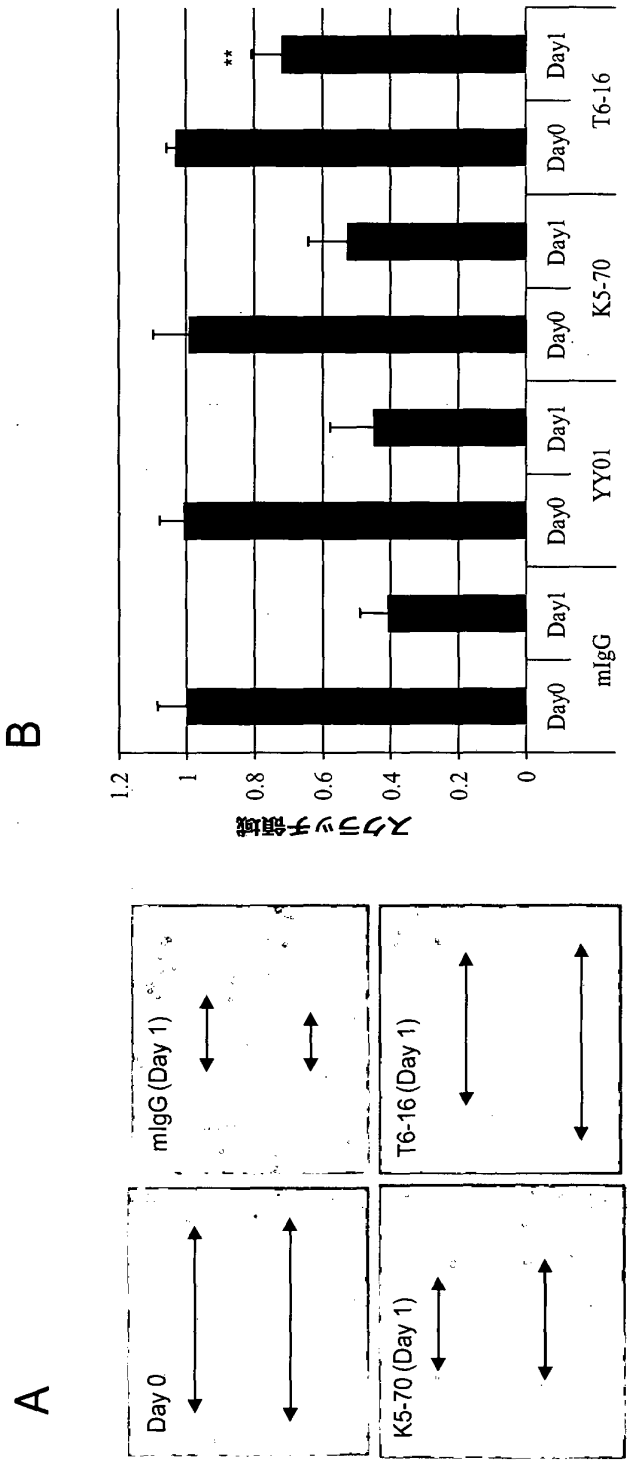
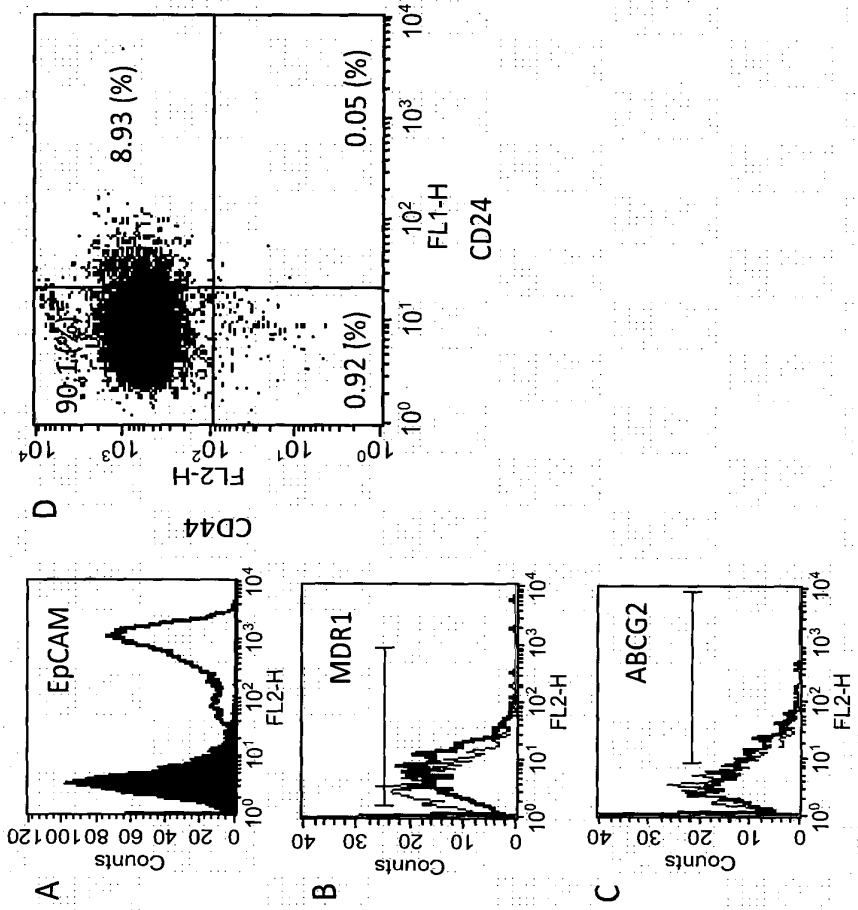

0 μg/mL
0.1 μg/mL
1 μg/mL
Ratio
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
mIgG
YY01
T6-16
T5-86
K5-70
K5-107
*
**
**
*
**

図10


A
Day 0
mIgG (Day 1)
K5-70 (Day 1)
T6-16 (Day 1)
B
クラゾシチ減
1.2
1
0.8
0.6
0.4
0.2
0
**
Day0
Day1
mIgG
YY01
K5-70
T6-16

OnCusp
Ex. 1002

図11



OnCusp
Ex. 1002

WO 2013/077458

K5-70 / Treatment

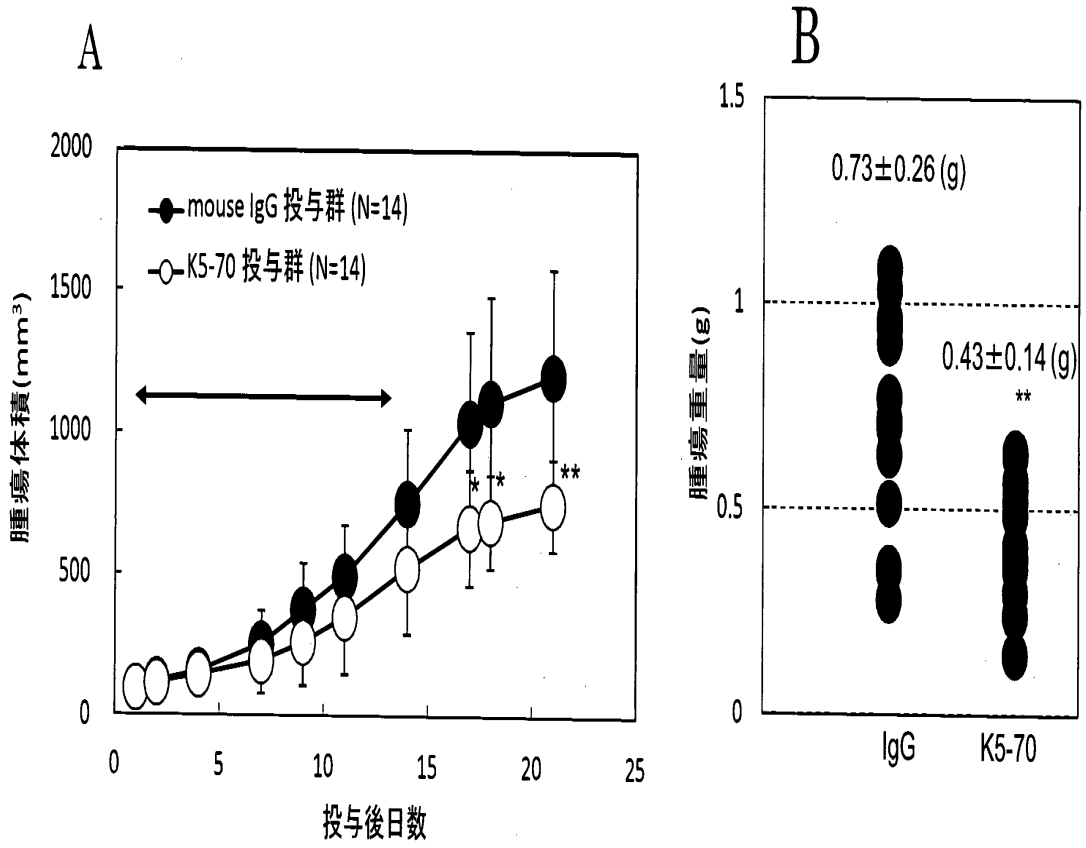


図12

PCT/JP2012/080800

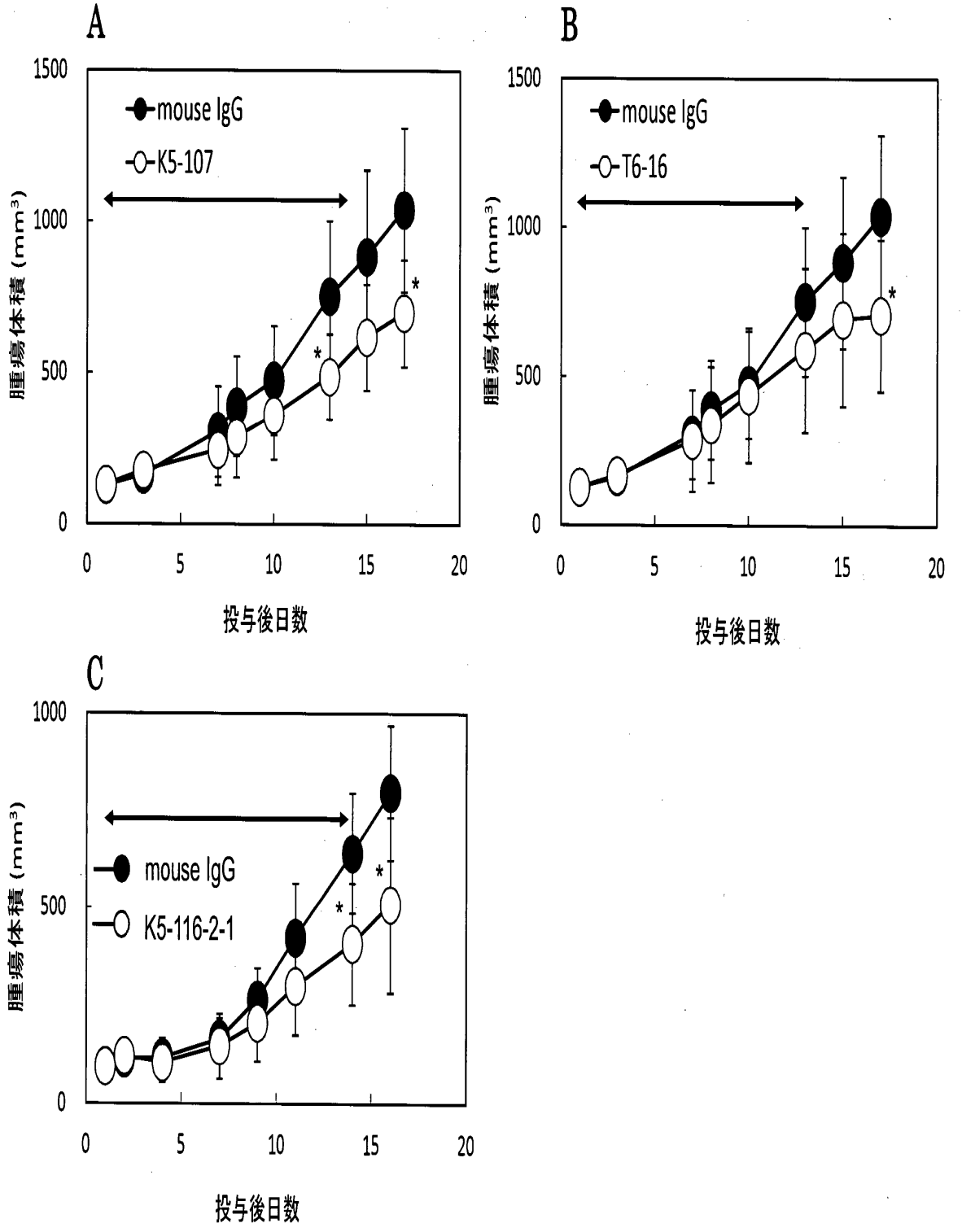

A
mouse IgG
K5-107
腫瘍体積 (mm³)
投与後日数
B
mouse IgG
T6-16
腫瘍体積 (mm³)
投与後日数
C
mouse IgG
K5-116-2-1
腫瘍体積 (mm³)
投与後日数

図13

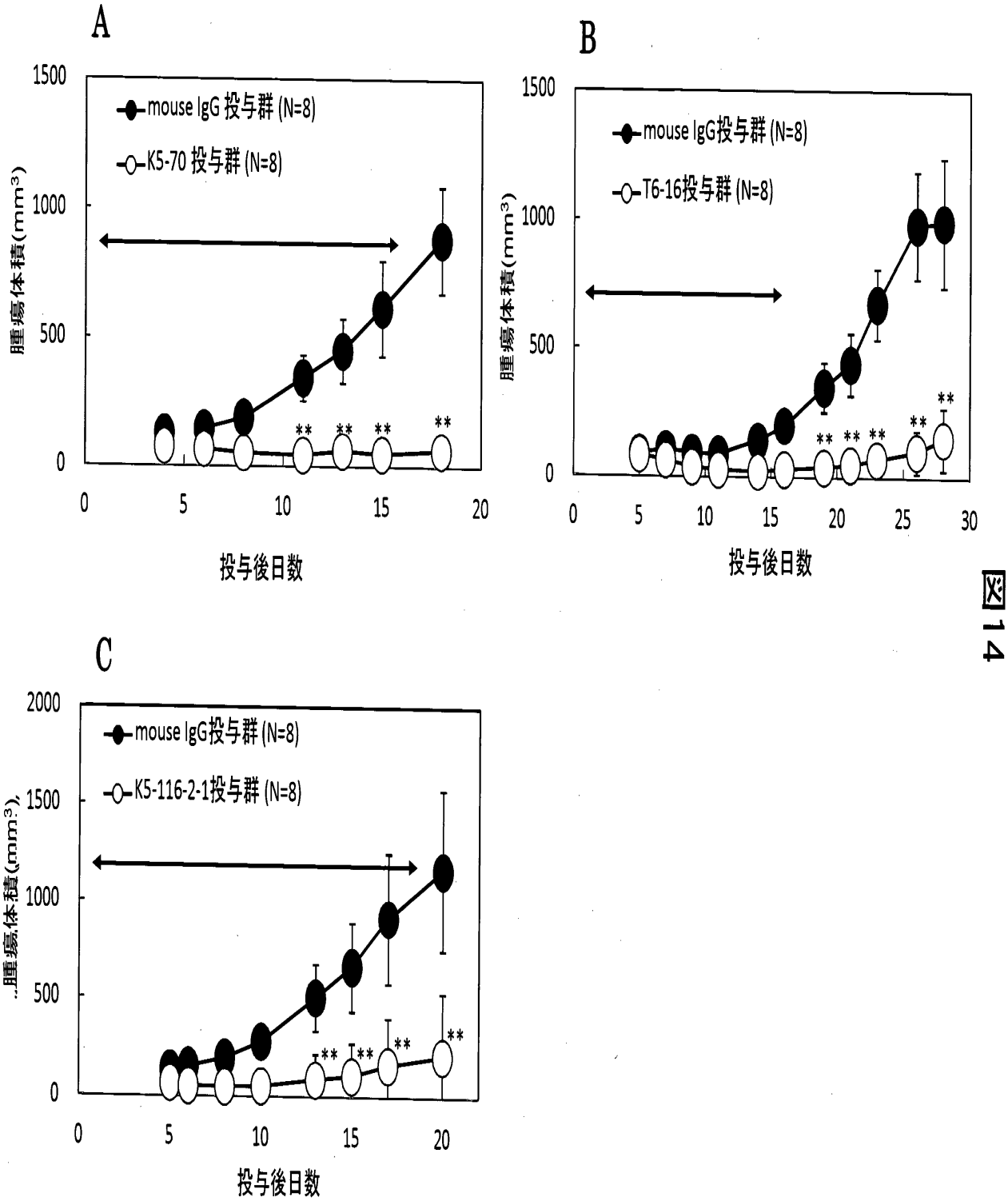

A
mouse IgG 投与群 (N=8)
K5-70 投与群 (N=8)
腫瘍体積(mm³)
投与後日数
B
mouse IgG投与群 (N=8)
T6-16投与群 (N=8)
腫瘍体積(mm³)
投与後日数
C
mouse IgG投与群 (N=8)
K5-116-2-1投与群 (N=8)
腫瘍体積(mm³)
投与後日数

図14

K5-70 /BxPC-3

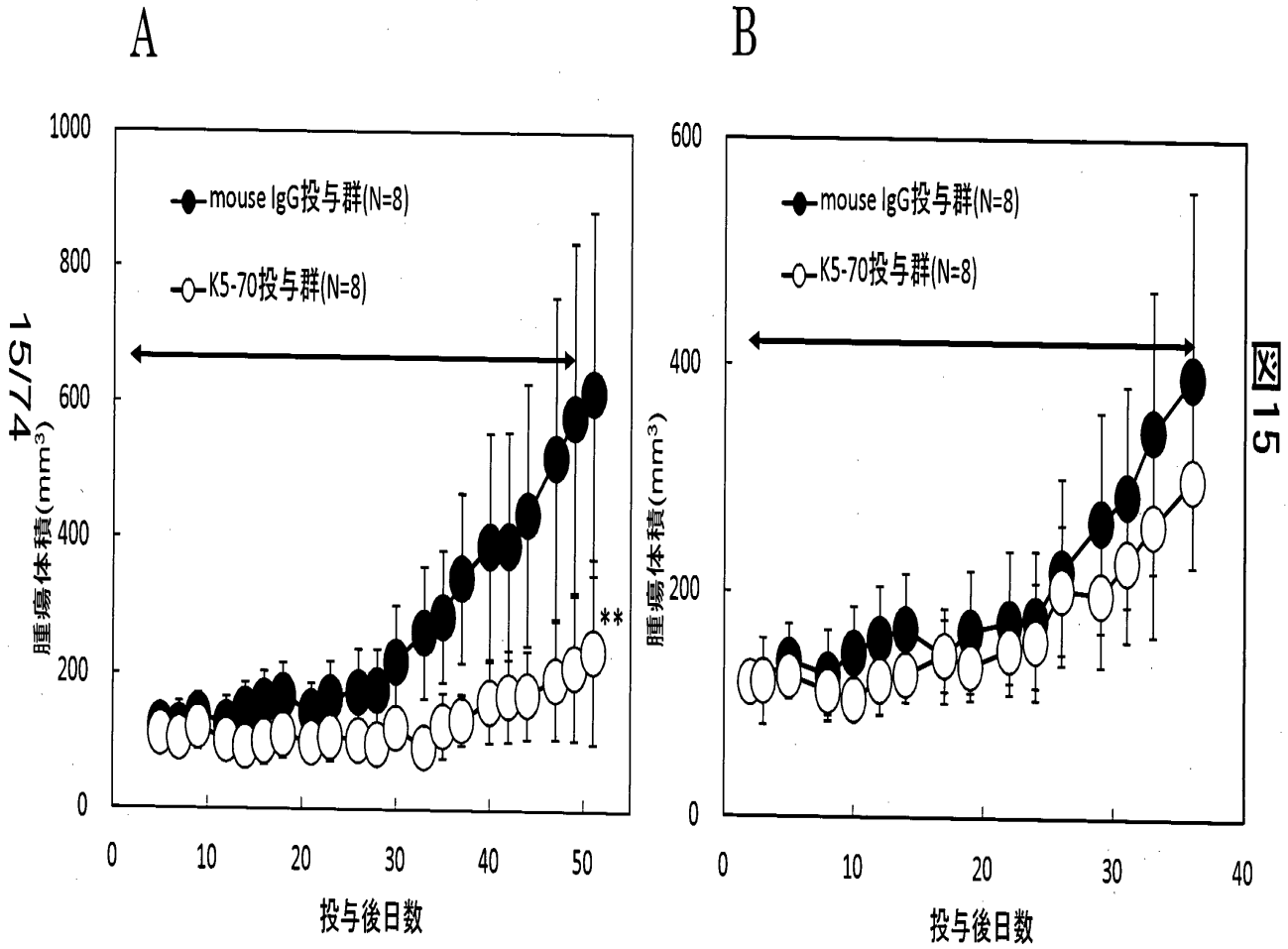
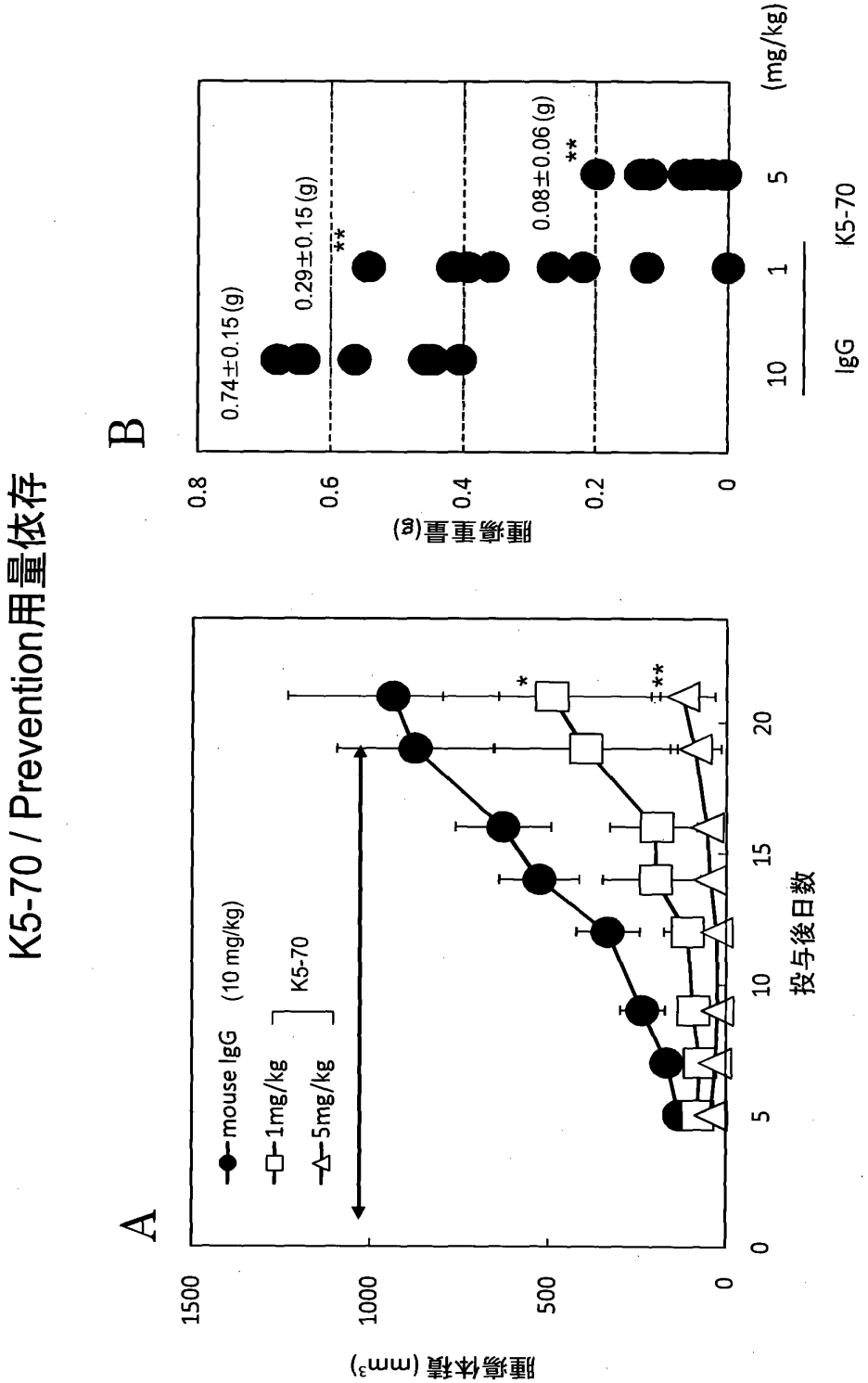


図15

図16


K5-70 / Prevention用量依存
A
mouse IgG (10 mg/kg)
1mg/kg
5mg/kg
K5-70
腫瘍体積 (mm³)
投与後日数
B
0.74±0.15 (g)
0.29±0.15 (g)
0.08±0.06 (g)
腫瘍重量(g)
(mg/kg)
IgG
K5-70

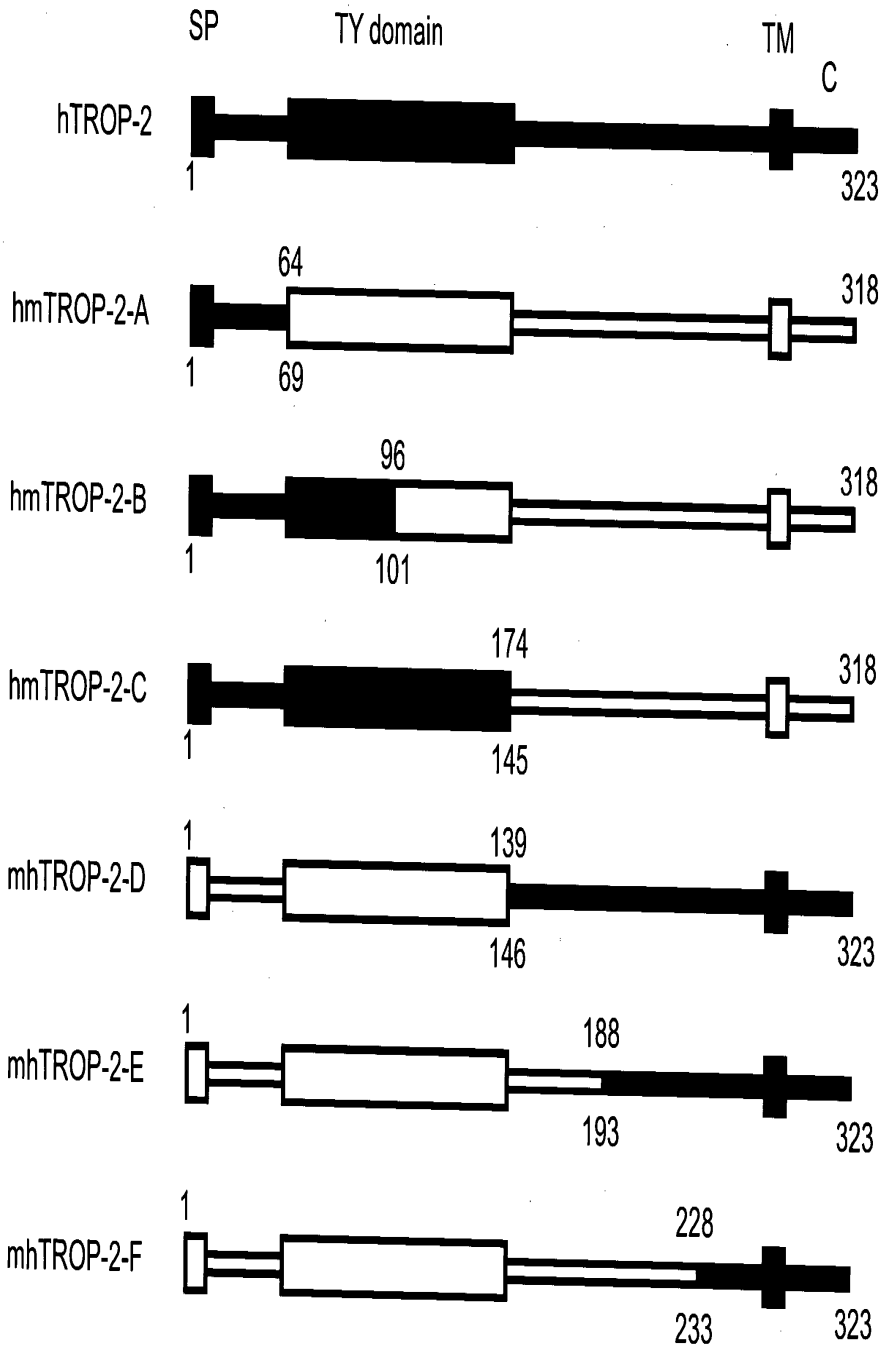
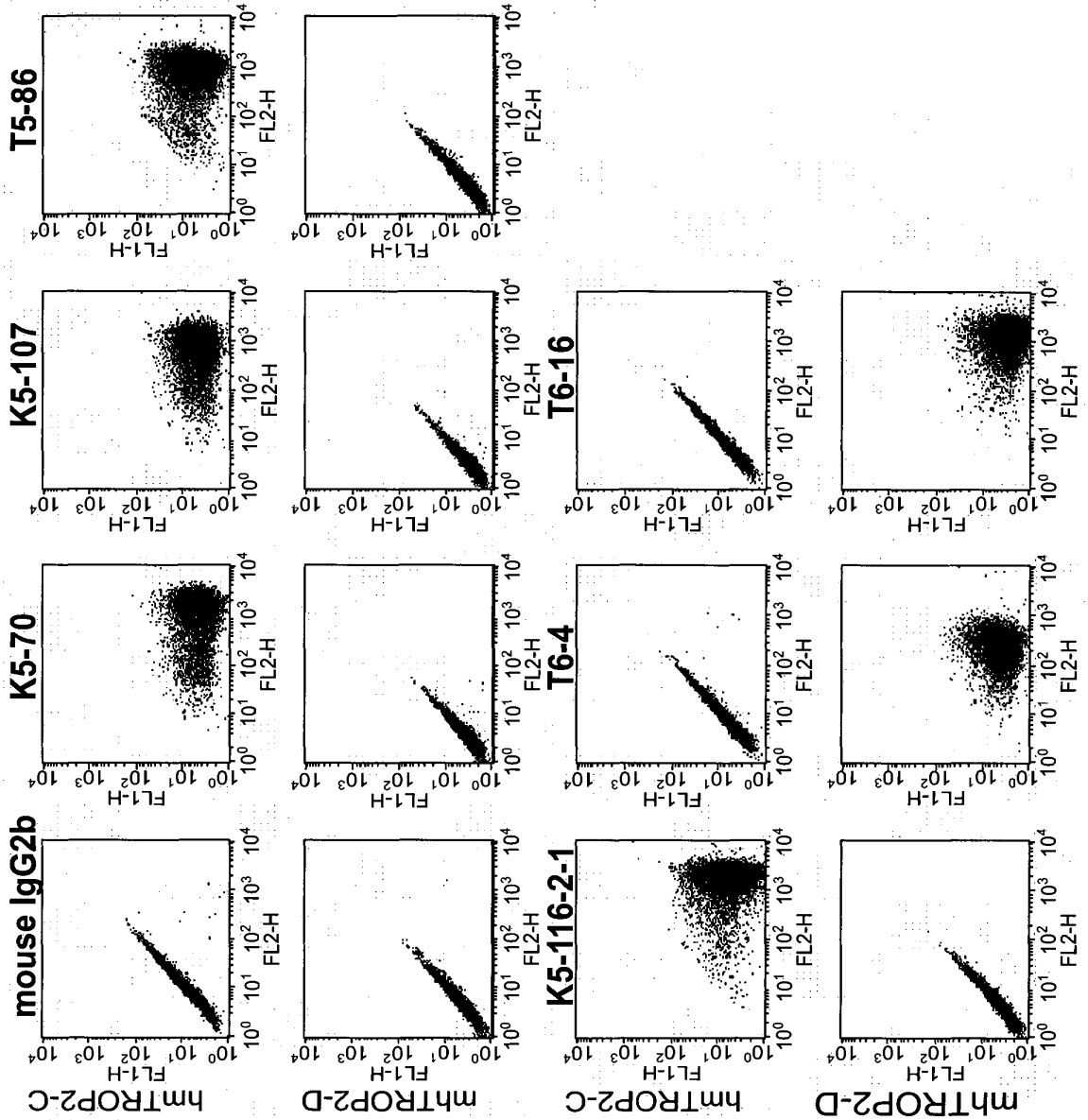

SP
TY domain
TM
C
hTROP-2
1
323
64
318
hmTROP-2-A
1
69
96
318
hmTROP-2-B
1
101
174
318
hmTROP-2-C
1
145
1
139
mhTROP-2-D
146
323
1
188
mhTROP-2-E
193
323
1
228
mhTROP-2-F
233
323

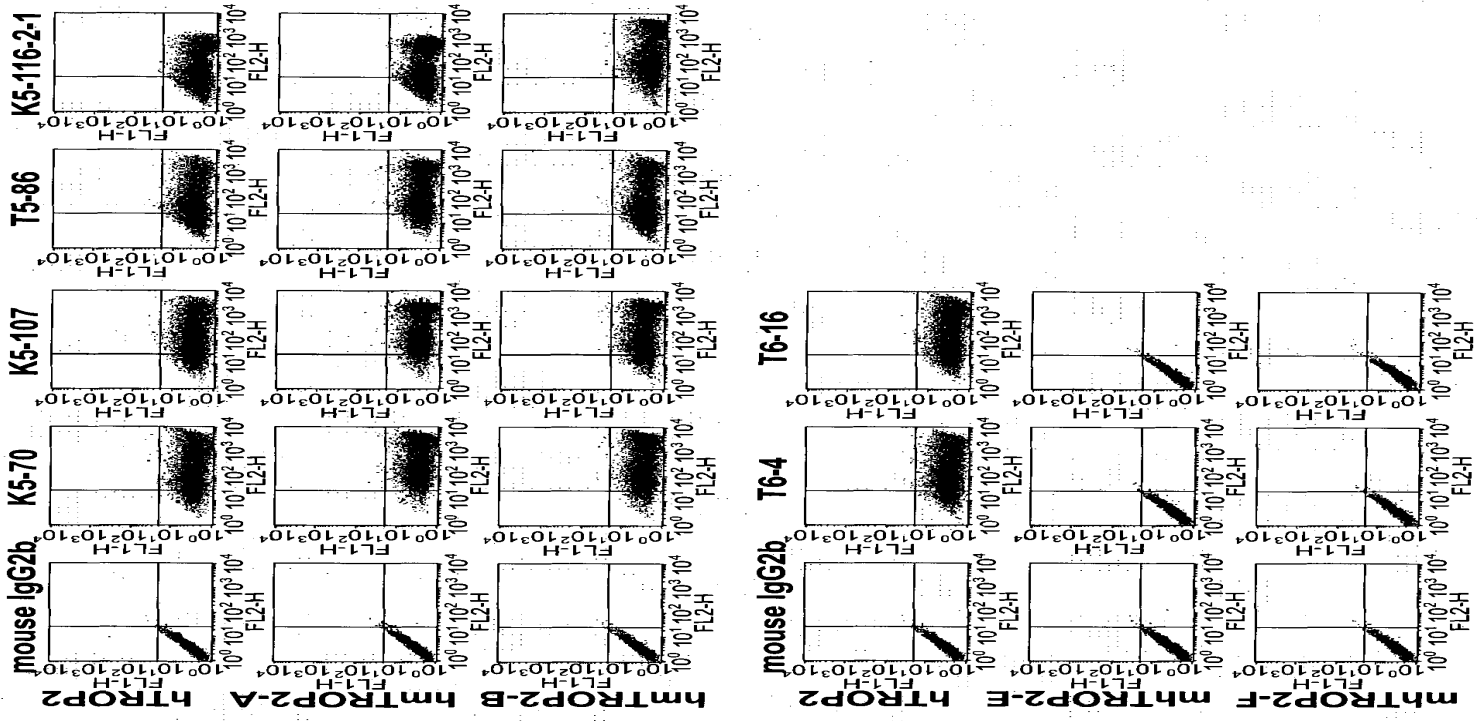
図17

図18


T5-86
K5-107
T6-16
K5-70
T6-4
mouse IgG2b
K5-116-2-1
mTROP2-D
hTROP2-C
mTROP2-D
hTROP2-C
FL1-H
FL2-H

OnCusp
Ex. 1002

図19



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PCT/JP2012/080800

図20

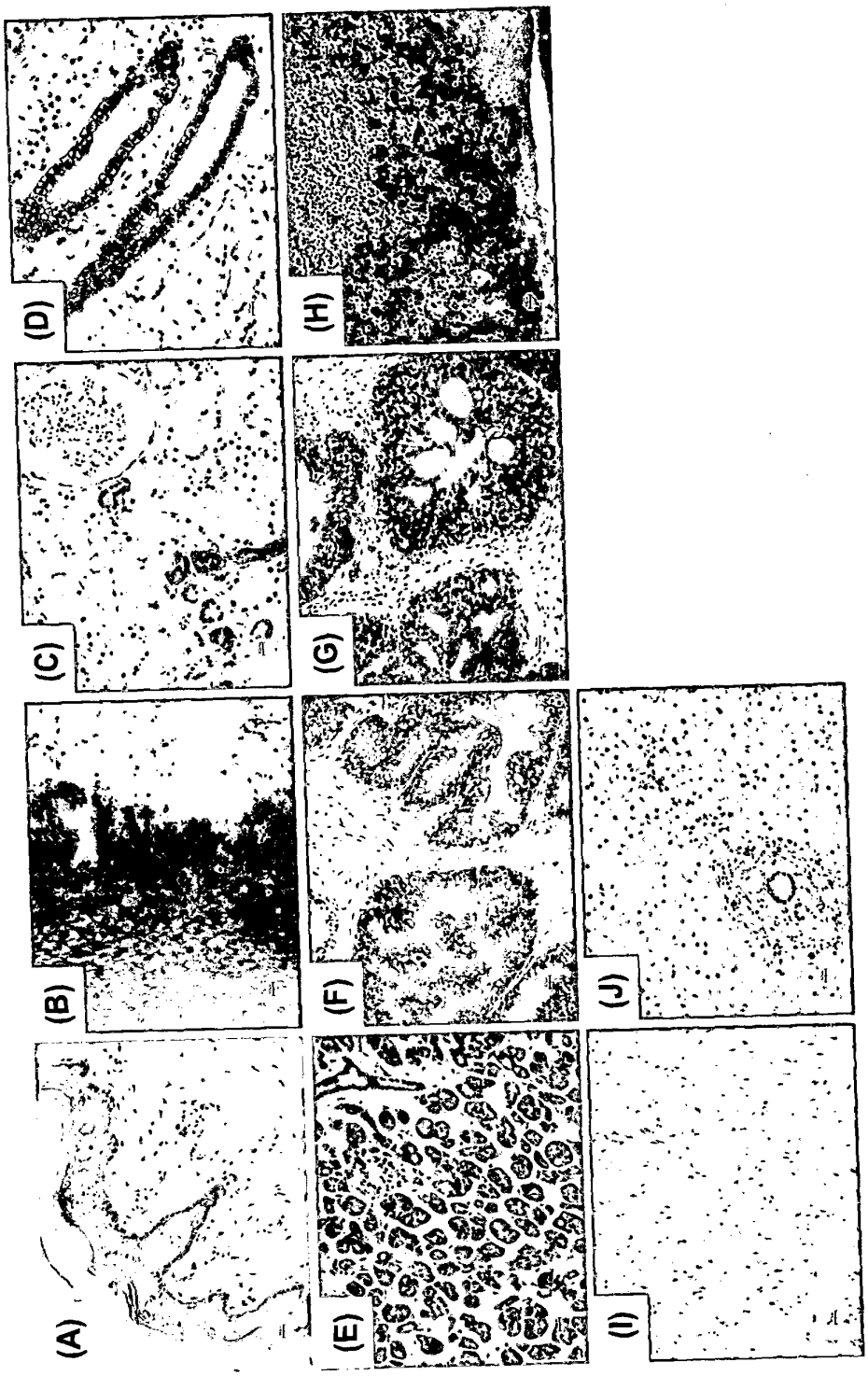


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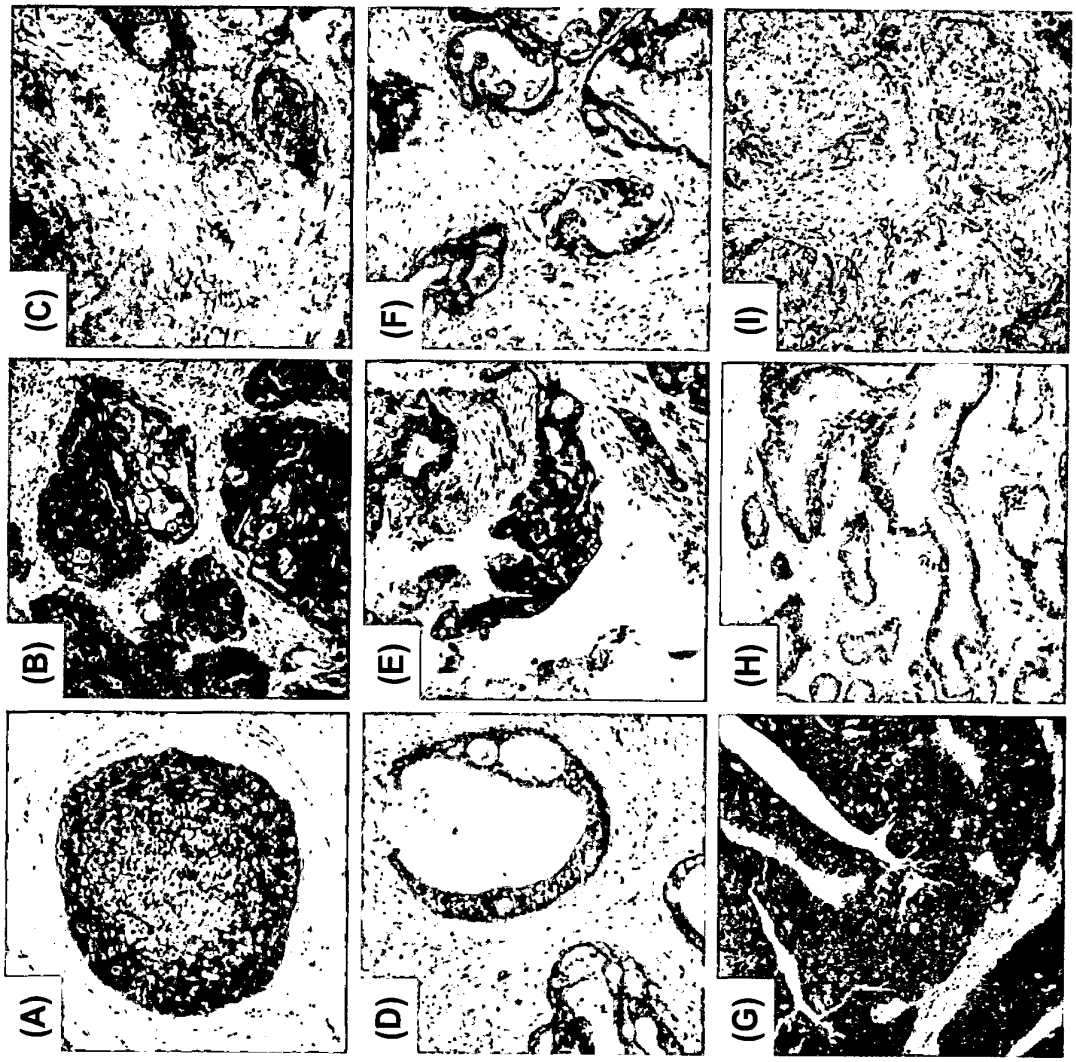


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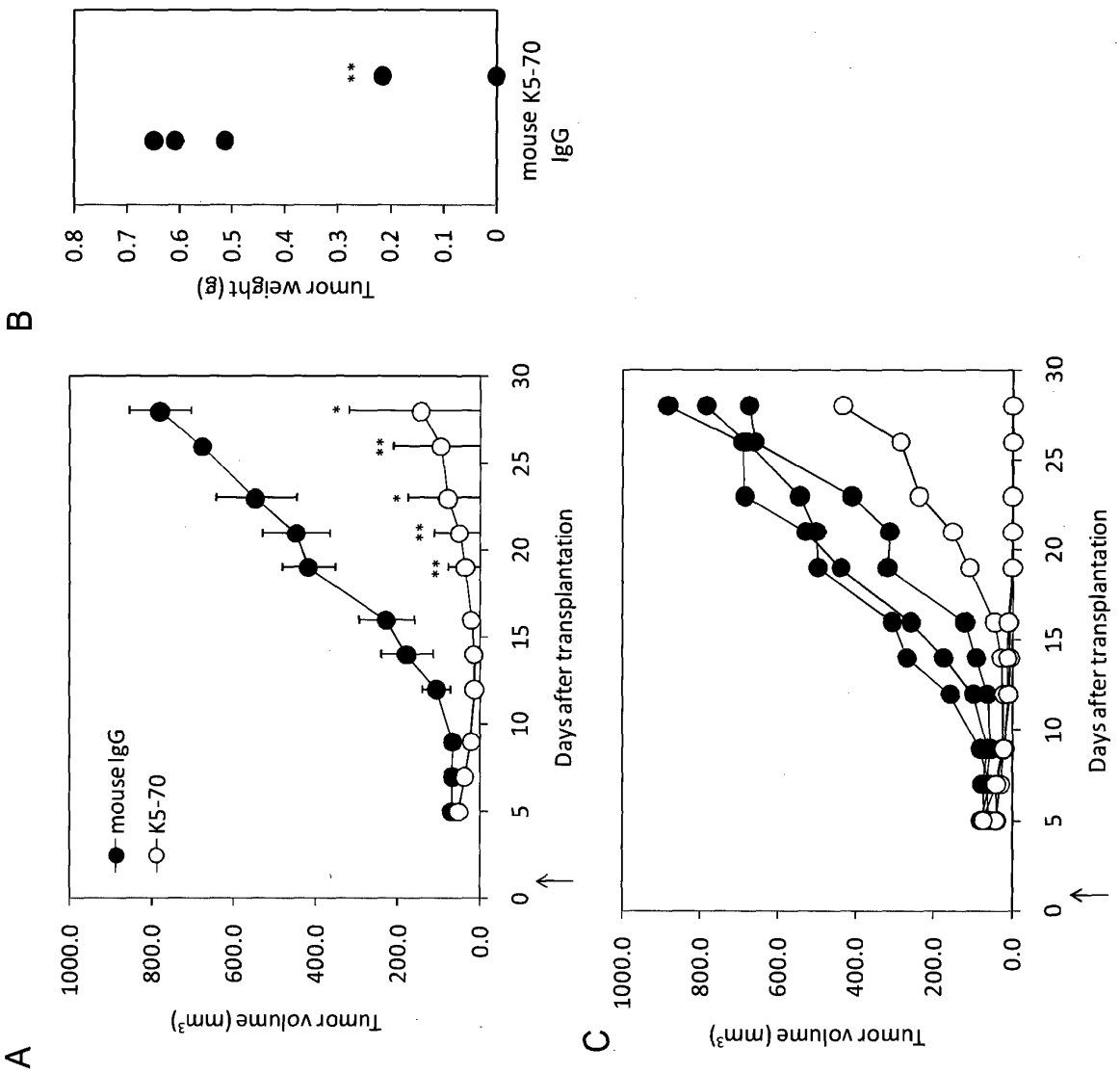
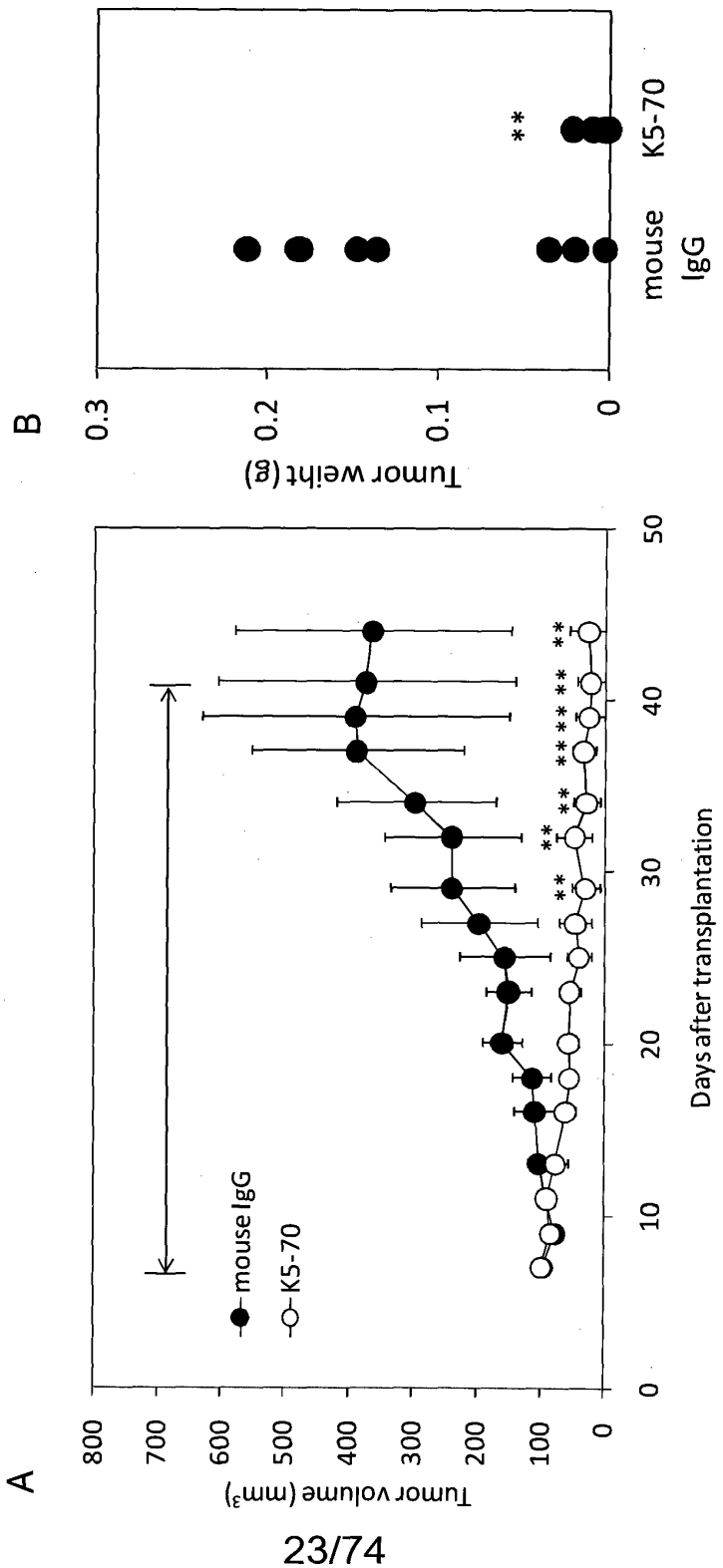

A
B
C
Tumor volume (mm³)
Days after transplantation
mouse IgG
K5-70
Tumor weight (g)
mouse K5-70 IgG
0.0
200.0
400.0
600.0
800.0
1000.0
0
5
10
15
20
25
30
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
*
**

図23


A
Tumor volume (mm3)
800
700
600
500
400
300
200
100
0
mouse IgG
K5-70
0
10
20
30
40
50
Days after transplantation
B
Tumor weight (g)
0.3
0.2
0.1
0
mouse IgG
K5-70

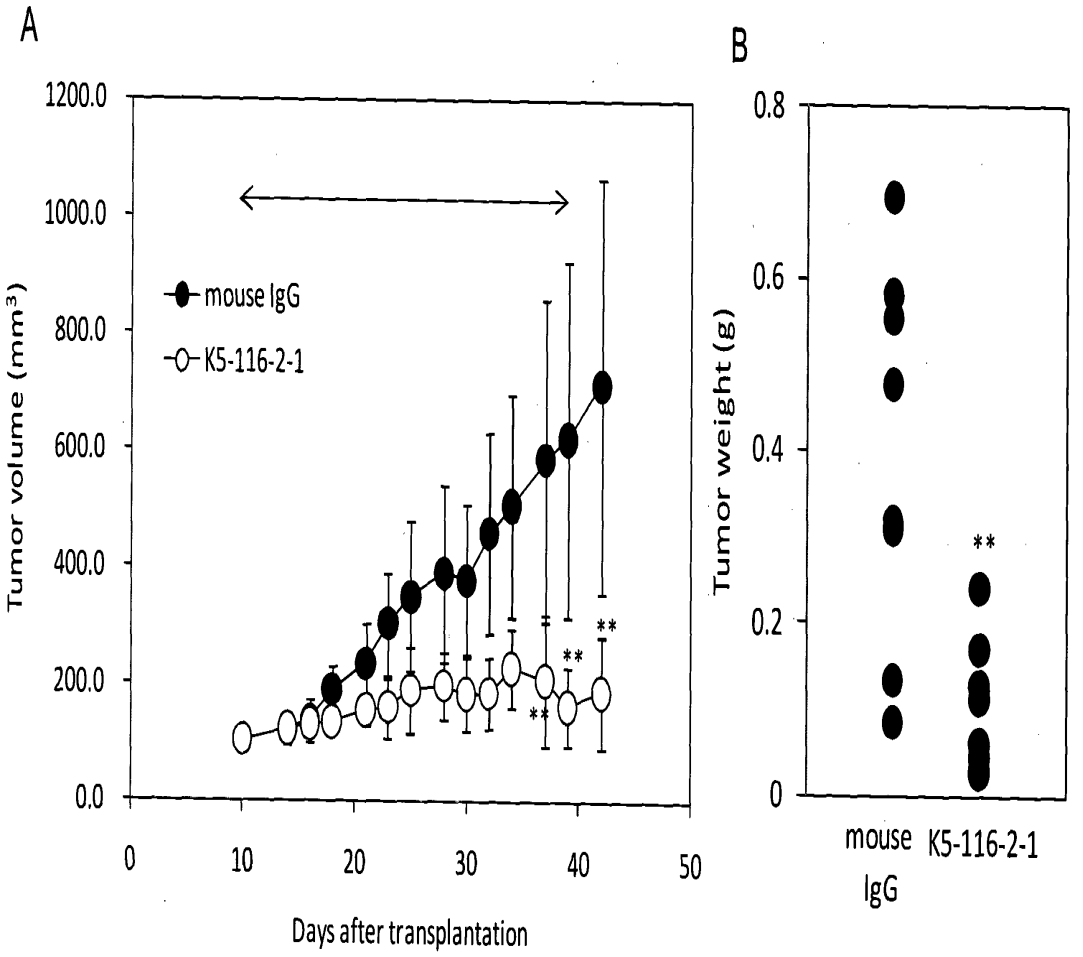

A
B
Tumor volume (mm³)
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
mouse IgG
K5-116-2-1
**
0
10
20
30
40
50
Days after transplantation
Tumor weight (g)
0.8
0.6
0.4
0.2
0
mouse IgG
K5-116-2-1

図24

図25

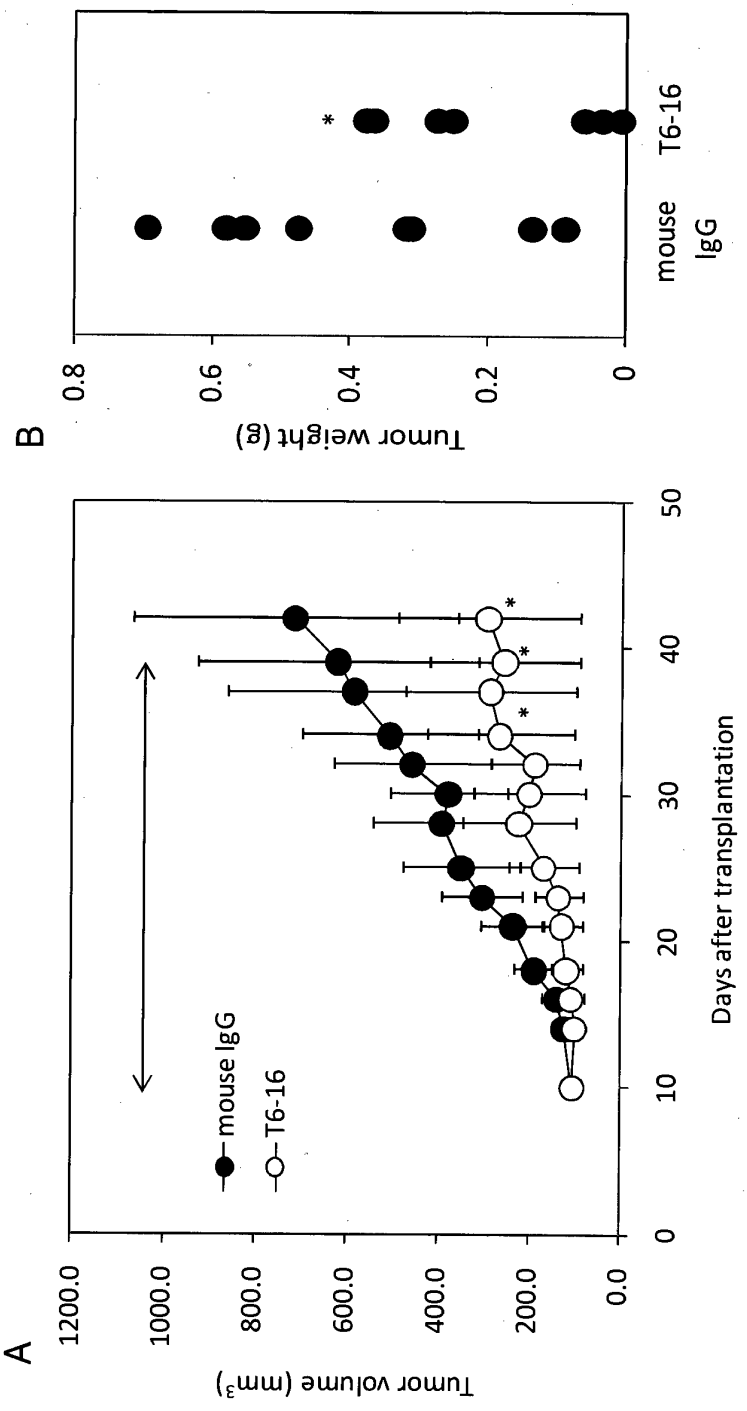

A
Tumor volume (mm3)
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
0
10
20
30
40
50
Days after transplantation
mouse IgG
T6-16
*
*
*
B
Tumor weight (g)
0.8
0.6
0.4
0.2
0
*
mouse IgG
T6-16

図26

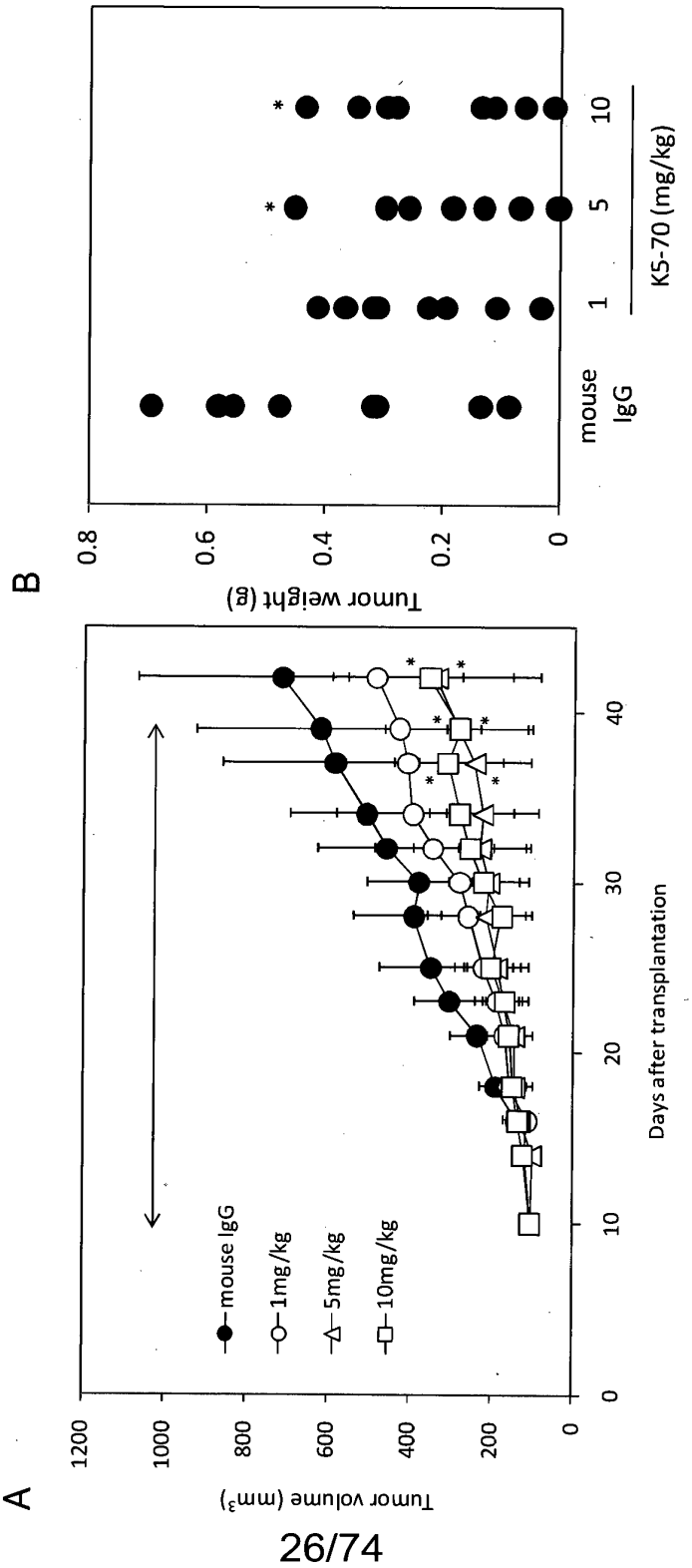

A
Tumor volume (mm3)
1200
1000
800
600
400
200
0
0
10
20
30
40
Days after transplantation
mouse IgG
1mg/kg
5mg/kg
10mg/kg
B
Tumor weight (g)
0.8
0.6
0.4
0.2
0
mouse IgG
1
5
10
K5-70 (mg/kg)

図27

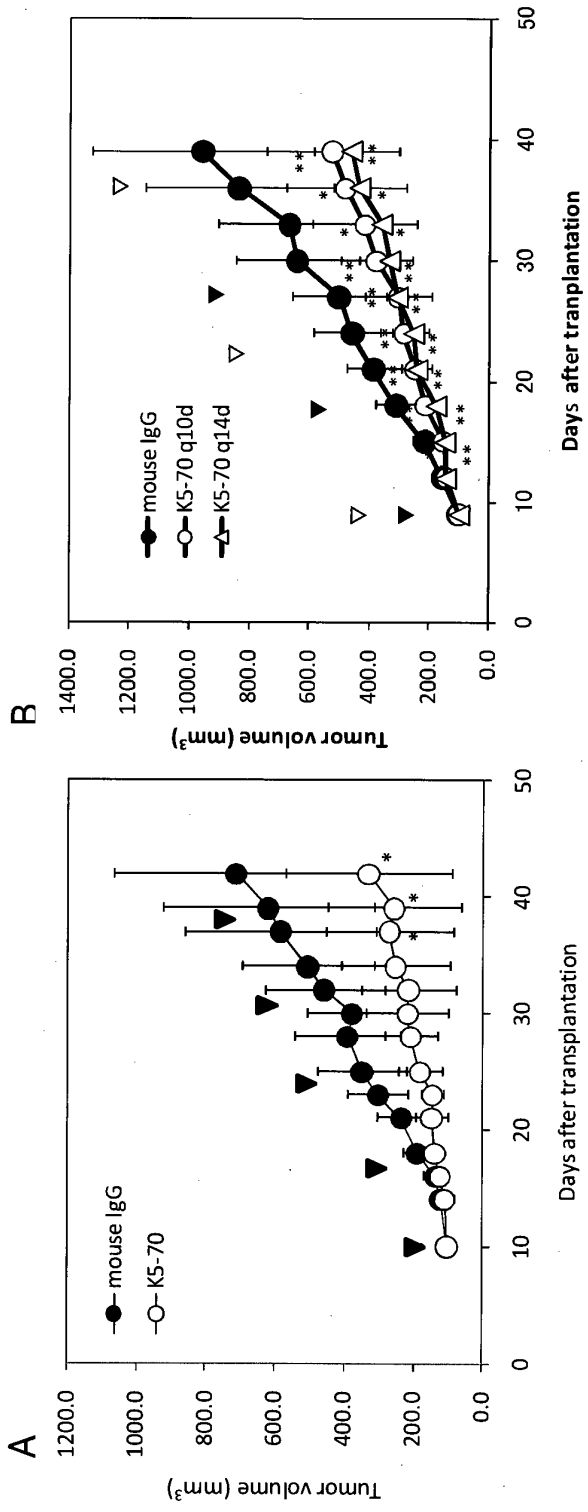
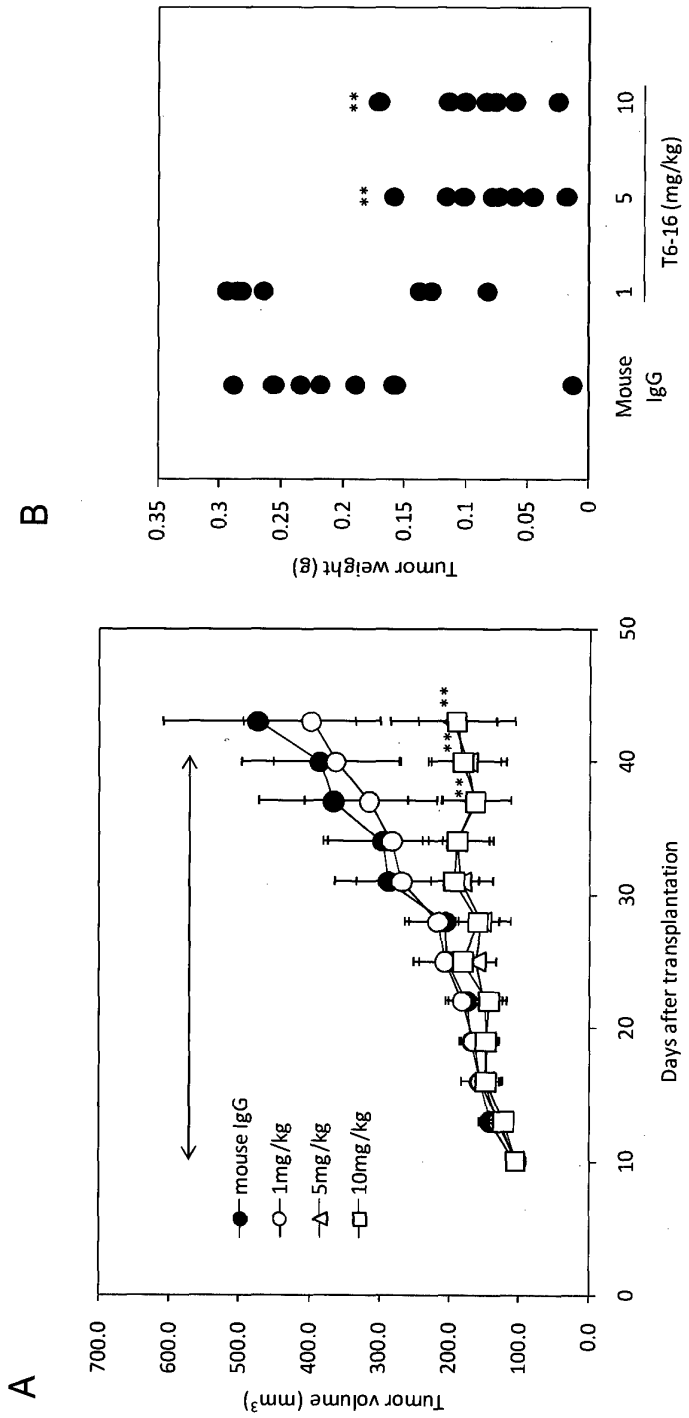

A
Tumor volume (mm3)
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
0
10
20
30
40
50
Days after transplantation
mouse IgG
K5-70
B
Tumor volume (mm3)
1400.0
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
0
10
20
30
40
50
Days after tranplantation
mouse IgG
K5-70 q10d
K5-70 q14d

図28


A
Tumor volume (mm3)
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
0
10
20
30
40
50
Days after transplantation
mouse IgG
1mg/kg
5mg/kg
10mg/kg
**
B
Tumor weight (g)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Mouse IgG
1
5
10
T6-16 (mg/kg)

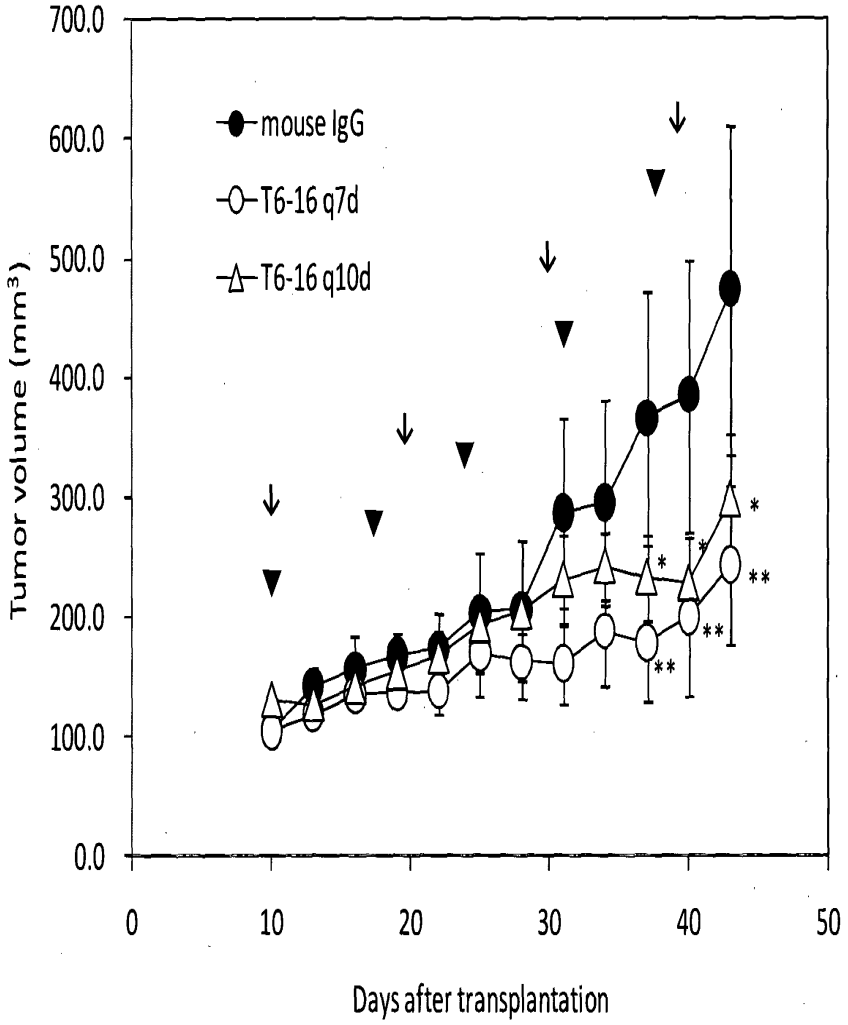
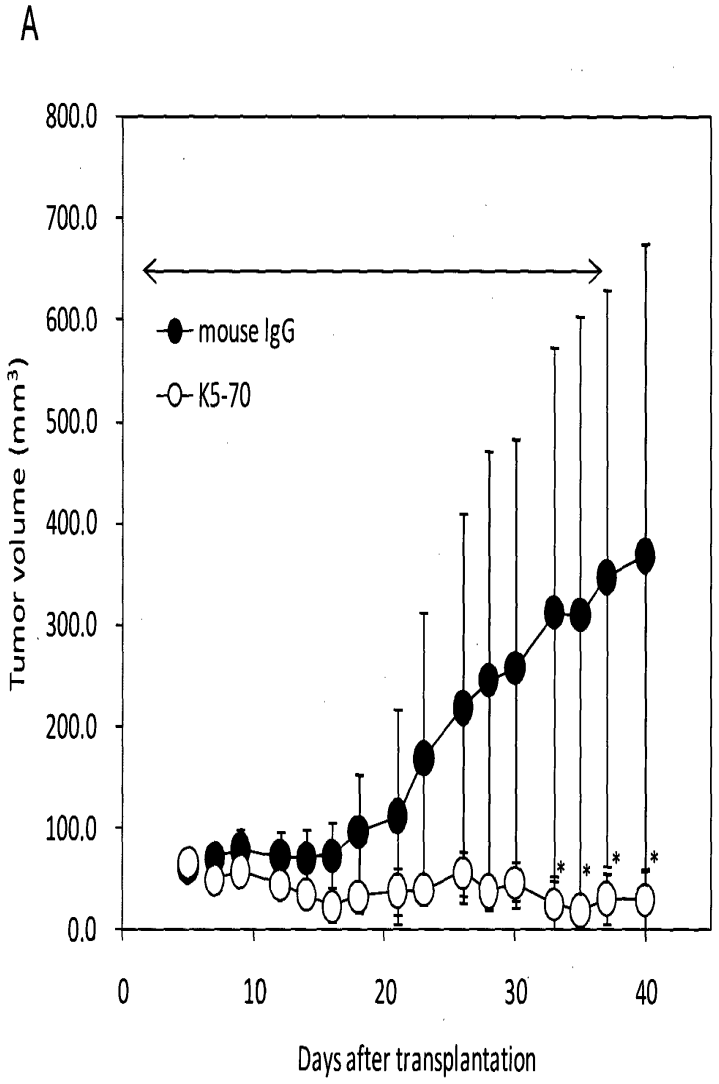

700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
Tumor volume (mm³)
mouse IgG
T6-16 q7d
T6-16 q10d
0
10
20
30
40
50
Days after transplantation
*
**

図29


A
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
Tumor volume (mm³)
mouse IgG
K5-70
0
10
20
30
40
Days after transplantation

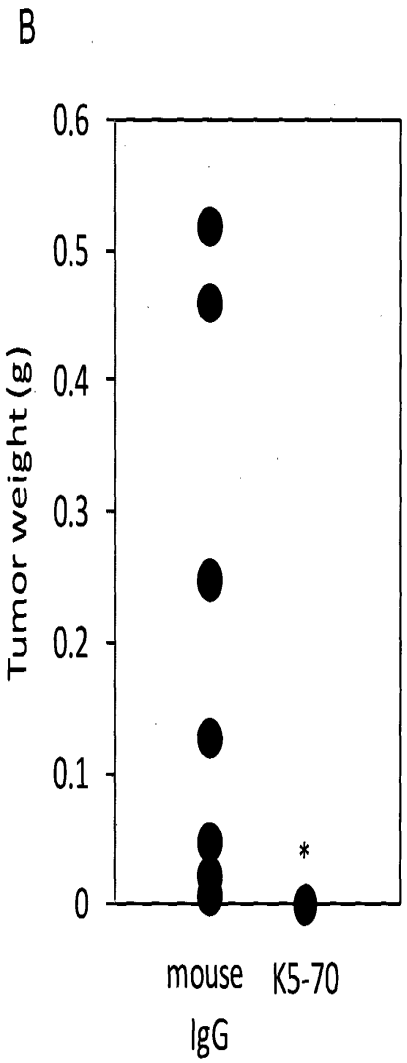
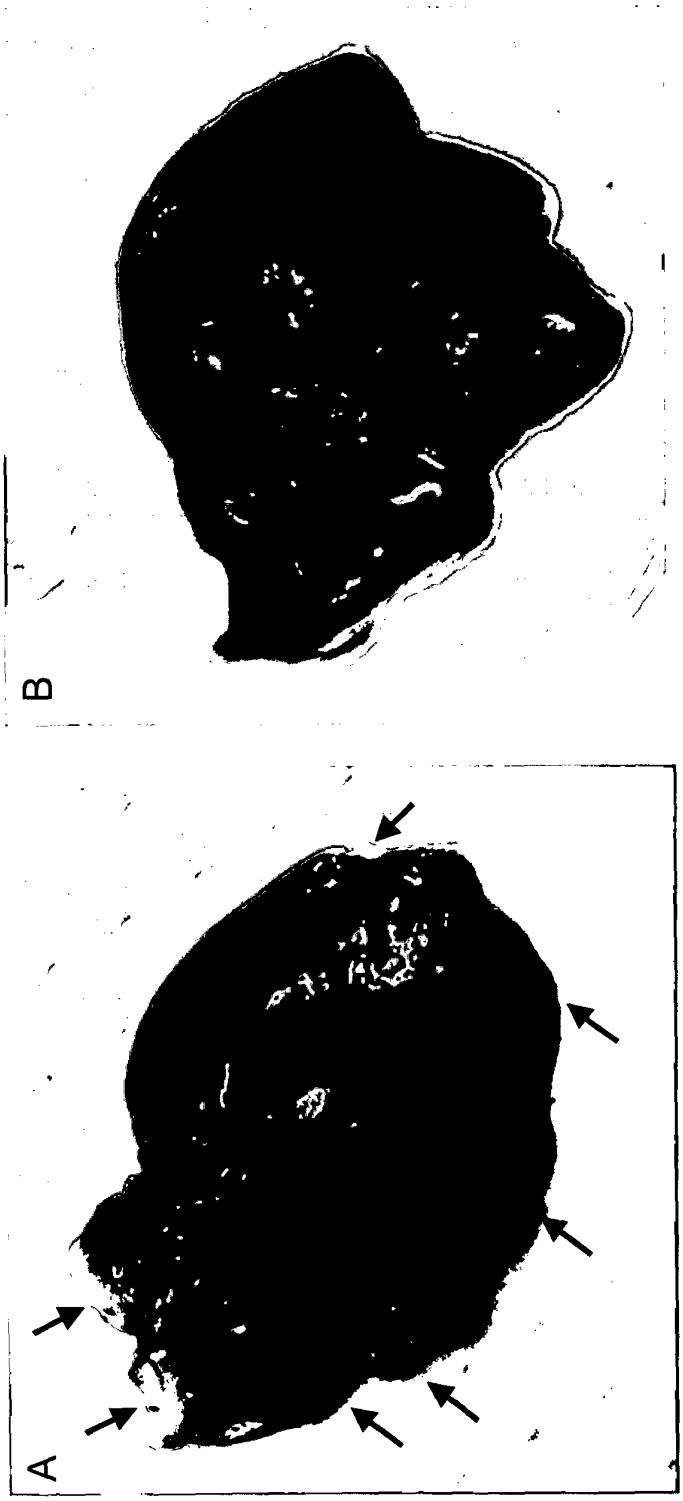

B
0.6
0.5
0.4
0.3
0.2
0.1
0
Tumor weight (g)
mouse IgG
K5-70

図30

図31



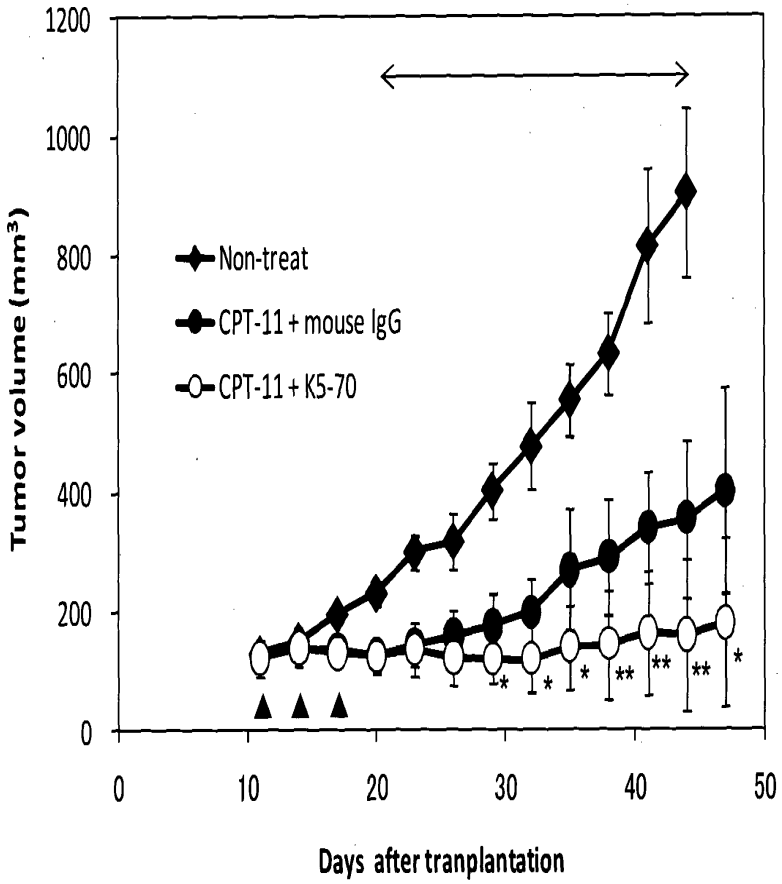

1200
1000
800
600
400
200
0
Tumor volume (mm³)
Non-treat
CPT-11 + mouse IgG
CPT-11 + K5-70
0
10
20
30
40
50
Days after tranplantation
*
*
*
**
**
**
*

図 32

K5-70
VH

ATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTGTCCACTCCCAG
M G W S C I I L F L V A T A T G V H S Q
GTCCAACTGCAGCAGCCTGGGGCTGAGCTGGTGAGGCCTGGGGCTTCAGTGAAGCTGTCC
V Q L Q Q P G A E L V R P G A S V K L S
TGCAAGGCTTCTGGCTACACCTTCACCATCTACTGGATAAACTGGGTGAAACAGAGGCCT
C K A S G Y T F T I Y W I N W V K Q R P
GGACAAGGCCTTGAGTGGATCGGAAATATTTATCCTTCTGATAGTTATACTAACTACAAT
G Q G L E W I G N I Y P S D S Y T N Y N
CAAAAGTTCAAGGACAAGGCCACATTGACTGTAGACAAATCCTCCAGCACAGCCTACATG
Q K F K D K A T L T V D K S S S T A Y M
CAGCTCAGCAGCCCGACATCTGAGGACTCTGCGGTCTATTACTGTACAAGAACGTCTATG
Q L S S P T S E D S A V Y Y C T R T S M
GCGGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
A D Y W G Q G T T L T V S S

図 33

K5-70
VL

ATGGTATCCACACCTCAGTTCCTTGTATTTTTGCTTTTCTGGATTCCAGCCTCCAGAGGT
M V S T P Q F L V F L L F W I P A S R G
GACATCTTGCTGACTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAAGAGTCAGT
D I L L T Q S P A I L S V S P G E R V S
TTCTCCTGCAGGGCCAGTCAGAGCATTGGCACAAGCATACACTGGTATCAGCAAAGAACA
F S C R A S Q S I G T S I H W Y Q Q R T
AATGGTTCTCCAAGGCTTCTCATAAAGTATGCTTCTGAGTCTATCTCTGGGATCCCTTCC
N G S P R L L I K Y A S E S I S G I P S
AGGTTTAGTGGCAGTGGATCAGGGACAGATTTTACTCTTAGCATCAACAGTGTGGAGTCT
R F S G S G S G T D F T L S I N S V E S
GAAGATATTGCAGATTATTACTGTCAACAAAGTAATAGCTGGCCATTCACGTTCGGCTCG
E D I A D Y Y C Q Q S N S W P F T F G S
GGGACAAAGTTGGAAATAAAA
G T K L E I K

図34

K5-107
VH

ATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTGTCCACTCCCAG
M G W S C I I L F L V A T A T G V H S Q
GTCCAACTGCAGCAACCTGGGTCTGAGCTGGTGAGGCCTGGAGCTTCAGTGAAGCTGTCC
V Q L Q Q P G S E L V R P G A S V K L S
TGCAAGGCTTCTGGCTACACATTCACCAGCTACTGGATGCACTGGGTGAAGCAGAGGCAT
C K A S G Y T F T S Y W M H W V K Q R H
GGACAAGGCCTTGAGTGGATTGGAAATATTTATCCTGGTGGTGGTTATACTAACTACGAT
G Q G L E W I G N I Y P G G G Y T N Y D
GAGAAGTTCAAGAGCAAGGGCACACTGACTGTAGACACATCCTCCAGCACAGCCTACATG
E K F K S K G T L T V D T S S S T A Y M
CACCTCAGCAGCCTGACATCTGAGGACTCTGCGGTCTATTACTGTACAAGATCATCCGTT
H L S S L T S E D S A V Y Y C T R S S V
TTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
F D Y W G Q G T T L T V S S

図35

K5-107
VL

ATGGTATCCACACCTCAGTTCCTTGTATTTTTGCTTTTCTGGATTCCAGCCTCCAGAGGT
M V S T P Q F L V F L L F W I P A S R G
GACATCTTGCTGACTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAAGAGTCAGT
D I L L T Q S P A I L S V S P G E R V S
TTCTCCTGCAGGGCCAGTCAGAACATTGGCACAAGCATACACTGGTTTCAGCAAAGAACA
F S C R A S Q N I G T S I H W F Q Q R T
AATGGTTCTCCAAGGCTTCTCATAAAGTATGCTTCTGAGTCTATCTCTGGGATCCCTTCC
N G S P R L L I K Y A S E S I S G I P S
AGGTTTAGTGGCAGTGGATCAGGGACAGATTTTACTCTTAGCATCAACAGTGTGGAGTCT
R F S G S G S G T D F T L S I N S V E S
GAAGATATTGCAGATTATTACTGTCAACAAAGTAATAGCTGGCCATTCACGTTCGGCTCG
E D I A D Y Y C Q Q S N S W P F T F G S
GGGACAAAGTTGGAAATAAAA
G T K L E I K

図36

K5-116-2-1
VH

ATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTGTCCACTCCCAG

M G W S C I I L F L V A T A T G V H S Q

GTCCAACTGCAGCAGCCTGGGGCTGAGCTGGTGAGGCCTGGGGCTTCAGTGAAGCTGTCC

V Q L Q Q P G A E L V R P G A S V K L S

TGCAAGGCTTCTGGCTACACCTTCACCAGCTACTGGATAACCTGGGTGAAGCAGAGGCCT

C K A S G Y T F T S Y W I T W V K Q R P

GGACAAGGCCTTGAGTGGATCGGAAATATTTATCCTTCTGATAGTTATACTAACTACAAT

G Q G L E W I G N I Y P S D S Y T N Y N

CAAAAGTTCAGGGACAAGGCCACATTGACTGTAGACAAATCCTCCAGTACAGCCTACATG

Q K F R D K A T L T V D K S S S T A Y M

CAGCTCAGCAGCCCGACATCTGAGGACTCTGCGGTCTATTACTGTTCAGCCCTCTTTGAC

Q L S S P T S E D S A V Y Y C S A L F D

TACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA

Y W G Q G T T L T V S S

図37

K5-116-2-1
VL

ATGGTATCCACACCTCAGTTCCTTGTATTTTTGCTTTTCTGGATTCCAGCCTCCAGAGGT
M V S T P Q F L V F L L F W I P A S R G
GACATCTTGCTGACTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAAGAGTCAGT
D I L L T Q S P A I L S V S P G E R V S
TTCTCCTGCAGGGCCAGTCAGAGCATTGGCACAAGCATACACTGGTATCAGCAAAGAACA
F S C R A S Q S I G T S I H W Y Q Q R T
AATGGTTCTCCAAGGCTTCTCATAAAGTATGCTTCTGAGTCTATCTCTGGGATCCCTTCC
N G S P R L L I K Y A S E S I S G I P S
AGGTTTAGTGGCAGTGGATCAGGGACAGATTTTATTCTTAGCATCAACAGTGTGGAGTCT
R F S G S G S G T D F I L S I N S V E S
GAAGATATTGCAGATTATTACTGTCAACAAAGTAATAGCTGGCCATTCACGTTCGGCTCG
E D I A D Y Y C Q Q S N S W P F T F G S
GGGACAAAGTTGGAAATAAAA
G T K L E I K

図38

T6-16
VH

ATGGGATGGAGCTGGATCTTTCTCTTCCTCCTGTCAGGAACTGCAGGCGTCCACTCTGAG
M G W S W I F L F L L S G T A G V H S E
GTCCAGCTTCAGCAGTCAGGACCTGAGCTGGTGAAACCTGGGGCCTCAGTGAAGATTTCC
V Q L Q Q S G P E L V K P G A S V K I S
TGCAAGGCTTCTGGATACACATTCACTGACTACAATATGCACTGGGTGAAGCAGAGCCAT
C K A S G Y T F T D Y N M H W V K Q S H
GGAAAGAACCTTGAATGGATTGGATATATTTATCCTTACAATGGTGGTACTGGCTACAAC
G K N L E W I G Y I Y P Y N G G T G Y N
CAGAGGTTCAAGAGCAGGGCCACAATGACTGTAGACAAATCCTCCAGCACAGCCTACATG
Q R F K S R A T M T V D K S S S T A Y M
GAGCTCCGCAGCCTGACATCTGATGACTCTGCAGTCTATTACTGTGCAAGAGAAGACTAC
E L R S L T S D D S A V Y Y C A R E D Y
GGTAGTAGCCCCTCTTATGCTATGGACTATTGGGGTCAAGGAACCTCAGTCATCGTCTCC
G S S P S Y A M D Y W G Q G T S V I V S
TCA
S

図 39

T6-16
VL

ATGAAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCCTGCTTCCAGCAGTGAT
M K L P V R L L V L M F W I P A S S S D
GTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAGGCCTCCATC
V V M T Q T P L S L P V S L G D Q A S I
TCTTGCAGATCTAGTCAGAGCCTTGTACACGGTAATGGAAACACCTATTTACATTGGTAC
S C R S S Q S L V H G N G N T Y L H W Y
CTGCAGAAGCCAGGCCAGTCTCCAAAGCTCCTGATCTACAAAGTTTCCAACCGATTTTCT
L Q K P G Q S P K L L I Y K V S N R F S
GGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACGGATTTCACACTCAAGATCAGC
G V P D R F S G S G S G T D F T L K I S
AGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGCTCTCAAACTACACATGTTCCCACG
R V E A E D L G V Y F C S Q T T H V P T
TTCGGCTCGGGGACAAAGTTGGAAATAAAA
F G S G T K L E I K

図40

図41

```
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DA980102 VH  QVQLVQSGA EVKKPGASVK VSCKASGYTF T-----WVRQ APGQRLEWMG
            5          6         7         8
            01223456789 0123456789 0123456789 0122223456789
              a                                    abc
  K5-70 VH  NIYPSDSYTNY NQKFKDKATL TVDKSSSTAY MQLSSPTSEDSAV
 HuK5-70 VH  NIYPSDSYTNY NQKFKDKATL TVDTSASTAY MELSSLRSEDTAV
DA980102 VH  ----------- ------RVTL TSDTSASTAY MEMSSLRSEDTAV
                      1         1
            9         0         1
            0123456789 0123456789 0123
  K5-70 VH  YYCTRTSMA- -DYWGQGTTL TVSS
 HuK5-70 VH  YYCTRTSMA- -DYWGQGTLV TVSS
DA980102 VH  VHYYCAR----- -WGQGTLV TVSS
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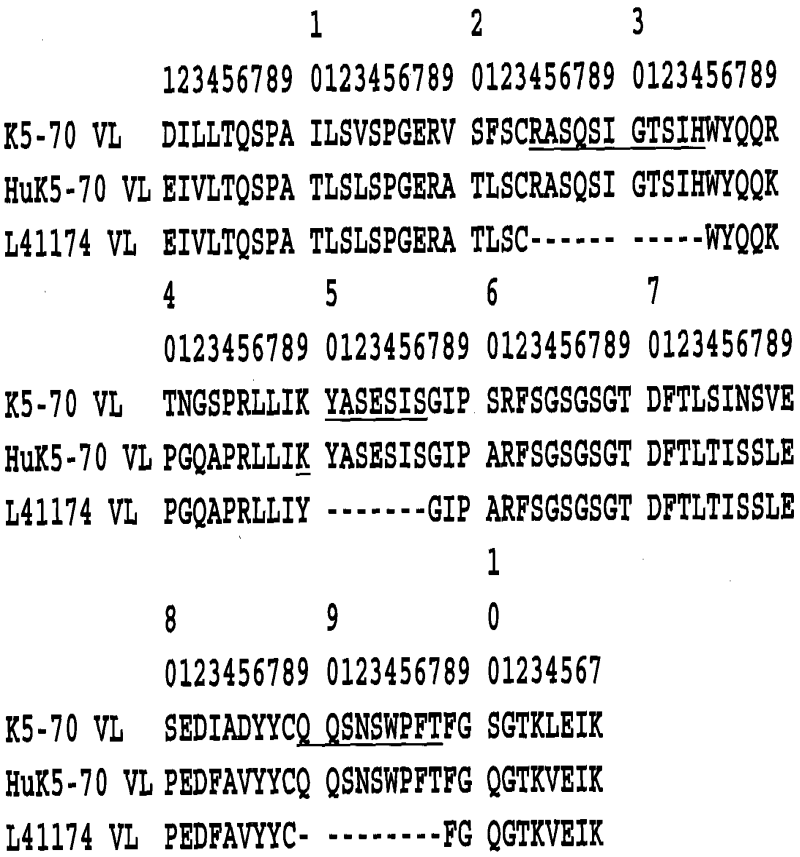

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HuK5-70 VL EIVLTQSPA TLSLSPGERA TLSCRASQSI GTSIHWYQQK
L41174 VL EIVLTQSPA TLSLSPGERA TLSC------ -----WYQQK
4 5 6 7
0123456789 0123456789 0123456789 0123456789
K5-70 VL TNGSPRLLIK YASESISGIP SRFSGSGSGT DFTLSINSVE
HuK5-70 VL PGQAPRLLIK YASESISGIP ARFSGSGSGT DFTLTISSLE
L41174 VL PGQAPRLLIY -------GIP ARFSGSGSGT DFTLTISSLE
1
8 9 0
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図 42

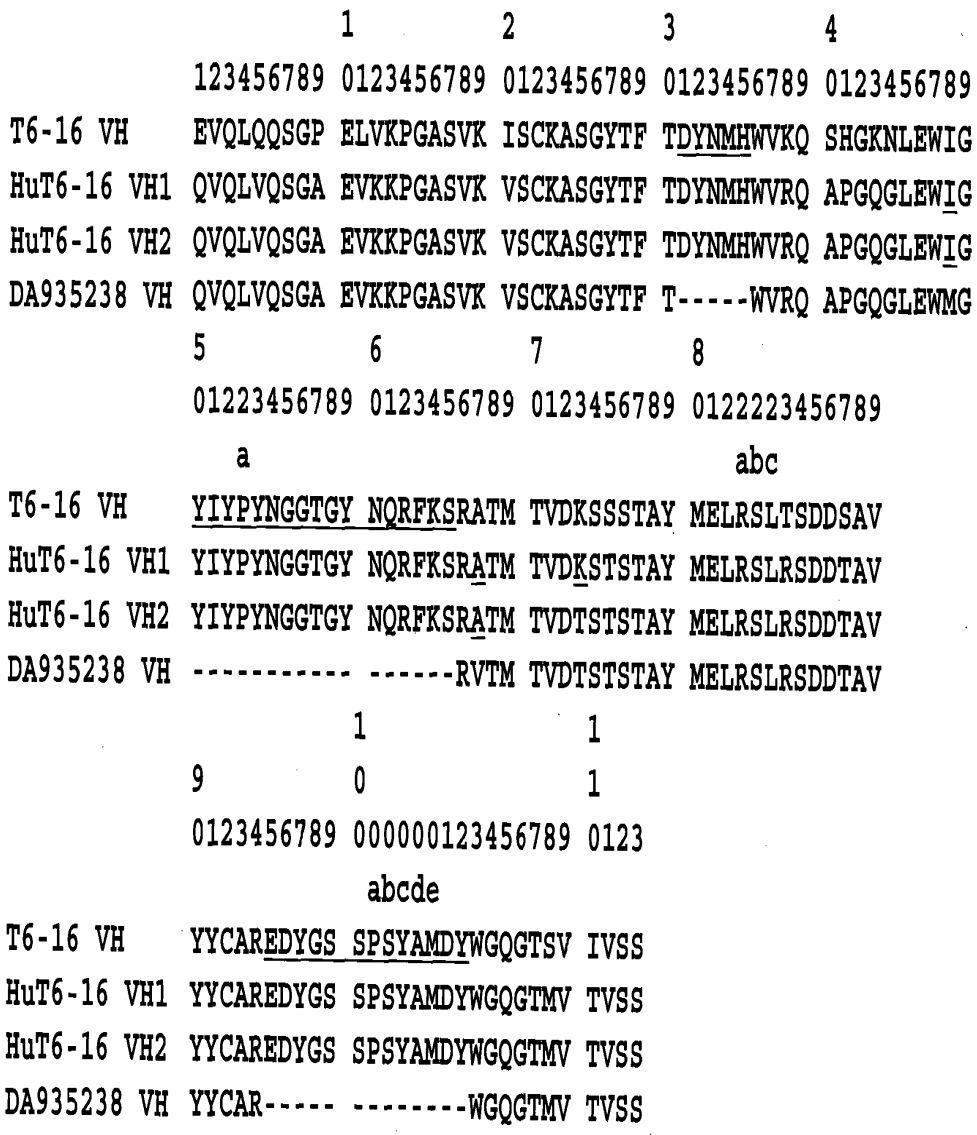

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HuT6-16 VH1 QVQLVQSGA EVKKPGASVK VSCKASGYTF TDYNMHWVRQ APGQGLEWIG
HuT6-16 VH2 QVQLVQSGA EVKKPGASVK VSCKASGYTF TDYNMHWVRQ APGQGLEWIG
DA935238 VH QVQLVQSGA EVKKPGASVK VSCKASGYTF T-----WVRQ APGQGLEWMG
5 6 7 8
01223456789 0123456789 0123456789 0122223456789
a abc
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HuT6-16 VH1 YIYPYNGGTGY NQRFKSRATM TVDKSTSTAY MELRSLRSDDTAV
HuT6-16 VH2 YIYPYNGGTGY NQRFKSRATM TVDTSTSTAY MELRSLRSDDTAV
DA935238 VH ---------- ------RVTM TVDTSTSTAY MELRSLRSDDTAV
1 1
9 0 1
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abcde
T6-16 VH YYCAREDYGS SPSYAMDYWGQGTSV IVSS
HuT6-16 VH1 YYCAREDYGS SPSYAMDYWGQGTMV TVSS
HuT6-16 VH2 YYCAREDYGS SPSYAMDYWGQGTMV TVSS
DA935238 VH YYCAR----- --------WGQGTMV TVSS

図 43

図44

```
                    1         2              3
          123456789 0123456789 012345677777789 0123456789
                                         abcde
T6-16 VL  DVVMTQTPL SLPVSLGDQA SISCRSSQSLVHGNG NTYLHWYLQK
HuT6-16 VL DIVMTQSPL SLPVTPGEPA SISCRSSQSLVHGNG NTYLHWYLQK
M99608 VL DIVMTQSPL SLPVTPGEPA SISC---------- -----WYLQK
          4         5         6         7
          0123456789 0123456789 0123456789 0123456789
T6-16 VL  PGQSPKLLIY KVSNRFSGVP DRFSGSGSGT DFTLKISRVE
HuT6-16 VL PGQSPQLLIY KVSNRFSGVP DRFSGSGSGT DFTLKISRVE
M99608 VL PGQSPQLLIY -------GVP DRFSGSGSGT DFTLKISRVE
                              1
          8         9         0
          0123456789 0123456789 01234567
T6-16 VL  AEDLGVYFCS QTTHVP-TFG SGTKLEIK
HuT6-16 VL AEDVGVYYCS QTTHVP-TFG GGTKVEIK
M99608 VL AEDVGVYYC----------FG GGTKVEIK
```

HuK5-70
VH

GAA TTC ACC ACC ATG GGG TGG AGC TGC ATT ATC CTG TTT CTT GTC GCA ACT GCA ACA GGC
M G W S C I I L F L V A T A T G

GTT CAC TCA CAG GTT CAG CTA GTC CAG TCT GGA GCT GAG GTG AAG AAA CCA GGG GCA TCT
V H S Q V Q L V Q S G A E V K K P G A S

GTC AAA GTG AGC TGT AAG GCC TCT GGC TAT ACG TTC ACG ATA TAC TGG ATC AAT TGG GTG
V K V S C K A S G Y T F T I Y W I N W V

AGG CAA GCT CCT GGA CAA CGG TTG GAA TGG ATT GGC AAC ATC TAT CCC TCA GAC TCC TAC
R Q A P G Q R L E W I G N I Y P S D S Y

ACC AAC TAC AAT CAG AAG TTC AAG GAC AAA GCC ACT CTC ACC GTA GAT ACC AGT GCC TCC
T N Y N Q K F K D K A T L T V D T S A S

ACA GCC TAT ATG GAG CTG AGC AGT TTA CGC AGC GAA GAT ACA GCG GTG TAC TAC TGC ACC
T A Y M E L S S L R S E D T A V Y Y C T

AGA ACC TCC ATG GCT GAC TAT TGG GGT CAG GGT ACA CTG GTG ACT GTG AGC TCC GCT AGC
R T S M A D Y W G Q G T L V T V S S

図45

図46

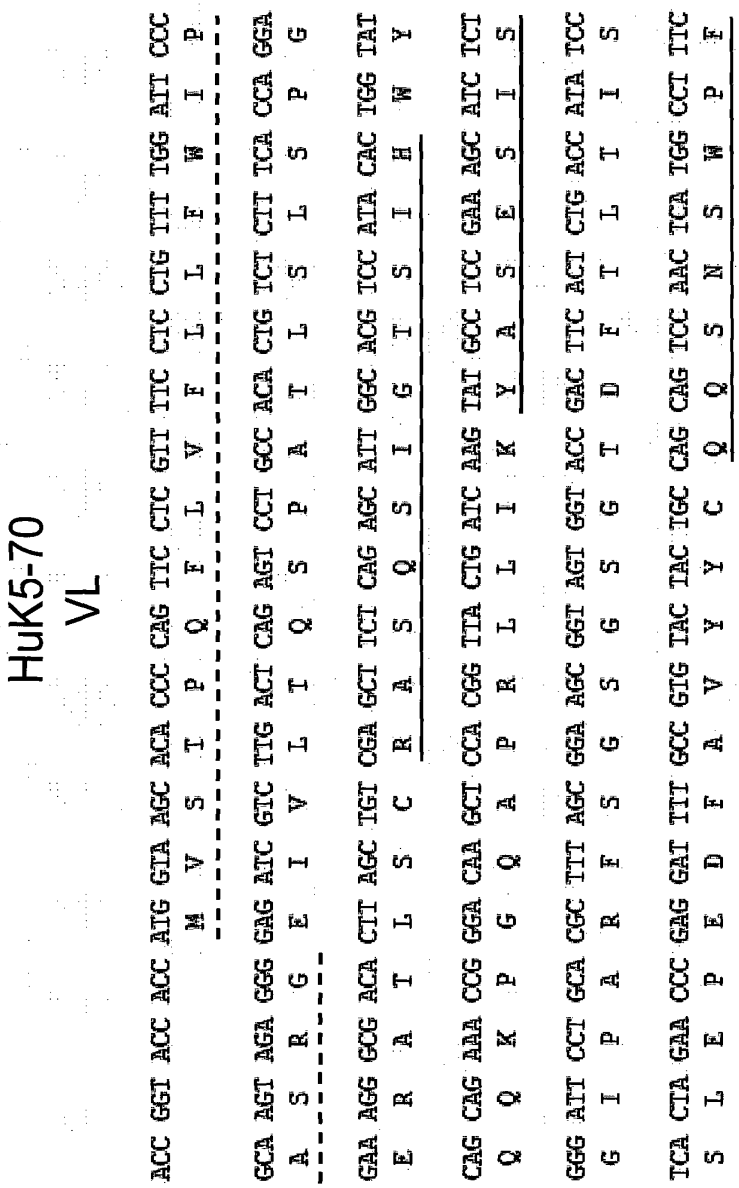

HuK5-70
VL
ACC GGT ACC ACC ATG GTA AGC ACA CCC CAG TTC CTC CTC GTT TTC CTC CTG TTT TGG ATT CCC
M V S T P Q F L L V F L L F W I P
GCA AGT AGA GGG GAG ATC GTC TTG ACT CAG AGT CCT GCC ACA CTG TCT CTT TCA CCA GGA
A S R G E I V L T Q S P A T L S L S P G
GAA AGG GCG ACA CTT AGC TGT CGA GCT TCT CAG AGC ATT GGC ACG TCC ATA CAC TGG TAT
E R A T L S C R A S Q S I G T S I H W Y
CAG CAG AAA CCG GGA CAA GCT CCA CGG TTA CTG ATC AAG TAT GCC TCC GAA AGC ATC TCT
Q Q K P G Q A P R L L I K Y A S E S I S
GGG ATT CCT GCA CGC TTT AGC GGA AGC GGT AGT GGT ACC GAC TTC ACT CTG ACC ATA TCC
G I P A R F S G S G S G T D F T L T I S
TCA CTA GAA CCC GAG GAT TTT GCC GTG TAC TAC TGC CAG CAG TCC AAC TCA TGG CCT TTC
S L E P E D F A V Y Y C Q Q S N S W P F
ACC TTT GGC CAA GGC ACC AAA GTG GAG ATC AAG CGT ACG
T F G Q G T K V E I K

図47

HuT6-16
VH1

```
GAA TTC ACC ACC ATG GGA TGG TCT TGG ATC TTT CTC TTC CTG CTG TCT GGC ACA GCT GGA
          M   G   W   S   W   I   F   L   F   L   L   S   G   T   A   G
          ----------------------------------------------------------------

GTG CAT TCC CAA GTT CAG CTG GTC CAG TCC GGA GCT GAA GTT AAA AAG CCC GGG GCC AGC
V   H   S   Q   V   Q   L   V   Q   S   G   A   E   V   K   K   P   G   A   S
----------.

GTC AAA GTC TCC TGC AAG GCA TCC GGG TAT ACT TTT ACC GAT TAT AAC ATG CAC TGG GTG
V   K   V   S   C   K   A   S   G   Y   T   F   T   D   Y   N   M   H   W   V

CGC CAA GCA CCC GGC CAG GGC CTG GAG TGG ATT GGC TAT ATC TAT CCT TAT AAT GGA GGG
R   Q   A   P   G   Q   G   L   E   W   I   G   Y   I   Y   P   Y   N   G   G

ACC GGC TAC AAC CAG AGA TTC AAG AGC AGA GCC ACC ATG ACA GTG GAT AAA TCT ACC AGC
T   G   Y   N   Q   R   F   K   S   R   A   T   M   T   V   D   K   S   T   S

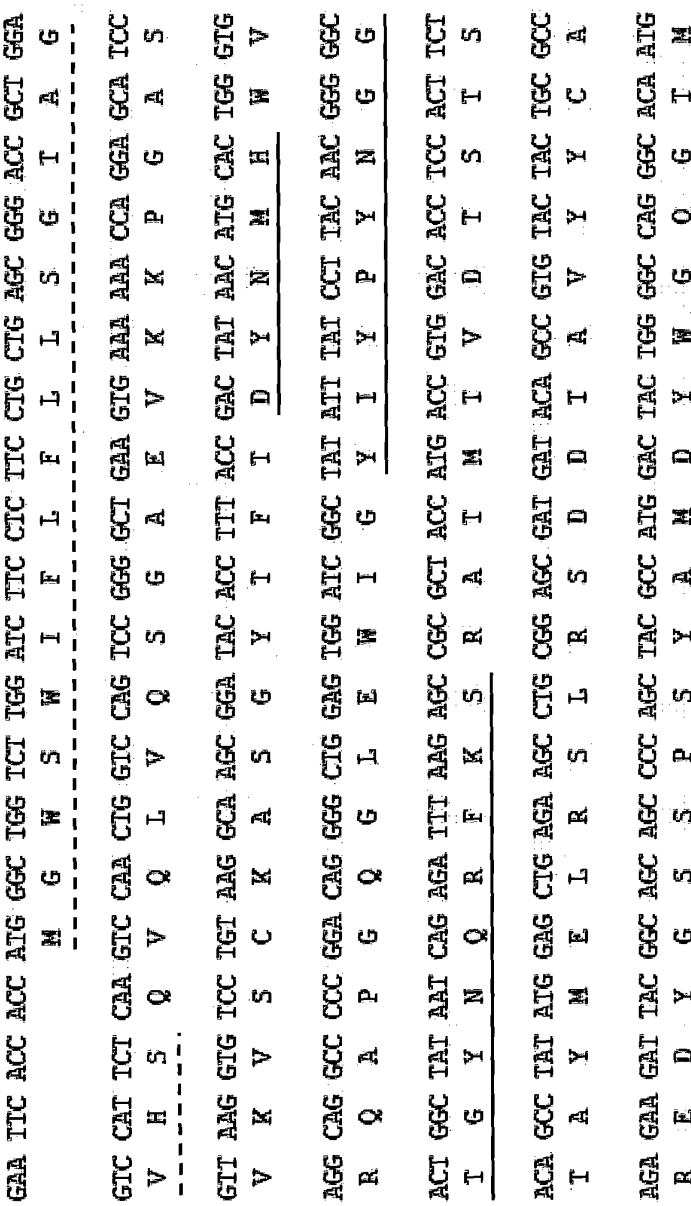
ACT GCC TAC ATG GAG CTG AGA AGC CTG CGG AGC GAC GAC ACA GCC GTG TAC TAC TGT GCC
T   A   Y   M   E   L   R   S   L   R   S   D   D   T   A   V   Y   Y   C   A

CGC GAG GAT TAC GGA AGC AGC CCA AGC TAC GCC ATG GAT TAC TGG GGC CAA GGC ACT ATG
R   E   D   Y   G   S   S   P   S   Y   A   M   D   Y   W   G   Q   G   T   M

GTG ACC GTG AGC AGC GCT AGC
V   T   V   S   S
```

図48

HuT6-16
VH2



HuT6-16
VL

```
ACC GGT ACC ACC ATG AAG CTC CCA GTG CGC CTC CTG GTC CTG ATG TTC TGG ATT CCC GCT
            M   K   L   P   V   R   L   L   V   L   M   F   W   I   P   A
            ----------------------------------------------------------------

TCC TCT AGC GAT ATC GTC ATG ACC CAA TCC CCA CTG TCT CTG CCT GTG ACA CCA GGC GAA
 S   S   S   D   I   V   M   T   Q   S   P   L   S   L   P   V   T   P   G   E
 ---------

CCT GCA TCT ATT AGC TGT AGA AGC AGC CAG TCC CTG GTG CAC GGA AAC GGA AAC ACC TAT
 P   A   S   I   S   C   R   S   S   Q   S   L   V   H   G   N   G   N   T   Y

CTG CAC TGG TAC CTG CAA AAA CCT GGA CAG AGC CCC CAG CTG CTG ATC TAC AAA GTC AGC
 L   H   W   Y   L   Q   K   P   G   Q   S   P   Q   L   L   I   Y   K   V   S

AAT AGA TTT AGC GGG GTG CCC GAC AGG TTT AGC GGC AGC GGC AGC GGC ACA GAT TTC ACC
 N   R   F   S   G   V   P   D   R   F   S   G   S   G   S   G   T   D   F   T

CTG AAA ATC TCC CGG GTG GAA GCC GAG GAC GTT GGG GTT TAC TAT TGC AGC CAG ACA ACC
 L   K   I   S   R   V   E   A   E   D   V   G   V   Y   Y   C   S   Q   T   T

CAT GTG CCC ACT TTC GGG GGC GGC ACT AAG GTG GAG ATC AAG CGT ACG
 H   V   P   T   F   G   G   G   T   K   V   E   I   K
```

(A)
1 2
122kDa
86kDa
H-chain
40kDa
32kDa
L-chain
WB: anti-human Ig-HRP
(B)
3 4
86kDa
H-chain
WB: anti-human IgG Fc
40kDa
32kDa
L-chain
WB: anti-human IgG F(ab')2

図 50

図51

図52

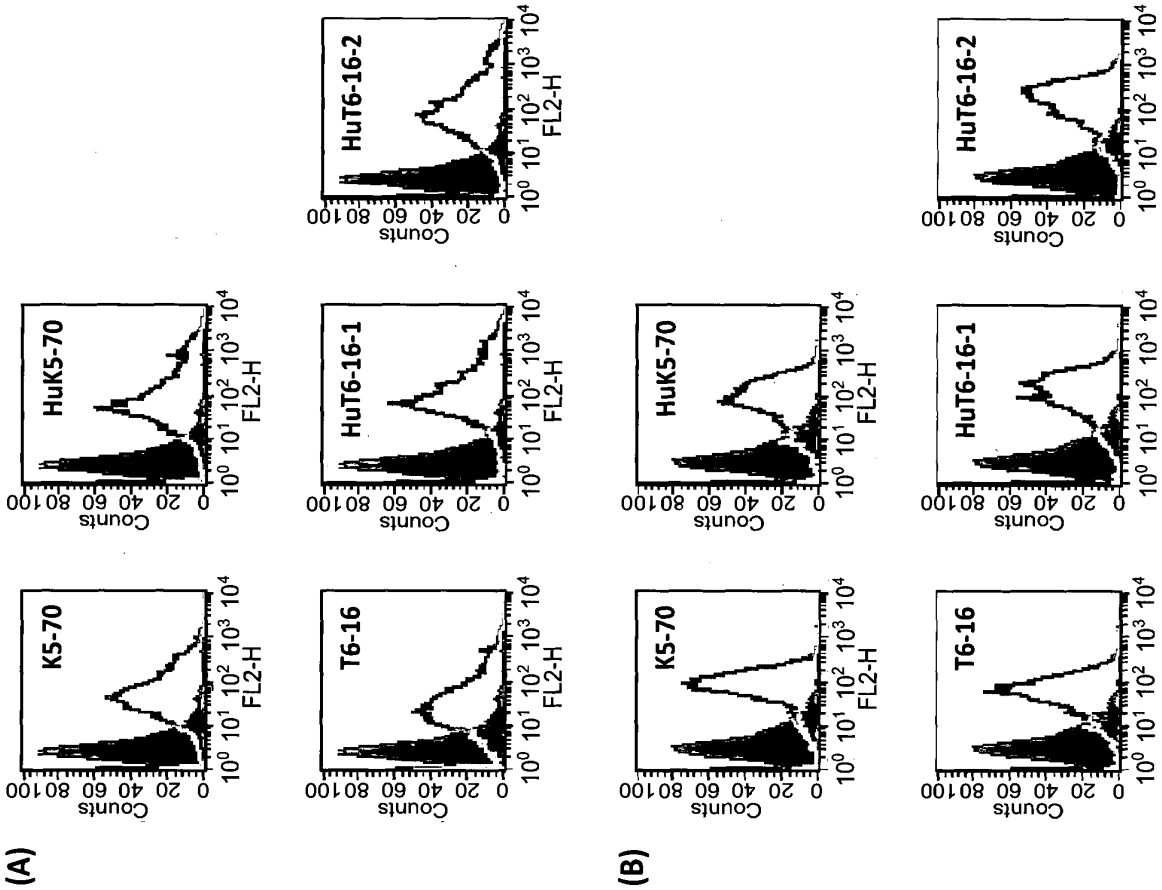


図53

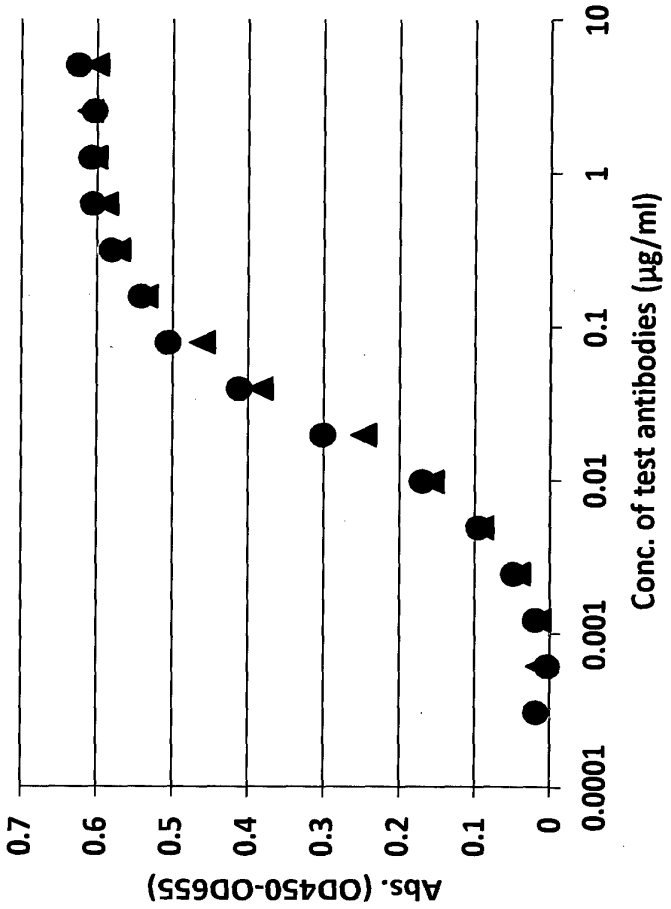

Abs. (OD450-OD655)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.0001
0.001
0.01
0.1
1
10
Conc. of test antibodies (μg/ml)

図54

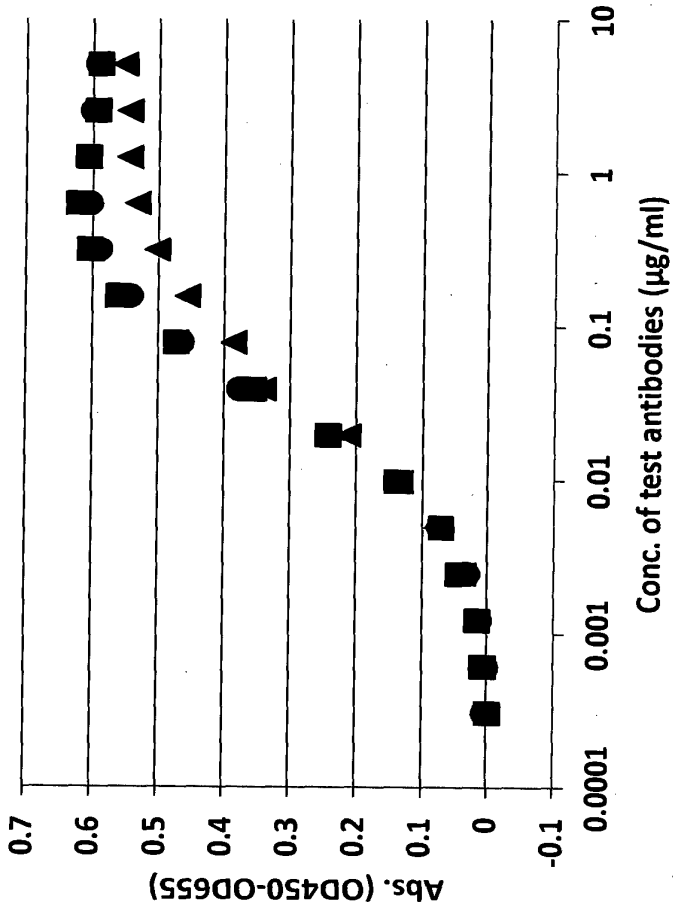

Abs. (OD450-OD655)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
0.0001
0.001
0.01
0.1
1
10
Conc. of test antibodies (μg/ml)

図55

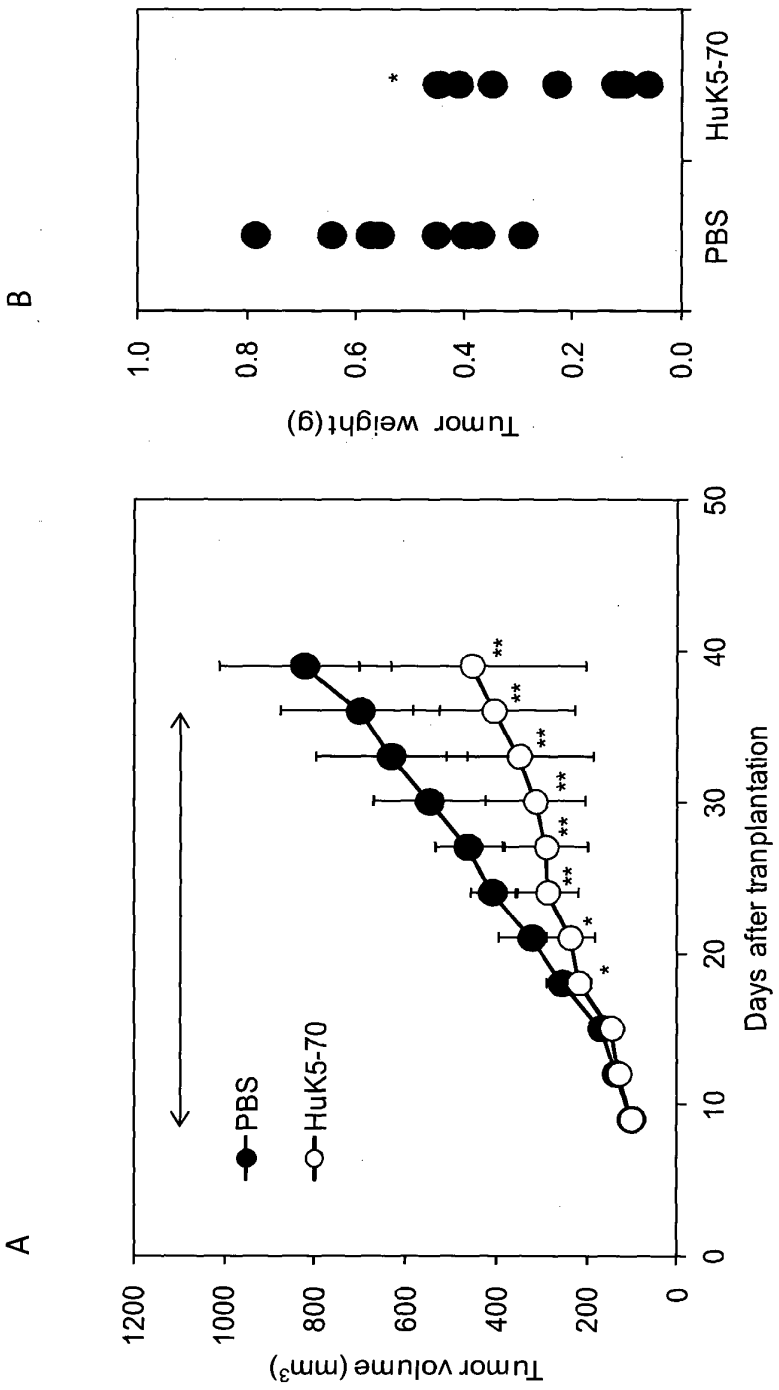

A
Tumor volume (mm3)
1200
1000
800
600
400
200
0
0
10
20
30
40
50
Days after tranplantation
PBS
HuK5-70
B
Tumor weight(g)
1.0
0.8
0.6
0.4
0.2
0.0
PBS
HuK5-70

図56

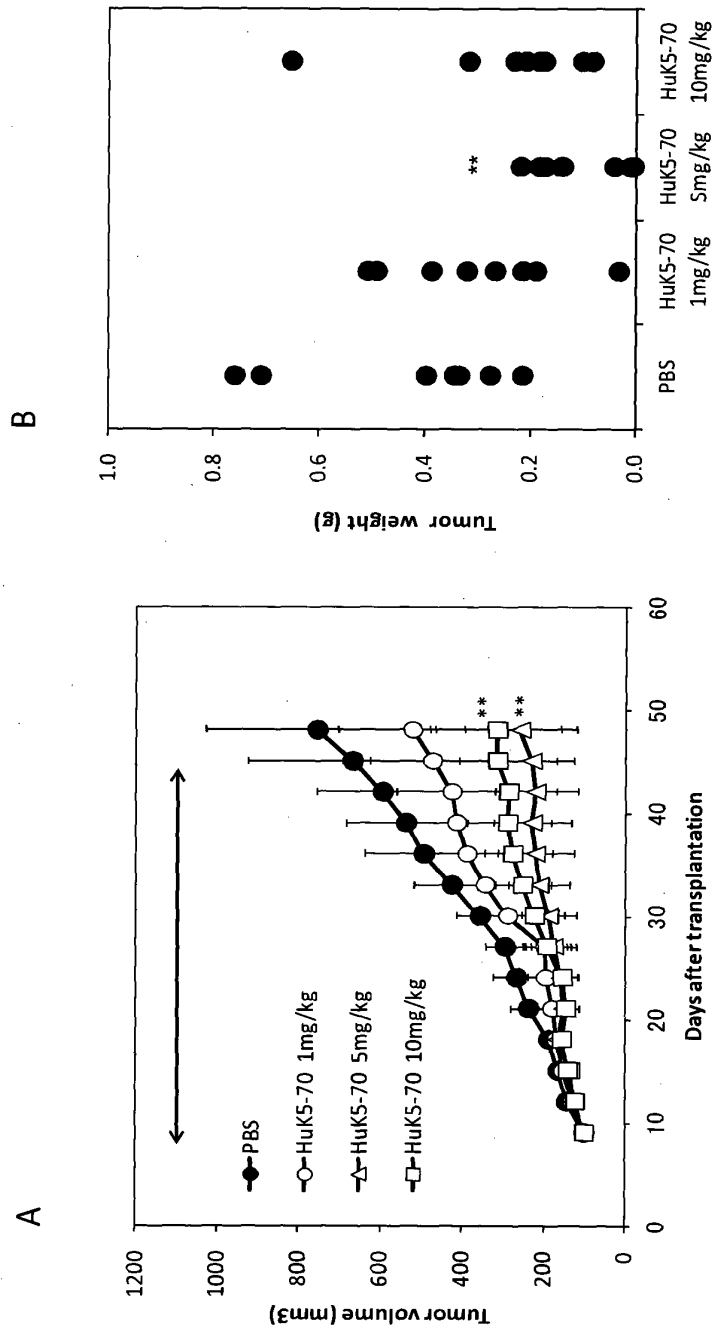

A
Tumor volume (mm3)
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
Days after transplantation
PBS
HuK5-70 1mg/kg
HuK5-70 5mg/kg
HuK5-70 10mg/kg
**
**
B
Tumor weight (g)
1.0
0.8
0.6
0.4
0.2
0.0
PBS
HuK5-70 1mg/kg
HuK5-70 5mg/kg
HuK5-70 10mg/kg
**

図57

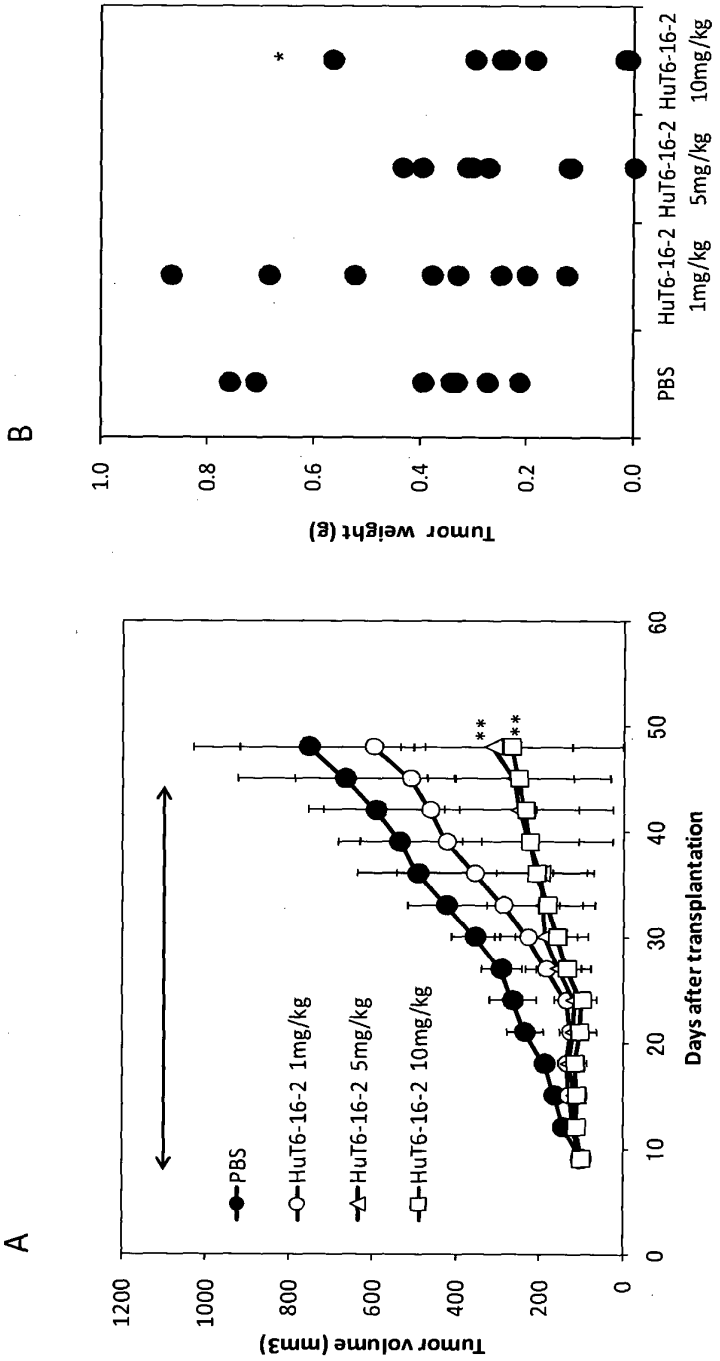

A
Tumor volume (mm3)
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
Days after transplantation
PBS
HuT6-16-2 1mg/kg
HuT6-16-2 5mg/kg
HuT6-16-2 10mg/kg
**
**
B
Tumor weight (g)
1.0
0.8
0.6
0.4
0.2
0.0
*
PBS
HuT6-16-2 1mg/kg
HuT6-16-2 5mg/kg
HuT6-16-2 10mg/kg

図58

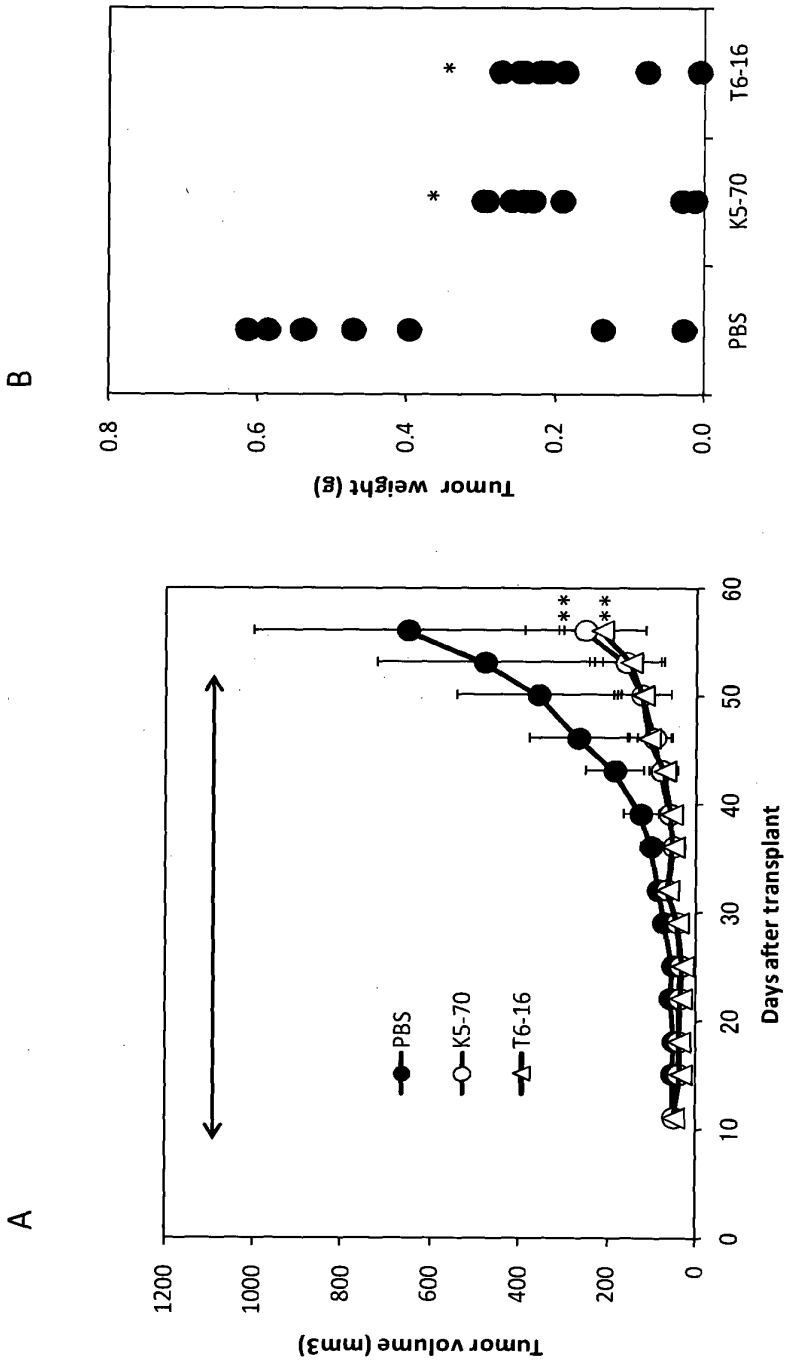

A
Tumor volume (mm3)
0
200
400
600
800
1000
1200
0
10
20
30
40
50
60
Days after transplant
PBS
K5-70
T6-16
**
**
B
Tumor weight (g)
0.0
0.2
0.4
0.6
0.8
PBS
K5-70
T6-16
*
*

図59

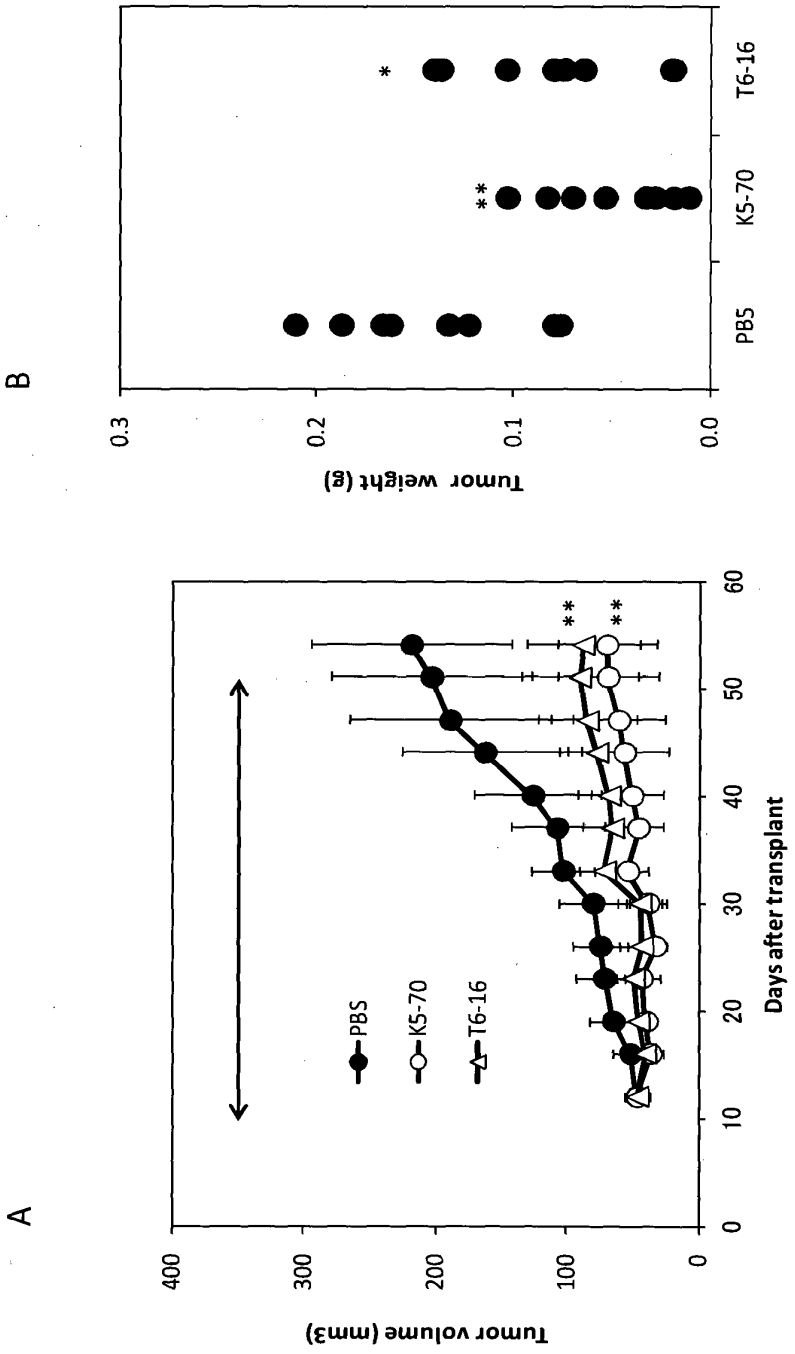

A
Tumor volume (mm3)
400
300
200
100
0
0
10
20
30
40
50
60
Days after transplant
PBS
K5-70
T6-16
**
**
B
Tumor weight (g)
0.3
0.2
0.1
0.0
PBS
K5-70
T6-16
*
**

図60

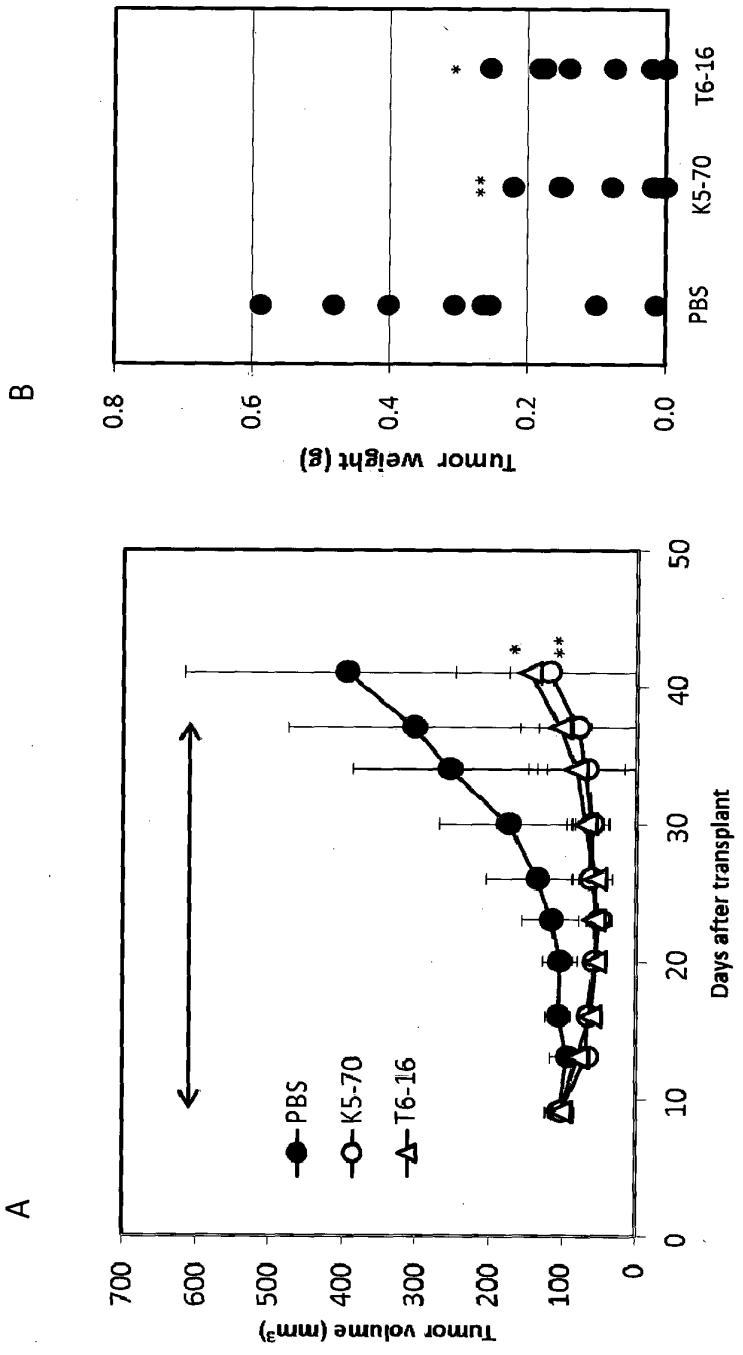
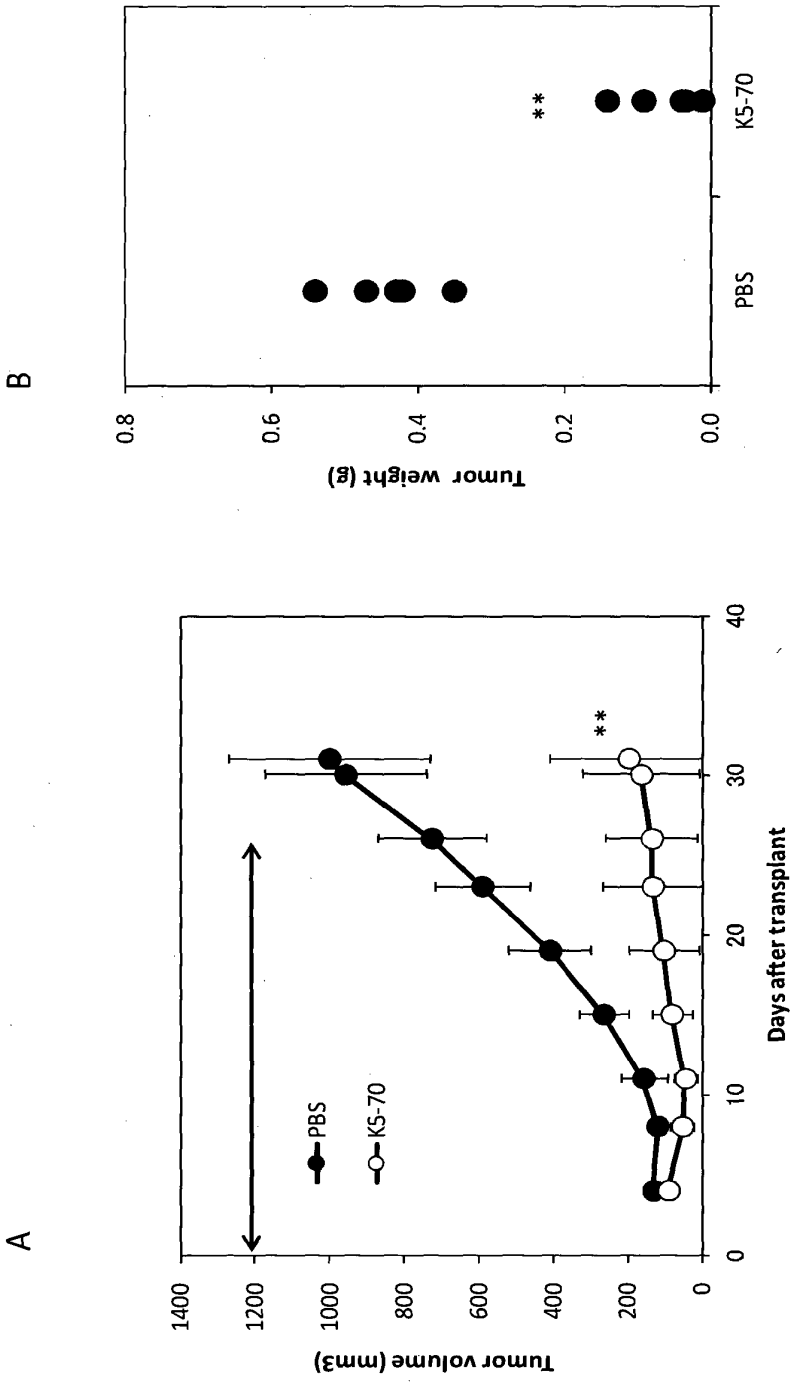

A
Tumor volume (mm3)
700
600
500
400
300
200
100
0
0
10
20
30
40
50
Days after transplant
PBS
K5-70
T6-16
*
**
B
Tumor weight (g)
0.8
0.6
0.4
0.2
0.0
PBS
K5-70
T6-16
*
**

図61


A
Tumor volume (mm3)
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
Days after transplant
PBS
K5-70
**
B
Tumor weight (g)
0.8
0.6
0.4
0.2
0.0
PBS
K5-70
**

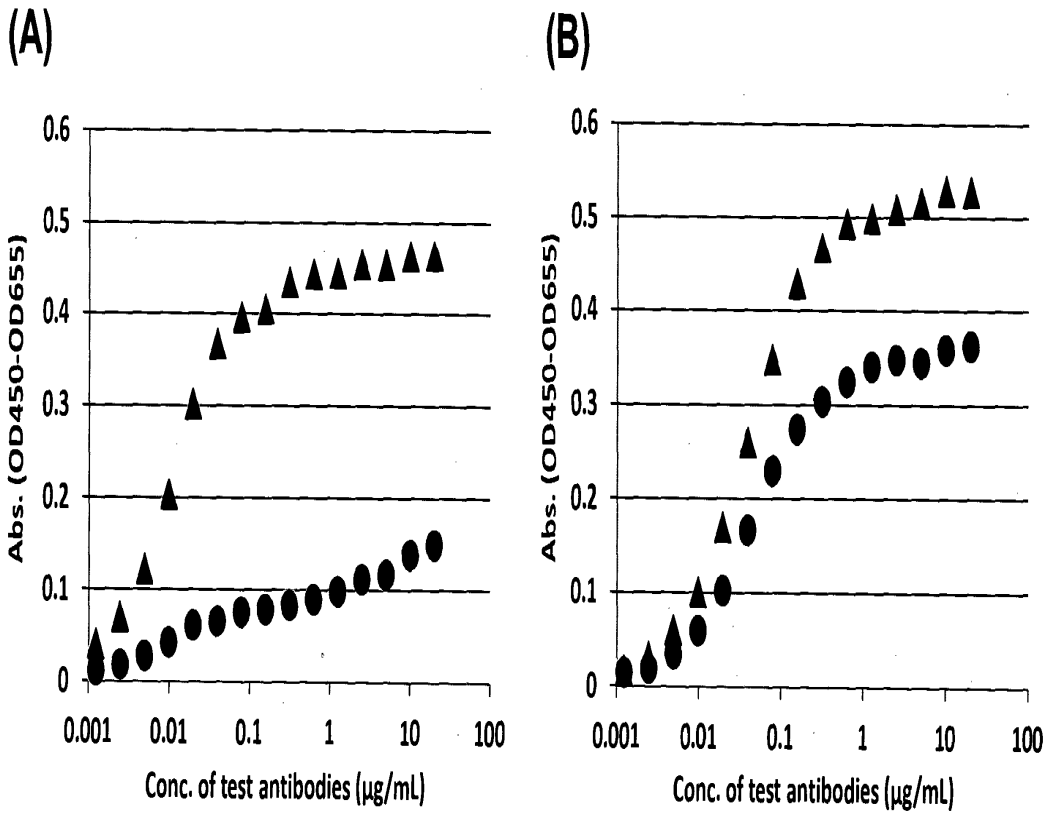

(A)
(B)
Abs. (OD450-OD655)
0.6
0.5
0.4
0.3
0.2
0.1
0
0.001
0.01
0.1
1
10
100
Conc. of test antibodies (μg/mL)

図62

図63

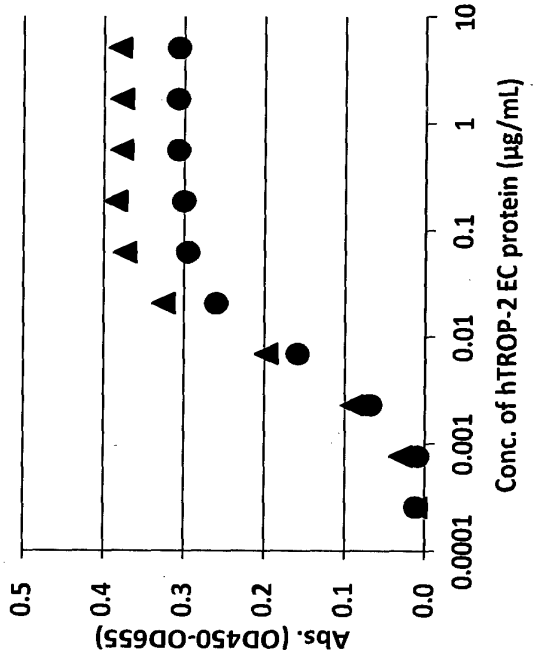

Abs. (OD450-OD655)
0.0
0.1
0.2
0.3
0.4
0.5
Conc. of hTROP-2 EC protein (μg/mL)
0.0001
0.001
0.01
0.1
1
10

図64

EcoRI Kozak

GAA TTC ACC ACC ATG GGA TGG TCC TGC ATT ATT CTC TTT CTC GTC GCC ACC GCC ACA GGC
M G W S C I I L F L V A T A T G

GTG CAC AGC CAG GTT CAA CTG CAG CAA CCT GGG GCA GAG CTG GTT CGG CCA GGG GCC TCC
V H S Q V Q L Q Q P G A E L V R P G A S

GTC AAA CTG TCC TGC AAA GCT TCT GGC TAC ACT TTC ACC ATC TAC TGG ATC AAC TGG GTG
V K L S C K A S G Y T F T I Y W I N W V

AAG CAG AGG CCC GGC CAG GGC CTG GAA TGG ATC GGA AAT ATC TAT CCT AGC GAT TCT TAC
K Q R P G Q G L E W I G N I Y P S D S Y

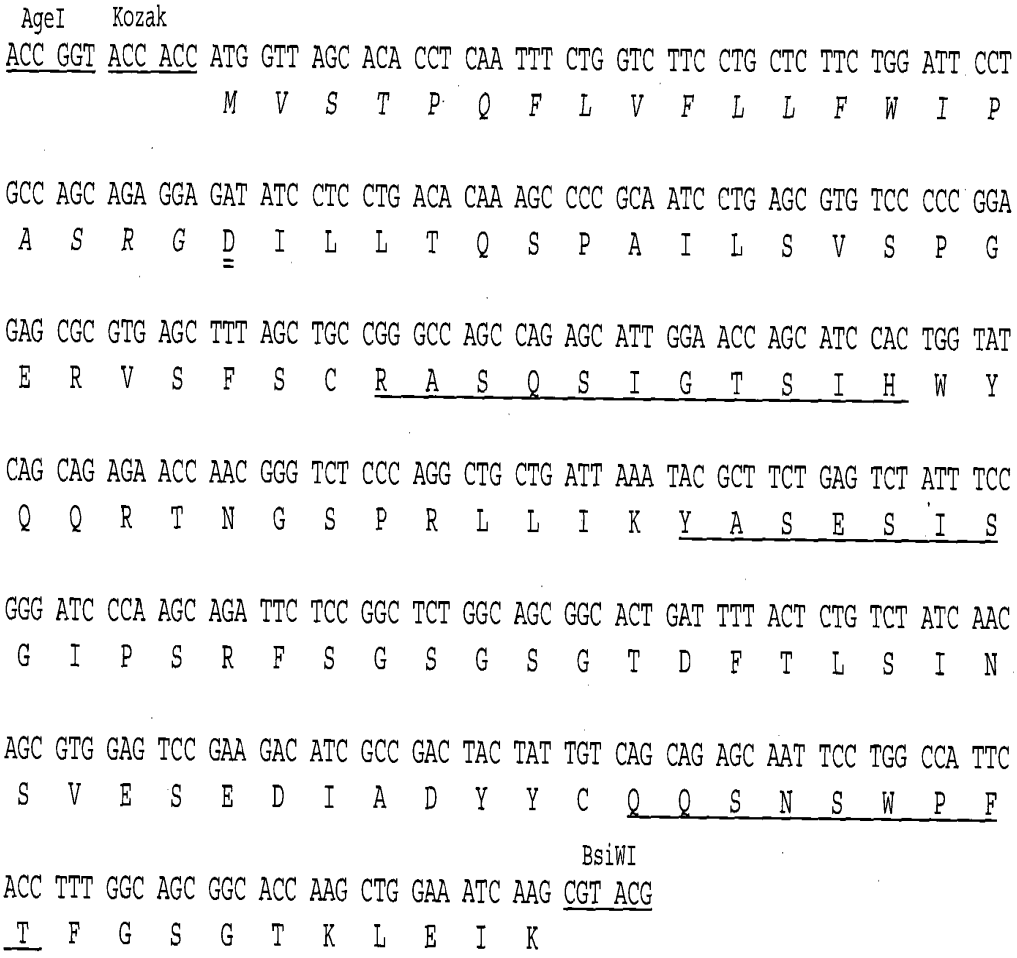
ACA AAT TAC AAC CAG AAG TTC AAG GAC AAG GCT ACC CTG ACC GTG GAC AAA TCT AGC TCC
T N Y N Q K F K D K A T L T V D K S S S

ACA GCC TAC ATG CAG CTG AGC AGC CCC ACT AGC GAG GAT AGC GCA GTG TAT TAT TGT ACC
T A Y M Q L S S P T S E D S A V Y Y C T

NheI

AGA ACC AGC ATG GCC GAC TAT TGG GGA CAG GGC ACA ACT CTG ACC GTG AGC AGC GCT AGC
R T S M A D Y W G Q G T T L T V S S

図65


AgeI
Kozak
ACC GGT ACC ACC ATG GTT AGC ACA CCT CAA TTT CTG GTC TTC CTG CTC TTC TGG ATT CCT
M V S T P Q F L V F L L F W I P
GCC AGC AGA GGA GAT ATC CTC CTG ACA CAA AGC CCC GCA ATC CTG AGC GTG TCC CCC GGA
A S R G D I L L T Q S P A I L S V S P G
GAG CGC GTG AGC TTT AGC TGC CGG GCC AGC CAG AGC ATT GGA ACC AGC ATC CAC TGG TAT
E R V S F S C R A S Q S I G T S I H W Y
CAG CAG AGA ACC AAC GGG TCT CCC AGG CTG CTG ATT AAA TAC GCT TCT GAG TCT ATT TCC
Q Q R T N G S P R L L I K Y A S E S I S
GGG ATC CCA AGC AGA TTC TCC GGC TCT GGC AGC GGC ACT GAT TTT ACT CTG TCT ATC AAC
G I P S R F S G S G S G T D F T L S I N
AGC GTG GAG TCC GAA GAC ATC GCC GAC TAC TAT TGT CAG CAG AGC AAT TCC TGG CCA TTC
S V E S E D I A D Y Y C Q Q S N S W P F
BsiWI
ACC TTT GGC AGC GGC ACC AAG CTG GAA ATC AAG CGT ACG
T F G S G T K L E I K

0.4
0.3
0.2
0.1
0
Abs. (OD450-OD655)
0.001
0.01
0.1
1
10
Conc. of test antibodies (μg/mL)
ChK5-70
MuVH/HuVL
HuVH/MuVL
HuK5-70

図66

図67

	123456789	1 0123456789	2 0123456789	3 0123456789	4 0123456789	5 01223456789
						a
HuK5-70 VH	QVQLVQSGA	EVKKPGASVK	VSCKASGYTF	TIYWINWVRQ	APGQRLEWIG	NIYPSDSYTNY
V5Q	----Q----	----------	----------	----------	----------	----------
S7P	------P--	----------	----------	----------	----------	----------
V11L	---------	-L--------	----------	----------	----------	----------
K12V	---------	--V-------	----------	----------	----------	----------
K13R	---------	---R------	----------	----------	----------	----------
V20L	---------	----------	L---------	----------	----------	----------
R38K	---------	----------	----------	--------K-	----------	----------
A40R	---------	----------	----------	----------	R---------	----------
R44G	---------	----------	----------	----------	----G-----	----------
T73K	---------	----------	----------	----------	----------	----------
A75S	---------	----------	----------	----------	----------	----------
E81Q	---------	----------	----------	----------	----------	----------
L82cP	---------	----------	----------	----------	----------	----------
R83T	---------	----------	----------	----------	----------	----------
T87S	---------	----------	----------	----------	----------	----------
L108T	---------	----------	----------	----------	----------	----------
V109L	---------	----------	----------	----------	----------	----------
V11L/R38K	---------	-L--------	----------	--------K-	----------	----------

	6 0123456789	7 0123456789	8 0122223456789	9 0123456789	10 0123456789	11 0123
		abc				
HuK5-70 VH	NQKFKDKATL	TVDTSASTAY	MELSSLRSEDTAV	YYCTRTSMA-	-DYWGQGTLV	TVSS
V5Q	----------	----------	----------	----------	----------	----
S7P	----------	----------	----------	----------	----------	----
V11L	----------	----------	----------	----------	----------	----
K12V	----------	----------	----------	----------	----------	----
K13R	----------	----------	----------	----------	----------	----
V20L	----------	----------	----------	----------	----------	----
R38K	----------	----------	----------	----------	----------	----
A40R	----------	----------	----------	----------	----------	----
R44G	----------	----------	----------	----------	----------	----
T73K	----------	---K------	----------	----------	----------	----
A75S	----------	-----S----	----------	----------	----------	----
E81Q	----------	----------	-Q----------	----------	----------	----
L82cP	----------	----------	-----P-------	----------	----------	----
R83T	----------	----------	------T------	----------	----------	----
T87S	----------	----------	----------S--	----------	----------	----
L108T	----------	----------	----------	----------	--------T-	----
V109L	----------	----------	----------	----------	---------L	----
V11L/R38K	----------	----------	----------	----------	----------	----

図68

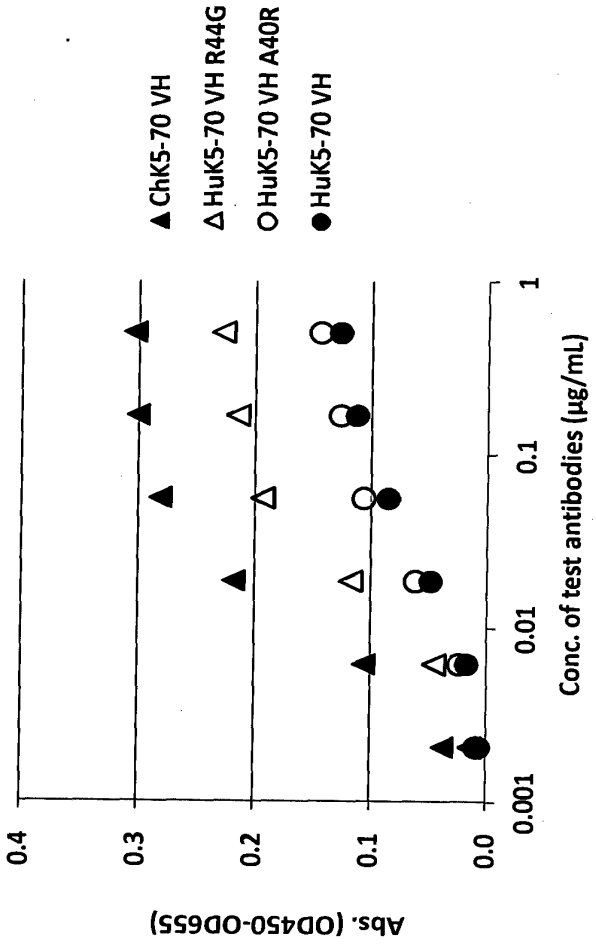

▲ChK5-70 VH
△HuK5-70 VH R44G
○HuK5-70 VH A40R
●HuK5-70 VH
Abs. (OD450-OD655)
0.4
0.3
0.2
0.1
0.0
0.001
0.01
0.1
1
Conc. of test antibodies (μg/mL)

図 69

EcoRI Kozak

GAA TTC ACC ACC ATG GGC TGG AGC TGT ATT ATT CTC TTC CTC GTC GCC ACT GCA ACC GGG
M G W S C I I L F L V A T A T G

GTC CAC TCC CAG GTC CAG CTG GTG CAG AGC GGA GCT GAG GTG AAG AAG CCC GGC GCC AGC
V H S Q V Q L V Q S G A E V K K P G A S

GTT AAG GTT TCT TGC AAA GCC TCT GGC TAT ACA TTC ACA ATC TAT TGG ATC AAT TGG GTG
V K V S C K A S G Y T F T I Y W I N W V

CGG CAG GCT CCA GGA CAA GGC CTG GAA TGG ATC GGC AAT ATC TAT CCT TCC GAC TCC TAC
R Q A P G Q G L E W I G N I Y P S D S Y

ACC AAC TAT AAC CAG AAA TTT AAG GAC AAA GCA ACA CTG ACC GTG GAC ACC TCT GCC AGC
T N Y N Q K F K D K A T L T V D T S A S

ACT GCT TAC ATG GAG CTG AGC AGC CTG AGA AGC GAA GAT ACT GCC GTG TAC TAC TGC ACC
T A Y M E L S S L R S E D T A V Y Y C T

NheI

AGG ACA AGC ATG GCC GAT TAC TGG GGA CAA GGG ACC CTG GTG ACC GTG AGC AGC GCT AGC
R T S M A D Y W G Q G T L V T V S S

図70

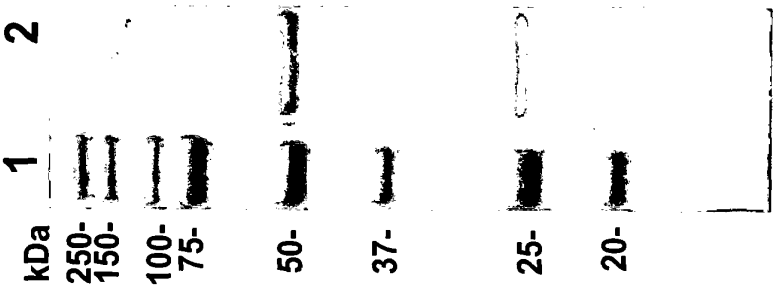
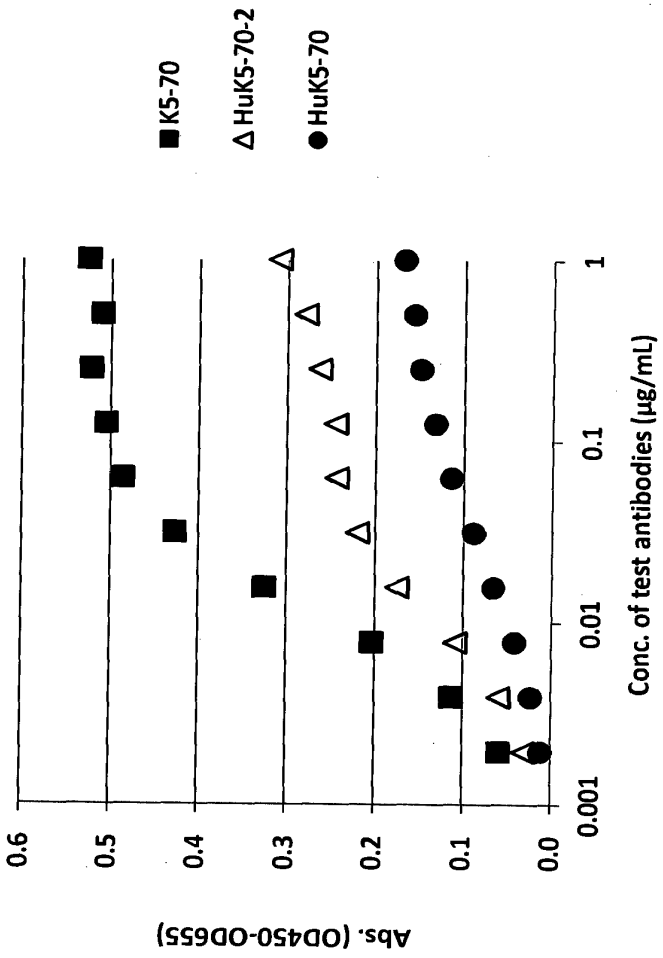


図71


■K5-70
△HuK5-70-2
●HuK5-70
Abs. (OD450-OD655)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.001
0.01
0.1
1
Conc. of test antibodies (μg/mL)

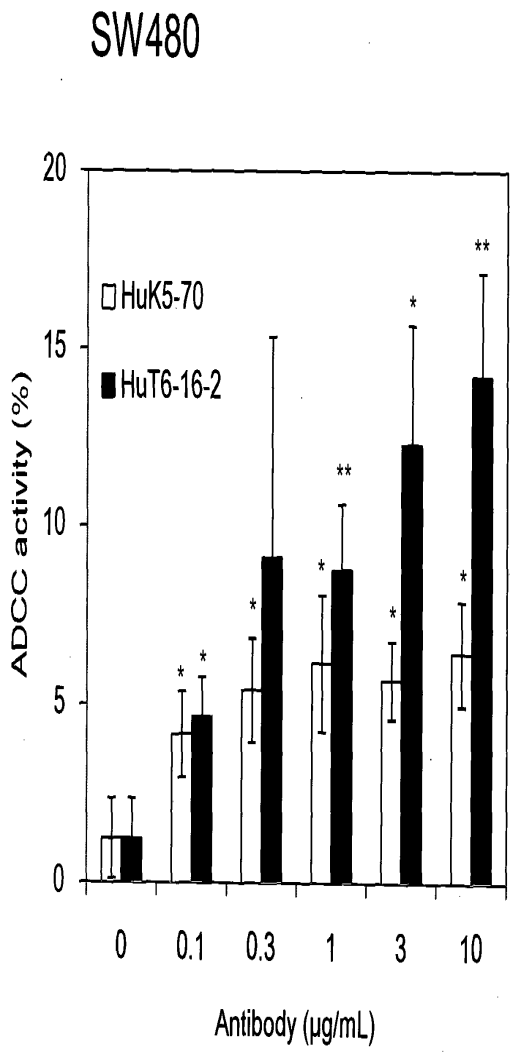

SW480
ADCC activity (%)
HuK5-70
HuT6-16-2
0
5
10
15
20
0
0.1
0.3
1
3
10
Antibody (μg/mL)
*P< 0.05, **P < 0.01

図72A

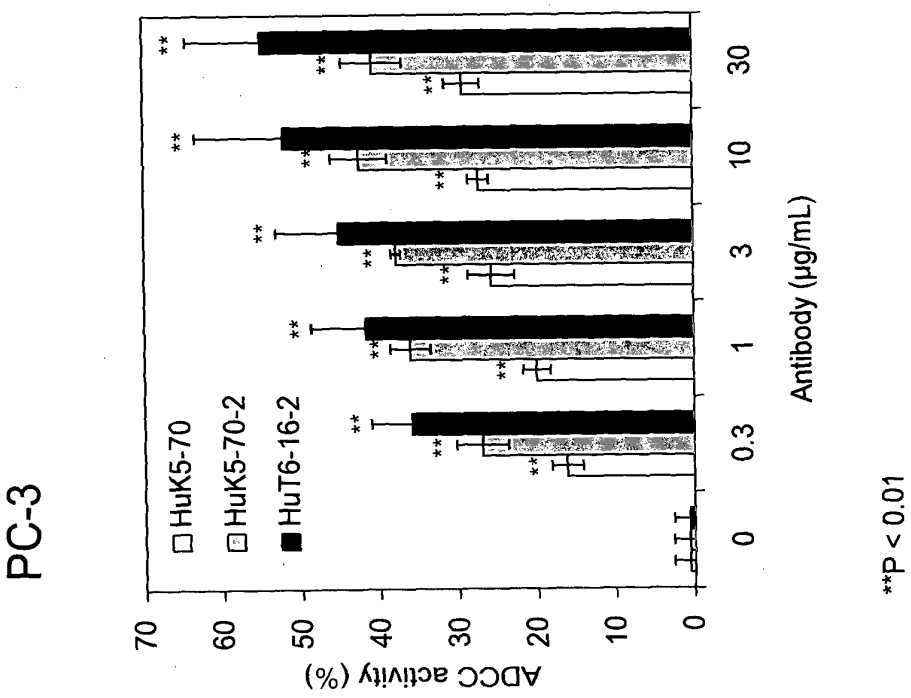
PK-59

**P < 0.01

図72B

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図72C



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2012/080800

A. CLASSIFICATION OF SUBJECT MATTER
C07K16/30(2006.01)i, *A61K39/395*(2006.01)i, *A61P35/00*(2006.01)i, *C12N15/09*(2006.01)i, *C12P21/08*(2006.01)i, *G01N33/53*(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K16/30, A61K39/395, A61P35/00, C12N15/09, C12P21/08, G01N33/53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2013
Kokai Jitsuyo Shinan Koho 1971-2013 Toroku Jitsuyo Shinan Koho 1994-2013

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
JSTPlus/JMEDPlus/JST7580(JDreamII), GenBank/EMBL/DDBJ/GeneSeq, UniProt/GeneSeq, WPI, BIOSIS(STN), CAplus(STN), MEDLINE(STN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X/A	US 2007/0202113 A1 (Arius Research, Inc.), 30 August 2007 (30.08.2007), & JP 2009-527230 A & EP 1996700 A & WO 2007/095748 A1	18-20,22-40, 42-44/1-17, 21,41
X/A	JP 2009-528995 A (Arius Research, Inc.), 13 August 2009 (13.08.2009), & US 2007/0202113 A1 & EP 1996700 A & WO 2007/095748 A1	18-20,22-40, 42-44/1-17, 21,41
X/A	JP 2009-527230 A (Arius Research, Inc.), 30 July 2009 (30.07.2009), & US 2007/0202043 A1 & EP 1996701 A & WO 2007/095749 A1	18-20,22-40, 42-44/1-17, 21,41

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 February, 2013 (26.02.13)	12 March, 2013 (12.03.13)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2012/080800

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X/A	JP 2006-502698 A (Immunomedics, Inc.), 26 January 2006 (26.01.2006), & US 2004/0001825 A1 & EP 1483295 A & WO 2003/074566 A2	18-20,22-40, 42-44/1-17, 21,41
X/A	JP 2010-528056 A (F. Hoffmann-La Roche AG.), 19 August 2010 (19.08.2010), & US 2008/0131428 A1 & EP 2155791 A & WO 2008/144891 A1	18-20,22-40, 42-44/1-17, 21,41
X/A	WO 2010/089782 A1 (Alberti Saverio), 12 August 2010 (12.08.2010), & JP 2012-516887 A & EP 2393512 A	18-20,22-40, 42-44/1-17, 21,41
P,X	WO 2011/145744 A1 (Livtech, Inc.), 24 November 2011 (24.11.2011), (Family: none)	1-44

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2012/080800

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

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

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
See extra sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2012/080800

Continuation of Box No.III of continuation of first sheet(2)

Each of the antibodies described in claims 1 and 3 is specified by an amino acid sequence for an H-chain V-region thereof and an amino acid sequence for an L-chain V-region thereof. Although these amino acid sequences have a certain degree of homology with each other in their framework regions, the sequences for these regions are derived from known sequences, as described in the description of the present application (see the statements in examples 32-33). Therefore, both of the antibodies have no significant homology with each other when compared with those in the prior art techniques, and do not share any common structure, i.e., any common essential structural factor.

Both of the antibodies are common in a matter that the antibodies are anti-human TROP-2 antibodies each having an anti-tumor activity *in vivo* and having a human-originated framework region. However, an mouse anti-human TROP-2 antibody having an anti-tumor activity *in vivo* is disclosed in a document known before the priority date of the present application (US 2007/0202113 A1) and the humanization by a CDR graft method or the like on the basis of an antibody of a mouse or the like is a technique known to persons skilled in the art, as described in the description of the present application (see page 42). When these matters are taken in to consideration, a matter that a humanized antibody having a human-originated framework region can be produced on the basis of a mouse anti-human TROP-2 antibody having an anti-tumor activity *in vivo* can be conceived easily by persons skilled in the art. Therefore, the common matter cannot be regarded as a special technical feature in the meaning within PCT Rule 13.2, second sentence.

Consequently, it is not considered that the inventions of claims 1-44 are relevant to a group of inventions which are so linked as to form a single general inventive concept.

Accordingly, two inventions, which comprise (A) the inventions of claims 1-2, and the parts of the inventions of claims 5-44 which are concerned with claims 1-2 and (B) the inventions of claims 3-4, and the parts of the inventions of claims 5-44 which are concerned with claims 3-4, are set forth in claims 1-44.

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国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２０１２／０８０８００

Ａ．　発明の属する分野の分類（国際特許分類（ＩＰＣ））

Int.Cl.　C07K16/30(2006.01)i, A61K39/395(2006.01)i, A61P35/00(2006.01)i, C12N15/09(2006.01)i, C12P21/08(2006.01)i, G01N33/53(2006.01)i

Ｂ．　調査を行った分野

調査を行った最小限資料（国際特許分類（ＩＰＣ））

Int.Cl.　C07K16/30, A61K39/395, A61P35/00, C12N15/09, C12P21/08, G01N33/53

最小限資料以外の資料で調査を行った分野に含まれるもの

日本国実用新案公報	１９２２－１９９６年
日本国公開実用新案公報	１９７１－２０１３年
日本国実用新案登録公報	１９９６－２０１３年
日本国登録実用新案公報	１９９４－２０１３年

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

JSTPlus/JMEDPlus/JST7580(JDreamII), GenBank/EMBL/DDBJ/GeneSeq, UniProt/GeneSeq, WPI, BIOSIS(STN), CAplus(STN), MEDLINE(STN)

Ｃ．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
X/A	US 2007/0202113 A1 (Arius Research, Inc.) 2007.08.30, & JP 2009-527230 A & EP 1996700 A & WO 2007/095748 A1	18-20, 22-40, 42-44/ 1-17, 21, 41
X/A	JP 2009-528995 A (アリアス　リサーチ、インコーポレイテッド) 2009.08.13, & US 2007/0202113 A1 & EP 1996700 A & WO 2007/095748 A1	18-20, 22-40, 42-44/ 1-17, 21, 41

☑ Ｃ欄の続きにも文献が列挙されている。　☐ パテントファミリーに関する別紙を参照。

＊　引用文献のカテゴリー
「Ａ」特に関連のある文献ではなく、一般的技術水準を示すもの
「Ｅ」国際出願日前の出願または特許であるが、国際出願日以後に公表されたもの
「Ｌ」優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献（理由を付す）
「Ｏ」口頭による開示、使用、展示等に言及する文献
「Ｐ」国際出願日前で、かつ優先権の主張の基礎となる出願の日の後に公表された文献
「Ｔ」国際出願日又は優先日後に公表された文献であって出願と矛盾するものではなく、発明の原理又は理論の理解のために引用するもの
「Ｘ」特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの
「Ｙ」特に関連のある文献であって、当該文献と他の１以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの
「＆」同一パテントファミリー文献

国際調査を完了した日 ２６．０２．２０１３	国際調査報告の発送日 １２．０３．２０１３
国際調査機関の名称及びあて先 日本国特許庁（ＩＳＡ／ＪＰ） 郵便番号１００－８９１５ 東京都千代田区霞が関三丁目４番３号	特許庁審査官（権限のある職員）　４Ｎ　３８４６ 伊藤　佑一 電話番号　０３－３５８１－１１０１　内線　３４８８

様式ＰＣＴ／ＩＳＡ／２１０（第２ページ）（２００９年７月）

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国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２０１２／０８０８００

Ｃ（続き）．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
X/ A	JP 2009-527230 A (アリアス　リサーチ、インコーポレイテッド) 2009.07.30, & US 2007/0202043 A1 & EP 1996701 A & WO 2007/095749 A1	18-20, 22-40, 42-44/ 1-17, 21, 41
X/ A	JP 2006-502698 A (イミューノメディクス、インコーポレイテッド) 2006.01.26, & US 2004/0001825 A1 & EP 1483295 A & WO 2003/074566 A2	18-20, 22-40, 42-44/ 1-17, 21, 41
X/ A	JP 2010-528056 A (エフ．ホフマンーラ　ロシュ　アーゲー) 2010.08.19, & US 2008/0131428 A1 & EP 2155791 A & WO 2008/144891 A1	18-20, 22-40, 42-44/ 1-17, 21, 41
X/ A	WO 2010/089782 A1 (Alberti Saverio) 2010.08.12, & JP 2012-516887 A & EP 2393512 A	18-20, 22-40, 42-44/ 1-17, 21, 41
P, X	WO 2011/145744 A1 (株式会社リブテック) 2011.11.24, (ファミリーなし)	1-44

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国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２０１２／０８０８００

第Ⅱ欄　請求の範囲の一部の調査ができないときの意見（第１ページの２の続き）

法第8条第3項（ＰＣＴ17条(2)(a)）の規定により、この国際調査報告は次の理由により請求の範囲の一部について作成しなかった。

１．☐ 請求項 _____ は、この国際調査機関が調査をすることを要しない対象に係るものである。
つまり、

２．☐ 請求項 _____ は、有意義な国際調査をすることができる程度まで所定の要件を満たしていない国際出願の部分に係るものである。つまり、

３．☐ 請求項 _____ は、従属請求の範囲であってＰＣＴ規則6.4(a)の第２文及び第3文の規定に従って記載されていない。

第Ⅲ欄　発明の単一性が欠如しているときの意見（第１ページの３の続き）

次に述べるようにこの国際出願に二以上の発明があるとこの国際調査機関は認めた。
特別ページ参照。

１．☑ 出願人が必要な追加調査手数料をすべて期間内に納付したので、この国際調査報告は、すべての調査可能な請求項について作成した。

２．☐ 追加調査手数料を要求するまでもなく、すべての調査可能な請求項について調査することができたので、追加調査手数料の納付を求めなかった。

３．☐ 出願人が必要な追加調査手数料を一部のみしか期間内に納付しなかったので、この国際調査報告は、手数料の納付のあった次の請求項のみについて作成した。

４．☐ 出願人が必要な追加調査手数料を期間内に納付しなかったので、この国際調査報告は、請求の範囲の最初に記載されている発明に係る次の請求項について作成した。

追加調査手数料の異議の申立てに関する注意

☐ 追加調査手数料及び、該当する場合には、異議申立手数料の納付と共に、出願人から異議申立てがあった。

☐ 追加調査手数料の納付と共に出願人から異議申立てがあったが、異議申立手数料が納付命令書に示した期間内に支払われなかった。

☑ 追加調査手数料の納付はあったが、異議申立てはなかった。

請求項１及び３に記載の抗体は、それぞれのＨ鎖Ｖ領域及びＬ鎖Ｖ領域のアミノ酸配列により特定されたものであるが、これらアミノ酸配列はフレームワーク領域において一定の相同性を有しているものの、本願明細書に記載されるように（実施例３２－３３の記載箇所参照）、当該領域の配列は公知配列に由来するものであるから、両抗体は先行技術との比較において互いに顕著な相同性がなく、共通の構造、すなわち、重要な構造要素を共有していない。

また、両抗体は in vivo において抗腫瘍活性を有し、ヒト由来のフレームワーク領域を有する抗ヒトＴＲＯＰ－２抗体であるという点で共通するものの、本願優先日前に公知の文献（US 2007/0202113 A1）において、in vivo において抗腫瘍活性を有するマウス抗ヒトＴＲＯＰ－２抗体が記載されており、また、本願明細書にも記載されるように（第４２頁参照）、マウス等の抗体を基にＣＤＲグラフト法などを用いてヒト化することは当業者の周知技術であったことを参酌すると、in vivo において抗腫瘍活性を有するマウス抗ヒトＴＲＯＰ－２抗体を基にヒト由来のフレームワーク領域を有するヒト化抗体を作製することは当業者が容易に想到し得ることであるから、ＰＣＴ規則１３．２の第２文の意味において、この共通事項は特別な技術的特徴であると認められない。

よって、請求項１－４４に係る発明は、単一の一般的発明概念を形成するように連関している一群の発明であるとは言えない。

したがって、請求項１－４４に係る発明は、

Ａ．請求項１－２に係る発明、及び、請求項５－４４に係る発明のうち請求項１－２に係る部分
Ｂ．請求項３－４に係る発明、及び、請求項５－４４に係る発明のうち請求項３－４に係る部分

の２発明が記載されている。

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
31 October 2013 (31.10.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/163229 A1

(51) International Patent Classification:
A61K 39/395 (2006.01)

(21) International Application Number:
PCT/US2013/037861

(22) International Filing Date:
23 April 2013 (23.04.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/637,808 24 April 2012 (24.04.2012) US

(71) Applicants: **SEATTLE GENETICS, INC.** [US/US]; 21823 - 30th Dr. S.E., Bothell, Washington 98021 (US). **DAIICHI SANKYO CO., LTD.** [JP/JP]; 3-5-1, Nihonbashi-honcho, Chuo-ku, Tokyo, Tokyo 103-8426 (JP).

(72) Inventors: **OHTSUKA, Toshiaki**; c/o Daiichi Sankyo Co., Ltd., Group II, Biologics Research Laboratories, 1-16-13 Kitakasai, Edogawa-ku, Tokyo, Tokyo 134-8630 (JP). **ICHIKAWA, Kimihisa**; c/o Daiichi Sankyo Co., Ltd., Group II, Oncology Research Laboratories, 1-16-13 Kitakasai, Edogawa-ku, Tokyo, Tokyo 134-8630 (JP). **YADA, Ayumi**; c/o Daiichi Sankyo Co., Ltd., Group III, Oncology Research Laboratories, 1-16-13 Kitakasai, Edogawa-ku, Tokyo, Tokyo 134-8630 (JP).

(74) Agents: **FLANAGAN, Lisa Dornbach** et al.; Kilpatrick Townsend & Stockton LLP, Eighth Floor, Two Embarcadero Center, San Francisco, California 94111 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, 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, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, 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, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,

[Continued on next page]

(54) Title: DR5 LIGAND DRUG CONJUGATES

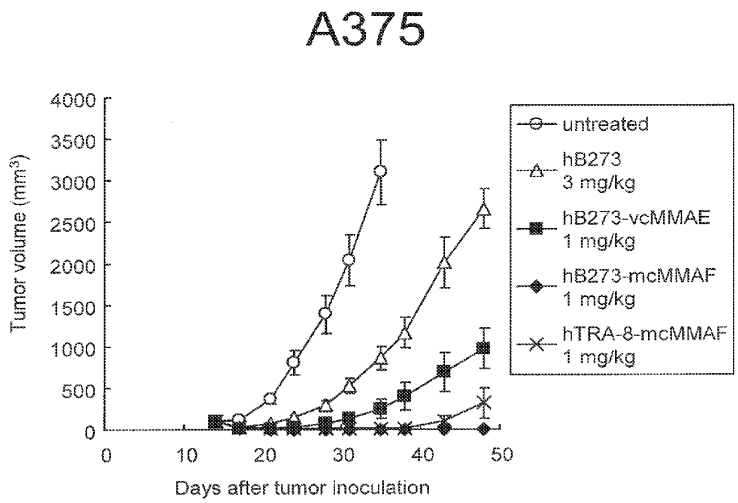


FIG. 13

(57) Abstract: Ligand Drug Conjugates are provided having a DRS binding moiety attached via linking groups and/or spacers to a therapeutic agent that are effective in treatment of various cancers. In some embodiments, the Ligand Drug Conjugate has the formula: L-(LU-D)p, where L is a Ligand unit, LU is a Linker unit and D is a Drug unit (or cytotoxic agent). The subscript p is an integer of from 1 to 20. Accordingly, the Ligand Drug Conjugates comprise a Ligand unit covalently linked to at least one Drug unit. The Drug units can be covalently linked directly or via a Linker unit (-LU-). The Ligand unit is a DR5 binding agent, such as an anti-DRS antibody.

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DR5 LIGAND DRUG CONJUGATES

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority benefit of U.S. Provisional Application Serial No. 61/637,808, filed April 24, 2012, the contents of which are incorporated herein by reference in their entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0002] NOT APPLICABLE

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED AS AN ASCII TEXT FILE

[0003] The Sequence Listing written in file –75-1PC.TXT, created on April 22, 2013, 20,480 bytes, machine format IBM-PC, MS-Windows operating system, is hereby incorporated by reference in its entirety for all purposes.

BACKGROUND OF THE INVENTION

[0004] Cell surface receptors involved in apoptosis induction, such as death domain-containing receptors, multimerize on the membrane of cells due to ligand binding and biologically trigger induction of apoptotic signals in the cells (Cell Death and Differentiation, 10:66-75 (2003)). Examples of such cell surface receptors include the tumor necrosis factor (hereinafter referred to as TNF)-related apoptosis-inducing ligand (hereinafter referred to as TRAIL) receptor family. TRAIL is a member of the TNF family of proteins, which includes Fas ligand and TNF-α (Wiley SR, et al. Immunity 1995 Dec; 3(6):673-82). These proteins are potent apoptosis inducers.

[0005] The receptors for the TNF family of proteins are characterized by a cysteine-rich repeat sequence in the extracellular domain. Among them, Fas, a receptor for Fas ligand, and

TNF receptor I (hereinafter referred to as TNFRI), a receptor for TNF-α, are collectively referred to as death domain-containing receptors. These receptors have an intracellular domain essential for apoptotic signaling which is called the death domain and homologous to the Drosophila suicide gene, reaper (Golstein, P., et al. (1995) Cell. 81, 185-186, White, K, et al. (1994) Science 264, 677-683).

[0006] Five TRAIL receptors have been identified, and two (DR4 [TRAIL-R1] and DR5 [TRAIL-R2]) of them can induce apoptotic signaling while the other three (DcR1 [TRAIL-R3], DcR2 [TRAIL-R4], and osteoprotegerin [OPG]) do not induce apoptotic signaling. Like Fas and TNFRI, intracellular segments of both DR4 and DR5 contain a death domain and induce apoptotic signaling by way of pathways including Fas-associated death domain protein (hereinafter referred to as FADD) and caspase-8 (Chaudhary PM, et al. Immunity 1997 Dec; 7(6): 821-30; Schneider P, et al. Immunity 1997 Dec; 7(6): 831-36).

[0007] For the above Fas, TNFRI, DR4, and DR5, agonistic antibodies which bind respectively to these molecules have an apoptosis-inducing activity on cells having the target molecules on their surface (Journal of Cellular Physiology, 209: 1021-1028 (2006); Leukemia, Apl; 21 (4): 805-812 (2007); Blood, 99: 1666-1675 (2002); Cellular Immunology, Jan; 153 (1): 184-193 (1994)). The efficacy of these agonistic antibodies is enhanced by cross-linking with a secondary antibody or effector cells (Journal of Immunology, 149: 3166-3173 (1992); European Journal of Immunology, Oct; 23 (10): 2676-2681 (1993)).

[0008] An anti-DR5 antibody having capacity to bind to a cell surface receptor involved in apoptosis induction is currently under clinical development as a therapeutic, and is expected to reveal the therapeutic effects and kill the cells (cancer cells and immune disease-related cells) expressing the receptor in a specific and agonistic manner. The mechanism of action of this antibody is proposed to be mediated by cross-linking of the antibody molecules together to form multimers before or after the binding of the antibody to the receptor. Such multimerization of the antibody subsequently causes multimerization of the antigen receptor (namely, apoptosis induction). It appears that in vitro experiments, artificial cross-linking by addition of a secondary antibody against the antibody is required to enhance the activity of the antibody and that in vivo, cross-linking by Fc receptors on immune effector cells is a mechanism of action required to produce the activity of the antibody. Recently, attempts have been made to further enhance the original function of an antibody by altering structures of the antibody. For example, it is reported that removal of a specific carbohydrate structure on an antibody improves the affinity for Fc receptors. Such a mechanism of action suggests that non-internalizing antibodies to the cell surface receptor are preferred.

[0009] There remains a need, however, for methods of treating DR5 expressing cancers.

BRIEF SUMMARY OF THE INVENTION

[0010] The present invention provides, *inter alia,* Ligand Drug Conjugates for targeted delivery of drug to DR5-expressing cells. The present inventors have conducted extensive studies and found that an antibody-drug conjugate containing an antibody that can induce apoptosis in cells has a more significant therapeutic effect on cancer than such antibody alone. By using the antibody-drug conjugate according to the present invention, the antibody itself exhibits an apoptosis-inducing effect and the drug conjugated to the antibody also exhibits a therapeutic effect. For these reasons, the antibody-drug conjugate has an effective therapeutic effect on patients who cannot be treated effectively by the antibody alone. The Ligand Drug Conjugates described herein have potent cytotoxic and/or cytostatic activity against cells expressing DR5, such as DR5-expressing cancer cells. In some embodiments, the Ligand Drug Conjugate has the formula:

$$L - (LU\text{-}D)_p \qquad (I)$$

wherein L is a Ligand unit, LU is a Linker unit and D is a Drug unit (or cytotoxic agent). The subscript p is an integer of from 1 to 20. Accordingly, the Ligand Drug Conjugates comprise a Ligand unit covalently linked to at least one Drug unit. The Drug units can be covalently linked directly or via a Linker unit (-LU-). The Ligand unit, described more fully below, is a DR5 binding agent, such as an anti-DR5 antibody. Accordingly, the present invention also provides methods for the treatment of, for example, various cancers. These methods encompass the use of Ligand Drug Conjugates wherein the Ligand unit is an anti-DR5 binding agent that specifically binds to DR5. The DR5 binding agent can be, for example, an anti-DR5 antibody, an anti-DR5 antigen-binding fragment, or other DR5 binding agent comprising the amino acid sequence of a humanized antibody heavy and/or light chain variable region, or derivative thereof.

[0011] In some embodiments, a Ligand Drug Conjugate comprises a DR5 binding agent covalently attached to a cytotoxic agent. In some embodiments, a Ligand Drug Conjugate has the formula:

$$L - (LU\text{-}D)_p \qquad (I)$$

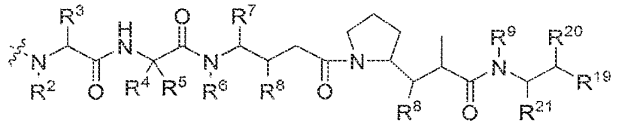
or a pharmaceutically acceptable salt thereof, having specificity for target cells expressing DR5, wherein:

L is a ligand unit which is a DR5 binding agent; and

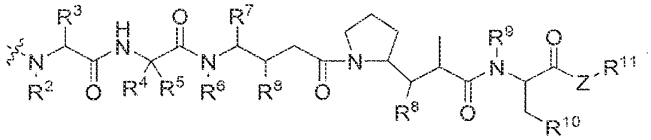
(LU-D) is a Linker unit-Drug unit moiety, wherein LU is a Linker unit, and D is a drug unit having cytostatic or cytotoxic activity against said target cells; and

the subscript p is an integer of from 1 to 20.

[0012] In some embodiments, a Ligand Drug Conjugate having Formula (I) comprises a Drug unit having the formula D_E or D_F:



D_E



D_F

or a pharmaceutically acceptable salt thereof, wherein, independently at each location:

the wavy line indicates the point of attachment to the remainder of the ligand drug conjugate;

R^2 is -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, or -C_2-C_{20} alkynyl;

R^3 is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, carbocycle, -C_1-C_{20} alkylene (carbocycle), -C_2-C_{20} alkenylene(carbocycle), -C_2-C_{20} alkynylene(carbocycle), -aryl, -C_1-C_{20} alkylene(aryl), -C_2-C_{20} alkenylene(aryl), -C_2-C_{20} alkynylene(aryl), -heterocycle, -C_1-C_{20} alkylene(heterocycle), -C_2-C_{20} alkenylene(heterocycle), or -C_2-C_{20} alkynylene(heterocycle);

R^4 is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, carbocycle, -C_1-C_{20} alkylene (carbocycle), -C_2-C_{20} alkenylene(carbocycle), -C_2-C_{20} alkynylene(carbocycle), -aryl, -C_1-C_{20} alkylene(aryl), -C_2-C_{20} alkenylene(aryl), -C_2-C_{20} alkynylene(aryl), -heterocycle, -C_1-C_{20} alkylene(heterocycle), -C_2-C_{20} alkenylene(heterocycle), or -C_2-C_{20} alkynylene(heterocycle);

R^5 is -H or -C_1-C_8 alkyl;

or $\mathbf{R^4}$ **and** $\mathbf{R^5}$ jointly form a carbocyclic ring and have the formula -$(CR^aR^b)_s$- wherein R^a and R^b are independently -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, or -carbocycle and s is 2, 3, 4, 5 or 6;

$\mathbf{R^6}$ is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, or -C_2-C_{20} alkynyl;

$\mathbf{R^7}$ is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, -carbocycle, -C_1-C_{20} alkylene (carbocycle), -C_2-C_{20} alkenylene(carbocycle), -C_2-C_{20} alkynylene(carbocycle), -aryl, -C_1-C_{20} alkylene(aryl), -C_2-C_{20} alkenylene(aryl), -C_2-C_{20} alkynylene(aryl), heterocycle, -C_1-C_{20} alkylene(heterocycle), -C_2-C_{20} alkenylene(heterocycle), or -C_2-C_{20} alkynylene(heterocycle);

each $\mathbf{R^8}$ is independently -H, -OH, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, -O-(C_1-C_{20} alkyl), -O-(C_2-C_{20} alkenyl), -O-(C_2-C_{20} alkynyl), or -carbocycle;

$\mathbf{R^9}$ is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, or -C_2-C_{20} alkynyl;

$\mathbf{R^{19}}$ is -aryl, -heterocycle, or -carbocycle;

$\mathbf{R^{20}}$ is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, -carbocycle, -O-(C_1-C_{20} alkyl), -O-(C_2-C_{20} alkenyl), -O-(C_2-C_{20} alkynyl), or $O\mathbf{R^{18}}$ wherein $\mathbf{R^{18}}$ is -H, a hydroxyl protecting group, or a direct bond where $O\mathbf{R^{18}}$ represents =O;

$\mathbf{R^{21}}$ is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, or -C_2-C_{20} alkynyl, -aryl, -heterocycle, or -carbocycle;

$\mathbf{R^{10}}$ is -aryl or -heterocycle;

Z is –O-, -S-, -NH-, or -NR^{12}-, wherein $\mathbf{R^{12}}$ is -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, or -C_2-C_{20} alkynyl;

$\mathbf{R^{11}}$ is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, -aryl, -heterocycle, -$(R^{13}O)_m$-R^{14}, or -$(R^{13}O)_m$-$CH(R^{15})_2$;

m is an integer ranging from 0-1000;

$\mathbf{R^{13}}$ is -C_2-C_{20} alkylene, -C_2-C_{20} alkenylene, or -C_2-C_{20} alkynylene;

$\mathbf{R^{14}}$ is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, or -C_2-C_{20} alkynyl;

each occurrence of $\mathbf{R^{15}}$ is independently -H, -COOH, –$(CH_2)_n$-$N(R^{16})_2$, –$(CH_2)_n$-SO_3H, –$(CH_2)_n$-SO_3-C_1-C_{20} alkyl, –$(CH_2)_n$-SO_3-C_2-C_{20} alkenyl, or –$(CH_2)_n$-SO_3-C_2-C_{20} alkynyl;

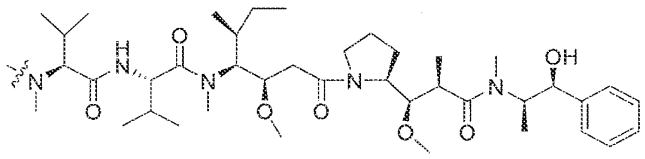
each occurrence of $\mathbf{R^{16}}$ is independently -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl or –$(CH_2)_n$-COOH; and

n is an integer ranging from 0 to 6; wherein said alkyl, alkenyl, alkynyl, alkylene, alkenylene, alkynyklene, aryl, carbocyle, and heterocycle radicals, whether alone or as part of another group, are optionally substituted.

[0013] In some embodiments, the Drug unit has formula D_E or a pharmaceutically acceptable salt form thereof. In some embodiments, D is D_E. In some embodiments, D is D_F. In some embodiments,

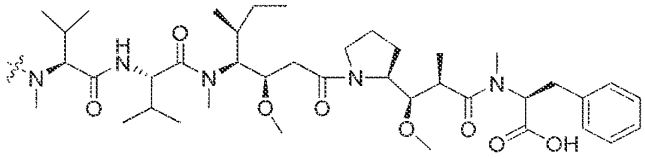
[0014] In some embodiments, the Drug unit has the formula:

[0015] In some embodiments, the Drug unit has the formula:



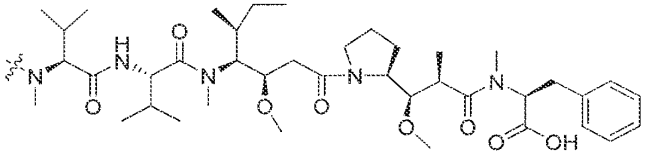
or a a pharmaceutically acceptable salt form thereof; the DR5 binding agent is an anti-DR5 antibody attached to the Linker unit through a sulfur atom of the antibody; the Linker unit comprises a –Val-Cit- moiety; and the subscript p is an integer of from 1 to 8.

[0016] In some embodiments, the Drug unit has the formula:



or a pharmaceutically acceptable salt form thereof; the DR5 binding agent is an anti-DR5 antibody attached to the Linker unit through a sulfur atom of the antibody; the Linker unit comprises a –Val-Cit- moiety; and the subscript p is an integer of from 1 to 8.

[0017] In some embodiments, the Drug unit has the formula:



or a pharmaceutically acceptable salt form thereof; the DR5 binding agent is an anti-DR5 antibody attached to the Linker unit through a sulfur atom of the antibody; the Linker unit comprises a --Succinimide-Caproic acid- moiety; and the subscript p is an integer of from 1 to 8.

[0018] In some embodiments, a Ligand Drug Conjugate having Formula (I) comprises a LU having the formula:

—A_a—W_w—Y_y—

or a pharmaceutically acceptable salt form thereof, wherein:

-A- is a Stretcher unit;

the subscript a is 0 or 1;

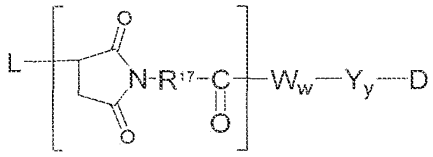
each W is independently an amino acid unit;

the subscript w is an integer of from 0 to 12;

-Y- is a Spacer unit; and

the subscript y is 0, 1 or 2.

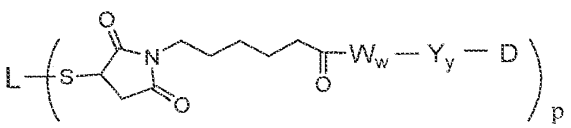
[0019] In some embodiments, a Ligand Drug Conjugate comprising a LU having the formula —A_a—W_w—Y_y— has the formula:



or a pharmaceutically acceptable salt form thereof, wherein:

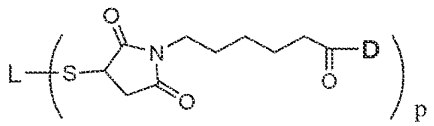
R^{17} is a member selected from the group consisting of -C_1-C_{10} alkylene-, -C_2-C_{10} alkenylene-, -C_2-C_{10} alkynylene-, -carbocyclo-, -O-(C_1-C_8 alkylene)-, O-(C_2-C_8 alkenylene)-, -O-(C_2-C_8 alkynylene)-, -arylene-, -C_1-C_{10} alkylene-arylene-, -C_2-C_{10} alkenylene-arylene, -C_2-C_{10} alkynylene-arylene, -arylene-C_1-C_{10} alkylene-, -arylene-C_2-C_{10} alkenylene-, -arylene-C_2-C_{10} alkynylene-, -C_1-C_{10} alkylene-(carbocyclo)-, -C_2-C_{10} alkenylene-(carbocyclo)-, -C_2-C_{10} alkynylene-(carbocyclo)-, -(carbocyclo)-C_1-C_{10} alkylene-, -(carbocyclo)-C_2-C_{10} alkenylene-, -(carbocyclo)-C_2-C_{10} alkynylene, heterocyclo-, -C_1-C_{10} alkylene-(heterocyclo)-, -C_2-C_{10} alkenylene-(heterocyclo)-, -C_2-C_{10} alkynylene-(heterocyclo)-, -(heterocyclo)-C_1-C_{10} alkylene-, -(heterocyclo)-C_2-C_{10} alkenylene-, -(heterocyclo)-C_2-C_{10} alkynylene-, -$(CH_2CH_2O)_r$-, and -$(CH_2CH_2O)_r$-CH_2-, wherein r is an integer of from 1 to 10, and wherein said alkyl, alkenyl, alkynyl, alkylene, alkenylene, alkynyklene, aryl, carbocyle, carbocyclo, heterocyclo, and arylene radicals, whether alone or as part of another group, are optionally substituted.

[0020] In some embodiments, a Ligand Drug Conjugate comprising a LU having the formula —A_a—W_w—Y_y— has the formula:



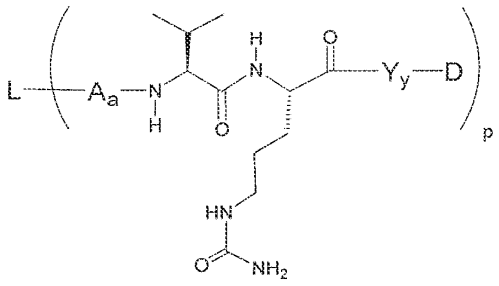
or a pharmaceutically acceptable salt form thereof, wherein S is a sulfur atom provided by the Ligand unit (L).

[0021] In some embodiments, a Ligand Drug Conjugate comprising a LU having the formula $-A_a-W_w-Y_y-$ has the formula:



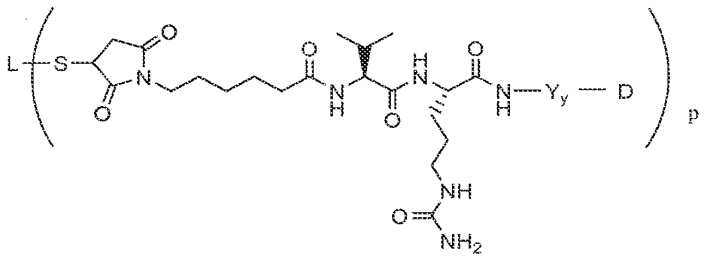
or a pharmaceutically acceptable salt form thereof, wherein S is a sulfur atom provided by the Ligand unit (L).

[0022] In some embodiments, a Ligand Drug Conjugate comprising a LU having the formula $-A_a-W_w-Y_y-$ has the formula:



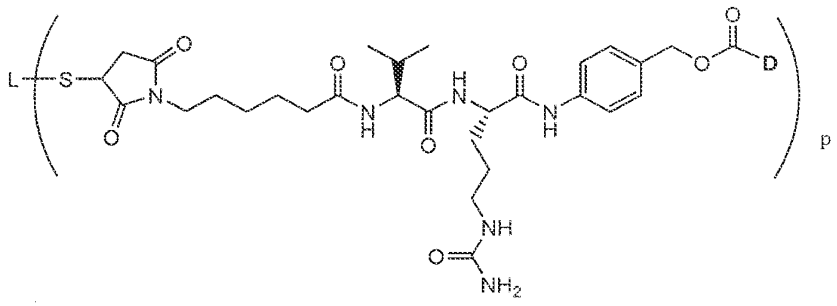
or a pharmaceutically acceptable salt form thereof.

[0023] In some embodiments, a Ligand Drug Conjugate comprising a LU having the formula $-A_a-W_w-Y_y-$ has the formula:



or a pharmaceutically acceptable salt form thereof.

[0024] In some embodiments, a Ligand Drug Conjugate comprising a LU having the formula $-A_a-W_w-Y_y-$ has the formula:



or a pharmaceutically acceptable salt form thereof.

[0025] In some embodiments, for the Ligand Drug Conjugate comprising a LU having the formula $-A_a-W_w-Y_y-$, w is an integer ranging from 2 to 12, and y is 1 or 2. In some embodiments, w is 2 and y is 1 or 2. In some embodiments, W_w is -valine-citrulline- and y is 1 or 2.

[0026] In some embodiments, a Ligand Drug Conjugate has the formula of Formula (I), wherein L is an anti-DR5 antibody. In some embodiments, an anti-DR5 antibody comprises (a) a heavy chain immunoglobulin having the CDR1 consisting of amino residues 1-5 of SEQ ID NO:11, the CDR2 consisting of amino acid residues 1-17 of SEQ ID NO:12, and the CDR3 consisting of amino acid residues 1-13 of SEQ ID NO:13; and (b) a light chain immunoglobulin having the CDR1 consisting of amino residues 1-16 of SEQ ID NO:14, the CDR2 consisting of amino acid residues 1-7 of SEQ ID NO:15, and the CDR3 consisting of amino acid residues 1-9 of SEQ ID NO:16.

[0027] In some embodiments, a Ligand Drug Conjugate has the formula:

$$L-(LU\text{-}D)_p \qquad \textbf{(I)}$$

or a pharmaceutically acceptable salt thereof, having specificity for target cells expressing DR5, wherein:

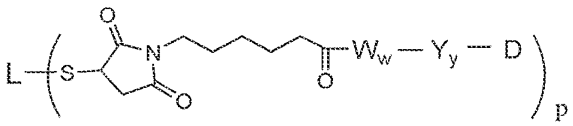
L is a Ligand unit which is an anti-DR5 antibody comprising (a) a heavy chain immunoglobulin having the CDR1 consisting of amino residues 1-5 of SEQ ID NO:11, the CDR2 consisting of amino acid residues 1-17 of SEQ ID NO:12, and the CDR3 consisting of amino acid residues 1-13 of SEQ ID NO:13; and (b) a light chain immunoglobulin having the CDR1 consisting of amino residues 1-16 of SEQ ID NO:14, the CDR2 consisting of amino acid residues 1-7 of SEQ ID NO:15, and the CDR3 consisting of amino acid residues 1-9 of SEQ ID NO:16; and

(LU-D) is a Linker unit-Drug unit moiety, wherein LU is a Linker unit, and D is a Drug unit, wherein the Drug unit is an auristatin; and

the subscript p is an integer of from 1 to 20.

[0028] In some embodiments, the auristatin is auristatin E, AEB, AEVB, AFP, MMAF, or MMAE. In some embodiments, the auristatin is MMAF. In some embodiments, the auristatin is MMAE.

[0029] In some embodiments, a Ligand Drug Conjugate has the formula:



or a pharmaceutically acceptable salt form thereof, wherein:

L is a Ligand unit which is an anti-DR5 antibody comprising (a) a heavy chain immunoglobulin having the CDR1 consisting of amino residues 1-5 of SEQ ID NO:11, the CDR2 consisting of amino acid residues 1-17 of SEQ ID NO:12, and the CDR3 consisting of amino acid residues 1-13 of SEQ ID NO:13; and (b) a light chain immunoglobulin having the CDR1 consisting of amino residues 1-16 of SEQ ID NO:14, the CDR2 consisting of amino acid residues 1-7 of SEQ ID NO:15, and the CDR3 consisting of amino acid residues 1-9 of SEQ ID NO:16;

S is a sulfur atom of the Ligand unit;

each W is independently an Amino Acid unit;

the subscript w is an integer of from 0 to 12;

Y is a Spacer unit; and

the subscript y is 0, 1, or 2.

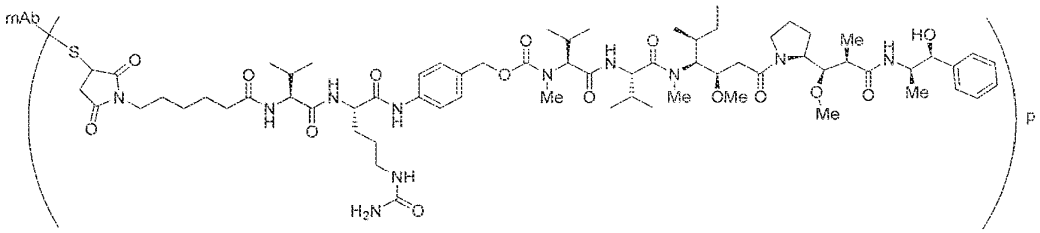
[0030] In some embodiments, W_w is valine-citrulline. In some embodiments, no Amino Acid unit is present. In some embodiments, no Spacer unit is present.

[0031] In some embodiments, a Ligand Drug Conjugate has a p value between 1 and 20. In some embodiments, the p value is 2, 4, 6, 8, 10, 12, 14, 16, 18, or 20. In some embodiments, the p value is between 1 and 8. In some embodiments, the p value is between 2 and 8. In some embodiments, the p value is 2, 4, 6, or 8.

[0032] In some embodiments, an anti-DR5 antibody comprises a heavy chain variable region comprising amino acid residues 1-122 of SEQ ID NO:9 and a light chain variable region comprising amino acid residues 1-114 of SEQ ID NO:10.

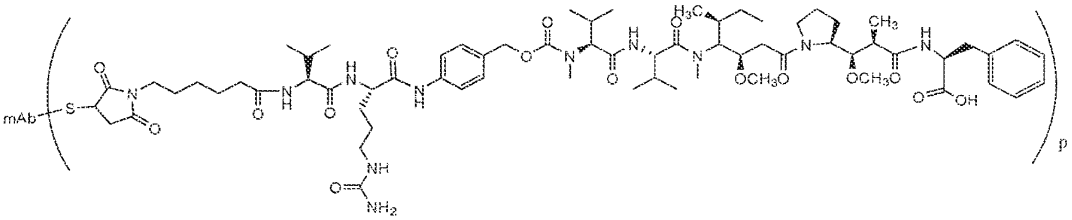
[0033] In some embodiments, an anti-DR5 antibody comprises a heavy chain consisting of amino residues 1-452 of SEQ ID NO:9 and a light chain consisting of amino acid residues 1-219 of SEQ ID NO:10. In some embodiments, an anti-DR5 antibody comprises a heavy chain consisting of amino residues 1-451 of SEQ ID NO:9 and a light chain consisting of amino acid residues 1-219 of SEQ ID NO:10.

[0034] In some embodiments, a Ligand Drug Conjugate has the formula:



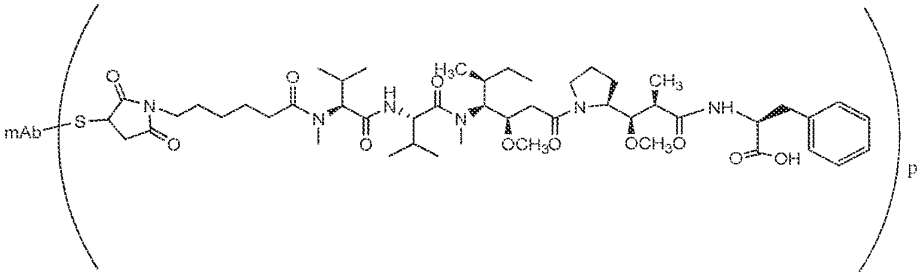
or a pharmaceutically acceptable salt form thereof, wherein mAb is an anti-DR5 antibody as described herein, S is a sulfur atom of the antibody, and p is an integer of from 1 to 8. In some embodiments, p is from 3 to 5. In some embodiments, p is from 1 to 3.

[0035] In some embodiments, a Ligand Drug Conjugate has the formula:



or a pharmaceutically acceptable salt form thereof, wherein mAb is an anti-DR5 antibody as described herein, S is a sulfur atom of the antibody, and p is an integer of from 1 to 8. In some embodiments, p is from 3 to 5. In some embodiments, p is from 1 to 3.

[0036] In some embodiments, the Ligand Drug Conjugate has the formula:



or a pharmaceutically acceptable salt form thereof, wherein mAb is an anti-DR5 antibody as described herein, S is a sulfur atom of the antibody, and p is an integer of from 1 to 8. In some embodiments, p is from 3 to 5. In some embodiments, p is from 1 to 3.

[0037] In another aspect, the present invention provides a composition comprising a mixture of Ligand Drug Conjugates as described herein. In some embodiments, the composition has an average p value that is between 1 and 20. In some embodiments, the composition has an average p value that is between 1 and 10. In some embodiments, the composition has an average p value that is from about 3.5 to about 4.5.

[0038] In another aspect, the present invention provides pharmaceutical compositions comprising a Ligand Drug Conjugate as described herein, in admixture with a pharmaceutically acceptable excipient.

[0039] In still another aspect, the present invention provides anti-tumor agents comprising a Ligand Drug Conjugate as described herein, as an effective ingredient.

[0040] In yet another aspect, the present invention provides methods of treating a cancer that expresses a DR5 protein or is a DR5 positive cancer. In some embodiments, the method comprises administering to a subject in need thereof an effective amount of a Ligand Drug Conjugate as described herein. In some embodiments, the DR5 positive or DR5 expressing cancer is selected from the group consisting of melanoma, glioblastoma, colorectal cancer, non-small cell lung carcinoma, uterine cancer, pancreatic cancer, prostate cancer, breast cancer, ovarian cancer, and hematological cancer. In some embodiments, the cancer is pancreatic cancer. In some embodiments, the cancer is melanoma. In some embodiments, the cancer is breast cancer. In some embodiments, the cancer is ovarian cancer. In some embodiments, the cancer is colorectal cancer. In some embodiments, the cancer is renal cancer. In some embodiments, the cancer is glioblastoma.

BRIEF DESCRIPTION OF THE DRAWINGS

[0041] **Figures 1-12** provide the results for 12 cell lines evaluated with hB273 Ligand Drug Conjugates of the present invention.

[0042] **Figures 13-23** provide *in vivo* results for the Ligand Drug Conjugates of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Definitions and Abbreviations

[0043] When a trade name is used herein, reference to the trade name also refers to the product formulation, the generic drug, and the active pharmaceutical ingredient(s) of the trade name product, unless otherwise indicated by context.

[0044] The terms "DR5 binding agent" and "anti-DR5 binding agent" as used herein refers to a molecule, *e.g.*, a protein, that specifically binds to DR5. Examples can include a full length anti-DR5 antibody, a fragment of a full length anti-DR5 antibody, or other agent that includes an antibody heavy and/or light chain variable region, and derivatives thereof.

[0045] The terms "inhibit" or "inhibition of" as used herein means to reduce by a measurable amount, or to prevent entirely.

[0046] The term "deplete," in the context of the effect of a DR5 binding agent on DR5-expressing cells, refers to a reduction in the number of or elimination of the DR5-expressing cells.

[0047] The term "compound" refers to and encompasses the chemical compound itself as well as, whether explicitly stated or not, and unless the context makes clear that the following are to be excluded: amorphous and crystalline forms of the compound, including polymorphic forms, where these forms may be part of a mixture or in isolation; free acid and free base forms of the compound, which are typically the forms shown in the structures provided herein; isomers of the compound, which refers to optical isomers, and tautomeric isomers, where optical isomers include enantiomers and diastereomers, chiral isomers and non-chiral isomers, and the optical isomers include isolated optical isomers as well as mixtures of optical isomers including racemic and non-racemic mixtures; where an isomer may be in isolated form or in a mixture with one or more other isomers; isotopes of the compound, including deuterium- and tritium-containing compounds, and including compounds containing radioisotopes, including therapeutically- and diagnostically-effective radioisotopes; multimeric forms of the compound, including dimeric, trimeric, etc. forms; salts of the compound, preferably pharmaceutically acceptable salts, including acid addition salts and base addition salts, including salts having organic counterions and inorganic counterions, and including zwitterionic forms, where if a compound is associated with two or more counterions, the two or more counterions may be the same or different; and solvates of the compound, including hemisolvates, monosolvates, disolvates, etc., including organic

solvates and inorganic solvates, said inorganic solvates including hydrates; where if a compound is associated with two or more solvent molecules, the two or more solvent molecules may be the same or different. In some instances, reference made herein to a compound of the invention will include an explicit reference to one or more of the above forms, *e.g.*, salts and/or solvates, however, this reference is for emphasis only, and is not to be construed as excluding other of the above forms as identified above.

[0048] Unless otherwise noted, the term "alkyl" refers to a saturated straight or branched hydrocarbon having from about 1 to about 20 carbon atoms (and all combinations and subcombinations of ranges and specific numbers of carbon atoms therein), with from about 1 to about 8 carbon atoms being preferred. Examples of alkyl groups are methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, *iso*-butyl, *sec*-butyl, *tert*-butyl, *n*-pentyl, 2-pentyl, 3-pentyl, 2-methyl-2-butyl, *n*-hexyl, *n*-heptyl, *n*-octyl, *n*-nonyl, *n*-decyl, 3-methyl-2-butyl, 3-methyl-1-butyl, 2-methyl-1-butyl, 1-hexyl, 2-hexyl, 3-hexyl, 2-methyl-2-pentyl, 3-methyl-2-pentyl, 4-methyl-2-pentyl, 3-methyl-3-pentyl, 2-methyl-3-pentyl, 2,3-dimethyl-2-butyl, and 3,3-dimethyl-2-butyl. In the structures herein, a bond without a label is taken is taken to be a methyl group, according to conventional practice in the art.

[0049] Alkyl groups, whether alone or as part of another group, may be referred to as "substituted." A substituted alkyl group is an alkyl group that is substituted with one or more groups, preferably 1 to 3 groups (and any additional substituents selected from halogen), including, but not limited to, -halogen, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH_2 , -C(O)NHR', -C(O)N(R')$_2$, -NHC(O)R', -SR', -SO_3R', -S(O)$_2$R', -S(O)R', -OH, =O, -N_3 , -NH_2, -NH(R'), -N(R')$_2$ and -CN, where each R' is independently selected from -H, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, or -aryl, and wherein said -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -aryl, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, and -C_2-C_8 alkynyl groups can be optionally further substituted with one or more groups including, but not limited to, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, -halogen, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -aryl, -C(O)R", -OC(O)R", -C(O)OR", -C(O)NH_2 , -C(O)NHR", -C(O)N(R")$_2$, -NHC(O)R", -SR", -SO_3R", -S(O)$_2$R", -S(O)R", -OH, -N_3 , -NH_2, -NH(R"), -N(R")$_2$ and -CN, where each R" is independently selected from -H, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, or -aryl.

[0050] Unless otherwise noted, the terms "alkenyl" and "alkynyl" refer to straight and branched carbon chains having from about 2 to about 20 carbon atoms (and all combinations and subcombinations of ranges and specific numbers of carbon atoms therein), with from about 2 to about 8 carbon atoms being preferred. An alkenyl chain has at least one double

bond in the chain and an alkynyl chain has at least one triple bond in the chain. Examples of alkenyl groups include, but are not limited to, ethylene or vinyl, allyl, -1-butenyl, -2-butenyl, -isobutylenyl, -1-pentenyl, -2-pentenyl, -3-methyl-1-butenyl, -2-methyl-2-butenyl, and -2,3–dimethyl–2–butenyl. Examples of alkynyl groups include, but are not limited to, acetylenic, propargyl, acetylenyl, propynyl, -1-butynyl, -2-butynyl, -1-pentynyl, -2-pentynyl, and -3-methyl-1 butynyl.

[0051] As with alkyl groups, alkenyl and alkynyl groups can be substituted. A "substituted" alkenyl or alkynyl group is one that is substituted with one or more groups, preferably 1 to 3 groups (and any additional substituents selected from halogen), including but not limited to, -halogen, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH_2, -C(O)NHR', -C(O)N(R')$_2$, -NHC(O)R', -SR', -SO_3R', -S(O)$_2$R', -S(O)R', -OH, =O, -N_3, -NH_2, -NH(R'), -N(R')$_2$ and -CN, where each R' is independently selected from -H, -C_1-C_8 alkyl, -C_2-C_8 alkyenl, -C_2-C_8 alkynyl, or -aryl and wherein said -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -aryl, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, and -C_2-C_8 alkynyl groups can be optionally further substituted with one or more substituents including, but not limited to, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, -halogen, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2C_8 alkynyl), -aryl, -C(O)R", -OC(O)R", -C(O)OR", -C(O)NH_2, -C(O)NHR", -C(O)N(R")$_2$, -NHC(O)R", -SR", -SO_3R", -S(O)$_2$R", -S(O)R", -OH, -N_3, -NH_2, -NH(R"), -N(R")$_2$ and -CN, where each R" is independently selected from -H, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, or -aryl.

[0052] Unless otherwise noted, the term "alkylene" refers to a saturated branched or straight chain hydrocarbon radical having from about 1 to about 20 carbon atoms (and all combinations and subcombinations of ranges and specific numbers of carbon atoms therein), with from about 1 to about 8 carbon atoms being preferred and having two monovalent radical centers derived by the removal of two hydrogen atoms from the same or two different carbon atoms of a parent alkane. Typical alkylenes include, but are not limited to, methylene, ethylene, propylene, butylene, pentylene, hexylene, heptylene, ocytylene, nonylene, decalene, 1,4-cyclohexylene, and the like. Alkylene groups, whether alone or as part of another group, can be optionally substituted with one or more groups, preferably 1 to 3 groups (and any additional substituents selected from halogen), including, but not limited to, -halogen, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH_2, -C(O)NHR', -C(O)N(R')$_2$, -NHC(O)R', -SR', -SO_3R', -S(O)$_2$R', -S(O)R', -OH, =O, -N_3, -NH_2, -NH(R'), -N(R')$_2$ and -CN, where each R' is independently selected from -H, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, or -aryl and wherein said -O-(C_1-C_8 alkyl), -O-

(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -aryl, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, and -C_2-C_8 alkynyl groups can be further optionally substituted with one or more substituents including, but not limited to, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, -halogen, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -aryl, -C(O)R", -OC(O)R", -C(O)OR", -C(O)NH_2, -C(O)NHR", -C(O)N(R")$_2$, -NHC(O)R", -SR", -SO_3R", -$S(O)_2$R", -S(O)R", -OH, -N_3, -NH_2, -NH(R"), -N(R")$_2$ and -CN, where each R" is independently selected from -H, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, or -aryl.

[0053] Unless otherwise noted, the term "alkenylene" refers to an optionally substituted alkylene group containing at least one carbon-carbon double bond. Exemplary alkenylene groups include, for example, ethenylene (-CH=CH-) and propenylene (-CH=CHCH$_2$-).

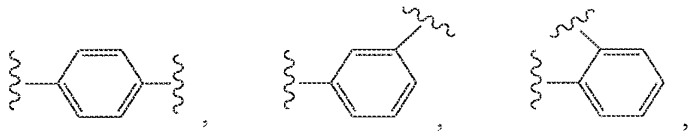
[0054] Unless otherwise noted, the term "alkynylene" refers to an optionally substituted alkylene group containing at least one carbon-carbon triple bond. Exemplary alkynylene groups include, for example, acetylene (-C≡C-), propargyl (-CH_2C≡C-), and 4-pentynyl (-$CH_2CH_2CH_2$C≡CH-).

[0055] Unless otherwise noted, the term "aryl" refers to a monovalent aromatic hydrocarbon radical of 6-20 carbon atoms (and all combinations and subcombinations of ranges and specific numbers of carbon atoms therein) derived by the removal of one hydrogen atom from a single carbon atom of a parent aromatic ring system. Some aryl groups are represented in the exemplary structures as "Ar". Typical aryl groups include, but are not limited to, radicals derived from benzene, substituted benzene, phenyl, naphthalene, anthracene, biphenyl, and the like.

[0056] An aryl group, whether alone or as part of another group, can be optionally substituted with one or more, preferably 1 to 5, or even 1 to 2 groups including, but not limited to, -halogen, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH_2, -C(O)NHR', -C(O)N(R')$_2$, -NHC(O)R', -SR', -SO_3R', -$S(O)_2$R', -S(O)R', -OH, -NO_2, -N_3, -NH_2, -NH(R'), -N(R')$_2$ and -CN, where each R' is independently selected from -H, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, or -aryl and wherein said -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), and -aryl groups can be further optionally substituted with one or more substituents including, but not limited to, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, -halogen, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -aryl, -C(O)R", -OC(O)R", -C(O)OR", -C(O)NH_2, -C(O)NHR", -C(O)N(R")$_2$, -NHC(O)R", -SR", -SO_3R", -$S(O)_2$R", -S(O)R", -OH, -N_3, -NH_2, -

NH(R"), -N(R")$_2$ and -CN, where each R" is independently selected from -H, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, or -aryl.

[0057] Unless otherwise noted, the term "arylene" refers to an optionally substituted aryl group which is divalent (*i.e.*, derived by the removal of two hydrogen atoms from the same or two different carbon atoms of a parent aromatic ring system) and can be in the ortho, meta, or para configurations as shown in the following structures with phenyl as the exemplary aryl group:



in which the aryl group (e.g., phenyl group) can be unsubstituted or substituted. In some embodiments, the arylene is a substituted arylene, wherein it is substituted with up to four groups including, but not limited to, -C_1-C_8 alkyl, -O-(C_1-C_8 alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH_2, -C(O)NHR', -C(O)N(R')$_2$, -NHC(O)R', -$S(O)_2$R', -S(O)R', -OH, -halogen, -N_3, -NH_2, -NH(R'), -N(R')$_2$ and -CN; wherein each R' is independently selected from H, -C_1-C_8 alkyl and aryl.

[0058] Unless otherwise noted, the term "heterocycle" refers to a monocyclic, bicyclic, or polycyclic ring system having from 3 to 14 ring atoms (also referred to as ring members) wherein at least one ring atom in at least one ring is a heteroatom selected from N, O, P, or S (and all combinations and subcombinations of ranges and specific numbers of carbon atoms and heteroatoms therein). The heterocycle can have from 1 to 4 ring heteroatoms independently selected from N, O, P, or S. One or more N, C, or S atoms in a heterocycle can be oxidized. A monocyclic heterocycle preferably has 3 to 7 ring members (e.g., 2 to 6 carbon atoms and 1 to 3 heteroatoms independently selected from N, O, P, or S), and a bicyclic heterocycle preferably has 5 to 10 ring members (*e.g.*, 4 to 9 carbon atoms and 1 to 3 heteroatoms independently selected from N, O, P, or S). The ring that includes the heteroatom can be aromatic or non-aromatic. Unless otherwise noted, the heterocycle is attached to its pendant group at any heteroatom or carbon atom that results in a stable structure.

[0059] Heterocycles are described in Paquette, "Principles of Modern Heterocyclic Chemistry" (W.A. Benjamin, New York, 1968), particularly Chapters 1, 3, 4, 6, 7, and 9; "The Chemistry of Heterocyclic Compounds, A series of Monographs" (John Wiley & Sons,

New York, 1950 to present), in particular Volumes 13, 14, 16, 19, and 28; and *J. Am. Chem. Soc.* 82:5566 (1960).

[0060] Unless otherwise noted, the term "heterocyclo" refers to an optionally substituted heterocycle group as defined above that is divalent (*i.e.*, derived by the removal of two hydrogen atoms from the same or two different carbon atoms of a parent heterocyclic ring system).

[0061] Examples of "heterocycle" groups include by way of example and not limitation pyridyl, dihydropyridyl, tetrahydropyridyl (piperidyl), thiazolyl, pyrimidinyl, furanyl, thienyl, pyrrolyl, pyrazolyl, imidazolyl, tetrazolyl, benzofuranyl, thianaphthalenyl, indolyl, indolenyl, quinolinyl, isoquinolinyl, benzimidazolyl, piperidinyl, 4-piperidonyl, pyrrolidinyl, 2-pyrrolidonyl, pyrrolinyl, tetrahydrofuranyl, bis-tetrahydrofuranyl, tetrahydropyranyl, bis-tetrahydropyranyl, tetrahydroquinolinyl, tetrahydroisoquinolinyl, decahydroquinolinyl, octahydroisoquinolinyl, azocinyl, triazinyl, 6H-1,2,5-thiadiazinyl, 2H,6H-1,5,2-dithiazinyl, thienyl, thianthrenyl, pyranyl, isobenzofuranyl, chromenyl, xanthenyl, phenoxathinyl, 2H-pyrrolyl, isothiazolyl, isoxazolyl, pyrazinyl, pyridazinyl, indolizinyl, isoindolyl, 3H-indolyl, 1H-indazolyl, purinyl, 4H-quinolizinyl, phthalazinyl, naphthyridinyl, quinoxalinyl, quinazolinyl, cinnolinyl, pteridinyl, 4H-carbazolyl, carbazolyl, β-carbolinyl, phenanthridinyl, acridinyl, pyrimidinyl, phenanthrolinyl, phenazinyl, phenothiazinyl, furazanyl, phenoxazinyl, isochromanyl, chromanyl, imidazolidinyl, imidazolinyl, pyrazolidinyl, pyrazolinyl, piperazinyl, indolinyl, isoindolinyl, quinuclidinyl, morpholinyl, oxazolidinyl, benzotriazolyl, benzisoxazolyl, oxindolyl, benzoxazolinyl, and isatinoyl. Preferred "heterocycle" groups include, but are not limited to, benzofuranyl, benzothiophenyl, indolyl, benzopyrazolyl, coumarinyl, isoquinolinyl, pyrrolyl, thiophenyl, furanyl, thiazolyl, imidazolyl, pyrazolyl, triazolyl, quinolinyl, pyrimidinyl, pyridinyl, pyridonyl, pyrazinyl, pyridazinyl, isothiazolyl, isoxazolyl and tetrazolyl.

[0062] A heterocycle group, whether alone or as part of another group, can be optionally substituted with one or more groups, preferably 1 to 2 groups, including but not limited to, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, -halogen, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH_2, -C(O)NHR', -C(O)N(R')$_2$, -NHC(O)R', -SR', -SO_3R', -S(O)$_2$R', -S(O)R', -OH, -N_3, -NH_2, -NH(R'), -N(R')$_2$ and -CN, where each R' is independently selected from -H, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, or -aryl and wherein said -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, and -aryl groups can be further optionally substituted with one or more substituents including, but not limited to, -C_1-C_8

alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, -halogen, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -aryl, -C(O)R", -OC(O)R", -C(O)OR", -$C(O)NH_2$, -C(O)NHR", -$C(O)N(R")_2$, -NHC(O)R", -SR", -SO_3R", -$S(O)_2R$", -S(O)R", -OH, -N_3, -NH_2, -NH(R"), -$N(R")_2$ and -CN, where each R" is independently selected from -H, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, or aryl.

[0063] By way of example and not limitation, carbon-bonded heterocycles can be bonded at the following positions: position 2, 3, 4, 5, or 6 of a pyridine; position 3, 4, 5, or 6 of a pyridazine; position 2, 4, 5, or 6 of a pyrimidine; position 2, 3, 5, or 6 of a pyrazine; position 2, 3, 4, or 5 of a furan, tetrahydrofuran, thiofuran, thiophene, pyrrole or tetrahydropyrrole; position 2, 4, or 5 of an oxazole, imidazole or thiazole; position 3, 4, or 5 of an isoxazole, pyrazole, or isothiazole; position 2 or 3 of an aziridine; position 2, 3, or 4 of an azetidine; position 2, 3, 4, 5, 6, 7, or 8 of a quinoline; or position 1, 3, 4, 5, 6, 7, or 8 of an isoquinoline. Still more typically, carbon bonded heterocycles include 2-pyridyl, 3-pyridyl, 4-pyridyl, 5-pyridyl, 6-pyridyl, 3-pyridazinyl, 4-pyridazinyl, 5-pyridazinyl, 6-pyridazinyl, 2-pyrimidinyl, 4-pyrimidinyl, 5-pyrimidinyl, 6-pyrimidinyl, 2-pyrazinyl, 3-pyrazinyl, 5-pyrazinyl, 6-pyrazinyl, 2-thiazolyl, 4-thiazolyl, or 5-thiazolyl.

[0064] By way of example and not limitation, nitrogen bonded heterocycles can be bonded at position 1 of an aziridine, azetidine, pyrrole, pyrrolidine, 2-pyrroline, 3-pyrroline, imidazole, imidazolidine, 2-imidazoline, 3-imidazoline, pyrazole, pyrazoline, 2-pyrazoline, 3-pyrazoline, piperidine, piperazine, indole, indoline, or 1H-indazole; position 2 of a isoindole, or isoindoline; position 4 of a morpholine; and position 9 of a carbazole, or β-carboline. Still more typically, nitrogen bonded heterocycles include 1-aziridyl, 1-azetedyl, 1-pyrrolyl, 1-imidazolyl, 1-pyrazolyl, and 1-piperidinyl.

[0065] Unless otherwise noted, the term "carbocycle," refers to a saturated or unsaturated non-aromatic monocyclic, bicyclic, or polycyclic ring system having from 3 to 14 ring atoms (and all combinations and subcombinations of ranges and specific numbers of carbon atoms therein) wherein all of the ring atoms are carbon atoms. Monocyclic carbocycles preferably have 3 to 6 ring atoms, still more preferably 5 or 6 ring atoms. Bicyclic carbocycles preferably have 7 to 12 ring atoms, *e.g.*, arranged as a bicyclo [4,5], [5,5], [5,6] or [6,6] system, or 9 or 10 ring atoms arranged as a bicyclo [5,6] or [6,6] system. The term "carbocycle" includes, for example, a monocyclic carbocycle ring fused to an aryl ring (*e.g.*, a monocyclic carbocycle ring fused to a benzene ring). Carbocyles preferably have 3 to 8 carbon ring atoms.

[0066] Carbocycle groups, whether alone or as part of another group, can be optionally substituted with, for example, one or more groups, preferably 1 or 2 groups (and any additional substituents selected from halogen), including, but not limited to, -halogen, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH_2 , -C(O)NHR', -C(O)N(R')$_2$, -NHC(O)R', -SR', -SO_3R', -S(O)$_2$R', -S(O)R', -OH, =O, -N_3, -NH_2, -NH(R'), -N(R')$_2$ and -CN, where each R' is independently selected from -H, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, or -aryl and wherein said -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), and -aryl groups can be further optionally substituted with one or more substituents including, but not limited to, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, -halogen, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -aryl, -C(O)R", -OC(O)R", -C(O)OR", -C(O)NH_2 , -C(O)NHR", -C(O)N(R")$_2$, -NHC(O)R", -SR", -SO_3R", -S(O)$_2$R", -S(O)R", -OH, -N_3 , -NH_2, -NH(R"), -N(R")$_2$ and -CN, where each R" is independently selected from -H, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, or -aryl.

[0067] Examples of monocyclic carbocylic substituents include -cyclopropyl, -cyclobutyl, -cyclopentyl, -1-cyclopent-1-enyl, -1-cyclopent-2-enyl, -1-cyclopent-3-enyl, cyclohexyl, -1-cyclohex-1-enyl, -1-cyclohex-2-enyl, -1-cyclohex-3-enyl, -cycloheptyl, -cyclooctyl. -1,3-cyclohexadienyl, -1,4-cyclohexadienyl, -1,3-cycloheptadienyl, -1,3,5-cycloheptatrienyl, and –cyclooctadienyl.

[0068] A "carbocyclo," whether used alone or as part of another group, refers to an optionally substituted carbocycle group as defined above that is divalent (i.e., derived by the removal of two hydrogen atoms from the same or two different carbon atoms of a parent carbocyclic ring system).

[0069] Unless otherwise indicated by context, a hyphen (-) designates the point of attachment to the pendant molecule. Accordingly, the term "-(C_1-C_8 alkylene)aryl" or "-C_1-C_8 alkylene(aryl)" refers to a C_1-C_8 alkylene radical as defined herein wherein the alkylene radical is attached to the pendant molecule at any of the carbon atoms of the alkylene radical and one of the hydrogen atoms bonded to a carbon atom of the alkylene radical is replaced with an aryl radical as defined herein. Typical "-(C_1-C_8 alkylene)aryl," "-(C_2-C_8 alkenylene)aryl", "and -(C_2-C_8 alkynylene)aryl" groups include, but are not limited to, benzyl, 2-phenylethan-1-yl, 2-phenylethen-1-yl, naphthylmethyl, 2-naphthylethan-1-yl, 2-naphthylethen-1-yl, naphthobenzyl, 2-naphthophenylethan-1-yl and the like.

[0070] When a particular group is "substituted", that group may have one or more substituents, preferably from one to five substituents, more preferably from one to three substituents, most preferably from one to two substituents, independently selected from the list of substituents. The group can, however, generally have any number of substituents selected from halogen. Groups that are substituted are so indicated.

[0071] It is intended that the definition of any substituent or variable at a particular location in a molecule be independent of its definitions elsewhere in that molecule. It is understood that substituents and substitution patterns on the compounds of this invention can be selected by one of ordinary skill in the art to provide compounds that are chemically stable and that can be readily synthesized by techniques known in the art as well as those methods set forth herein.

[0072] Protective groups as used herein refer to groups which selectively block, either temporarily or permanently, one reactive site in a multifunctional compound. Suitable hydroxy-protecting groups for use in the present invention are pharmaceutically acceptable and may or may not need to be cleaved from the parent compound after administration to a subject in order for the compound to be active. Cleavage is through normal metabolic processes within the body. Hydroxy protecting groups are well known in the art, see, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS by T. W. *Greene* and P. G. M. Wuts (John Wiley & sons, 3rd Edition) incorporated herein by reference in its entirety and for all purposes and include, for example, ether (e.g., alkyl ethers and silyl ethers including, for example, dialkylsilylether, trialkylsilylether, dialkylalkoxysilylether), ester, carbonate, carbamates, sulfonate, and phosphate protecting groups. Examples of hydroxy protecting groups include, but are not limited to, methyl ether; methoxymethyl ether, methylthiomethyl ether, (phenyldimethylsilyl)methoxymethyl ether, benzyloxymethyl ether, p-methoxybenzyloxymethyl ether, p-nitrobenzyloxymethyl ether, o-nitrobenzyloxymethyl ether, (4-methoxyphenoxy)methyl ether, guaiacolmethyl ether, t-butoxymethyl ether, 4-pentenyloxymethyl ether, siloxymethyl ether, 2-methoxyethoxymethyl ether, 2,2,2-trichloroethoxymethyl ether, bis(2-chloroethoxy)methyl ether, 2-(trimethylsilyl)ethoxymethyl ether, menthoxymethyl ether, tetrahydropyranyl ether, 1-methoxycylcohexyl ether, 4-methoxytetrahydrothiopyranyl ether, 4-methoxytetrahydrothiopyranyl ether S,S-Dioxide, 1-[(2-choro-4-methyl)phenyl]-4-methoxypiperidin-4-yl ether, 1-(2-fluorophneyl)-4-methoxypiperidin-4-yl ether, 1,4-dioxan-2-yl ether, tetrahydrofuranyl ether, tetrahydrothiofuranyl ether; substituted ethyl ethers such as 1-ethoxyethyl ether, 1-(2-chloroethoxy)ethyl ether, 1-[2-(trimethylsilyl)ethoxy]ethyl ether, 1-methyl-1-methoxyethyl

ether, 1-methyl-1-benzyloxyethyl ether, 1-methyl-1-benzyloxy-2-fluoroethyl ether, 1-methyl-1phenoxyethyl ether, 2-trimethylsilyl ether, t-butyl ether, allyl ether, propargyl ethers, p-chlorophenyl ether, p-methoxyphenyl ether, benzyl ether, p-methoxybenzyl ether 3,4-dimethoxybenzyl ether, trimethylsilyl ether, triethylsilyl ether, tripropylsilylether, dimethylisopropylsilyl ether, diethylisopropylsilyl ether, dimethylhexylsilyl ether, t-butyldimethylsilyl ether, diphenylmethylsilyl ether, benzoylformate ester, acetate ester, chloroacetate ester, dichloroacetate ester, trichloroacetate ester, trifluoroacetate ester, methoxyacetate ester, triphneylmethoxyacetate ester, phenylacetate ester, benzoate ester, alkyl methyl carbonate, alkyl 9-fluorenylmethyl carbonate, alkyl ethyl carbonate, alkyl 2,2,2,-trichloroethyl carbonate, 1,1,-dimethyl-2,2,2-trichloroethyl carbonate, alkylsulfonate, methanesulfonate, benzylsulfonate, tosylate, methylene acetal, ethylidene acetal, and t-butylmethylidene ketal. Preferred protecting groups are represented by the formulas -R, -Si(R)(R)(R), -C(O)R, -C(O)OR, -C(O)NH(R), -$S(O)_2$R, -$S(O)_2$OH, P(O)$(OH)_2$, and -P(O)(OH)OR, wherein R is C_1-C_{20} alkyl, C_2-C_{20} alkenyl, C_2-C_{20} alkynyl, -C_1-C_{20} alkylene(carbocycle), -C_2-C_{20} alkenylene(carbocycle), -C_2-C_{20} alkynylene(carbocycle), -C_6-C_{10} aryl, -C_1-C_{20} alkylene(aryl), -C_2-C_{20} alkenylene(aryl), -C_2-C_{20} alkynylene(aryl), -C_1-C_{20} alkylene(heterocycle), -C_2-C_{20} alkenylene(heterocycle), or -C_2-C_{20} alkynylene(heterocycle) wherein said alkyl, alkenyl, alkynyl, alkylene, alkenylene, alkynylene, aryl, carbocycle, and heterocycle radicals whether alone or as part of another group are optionally substituted.

[0073] The abbreviation "AFP" refers to dimethylvaline-valine-dolaisoleuine- dolaproine-phenylalanine-p-phenylenediamine (*see* Formula XVIII *infra*).

[0074] The abbreviation "MMAE" refers to monomethyl auristatin E (*see* Formula XIII *infra*).

[0075] The abbreviation "AEB" refers to an ester produced by reacting auristatin E with paraacetyl benzoic acid (*see* Formula XXII *infra*).

[0076] The abbreviation "AEVB" refers to an ester produced by reacting auristatin E with benzoylvaleric acid (*see* Formula XXIII *infra*).

[0077] The abbreviation "MMAF" refers to dovaline-valine-dolaisoleuine-dolaproine-phenylalanine (*see* Formula XXI *infra*).

[0078] 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

"pharmaceutically compatible ingredient" refers to a pharmaceutically acceptable diluent, adjuvant, excipient, or vehicle with which the antibody or antibody derivative is administered.

[0079] The term "animal" refers to humans, non-human mammals (*e.g.,* dogs, cats, rabbits, cattle, horses, sheep, goats, swine, deer, and the like) and non-mammals (*e.g.,* birds, and the like).

GENERAL

[0080] The methods described herein encompass the use of Ligand Drug Conjugates wherein the Ligand unit is an anti-DR5 binding agent that specifically binds to DR5. The DR5 binding agent can be, for example, an anti-DR5 antibody, an anti-DR5 antigen-binding fragment, or other DR5 binding agent comprising the amino acid sequence of a humanized antibody heavy and/or light chain variable region, or derivative thereof.

LIGAND DRUG CONJUGATE

[0081] The present invention provides, *inter alia,* Ligand Drug Conjugates for targeted delivery of drugs. The inventors have made the discovery that the Ligand Drug Conjugates have potent cytotoxic and/or cytostatic activity against cells expressing DR5. The Ligand Drug Conjugates comprise a Ligand unit covalently linked to at least one Drug unit. The Drug units can be covalently linked directly or via a Linker unit (-LU-).

[0082] In some embodiments, the Ligand Drug Conjugate has the following formula:

$$L - (LU-D)_p \qquad (I)$$

or a pharmaceutically acceptable salt thereof; wherein:
L is the Ligand unit, i.e., a DR5 binding agent of the present invention, and
(LU-D) is a Linker unit-Drug unit moiety, wherein:
LU- is a Linker unit, and
-D is a drug unit having cytostatic or cytotoxic activity against a target cell; and
p is from 1 to 20.

[0083] In some embodiments, the Ligand Drug Conjugate has a p value (number of drug units loaded per ligand) from 1 to 20. In some embodiments, p ranges from 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, or 1 to 2. In some embodiments, p ranges from 2 to

10, 2 to 9, 2 to 8, 2 to 7, 2 to 6, 2 to 5, 2 to 4 or 2 to 3. In some embodiments, the p value is 2, 4, 6, or 8. In other embodiments, p is 1, 2, 3, 4, 5 or 6.

[0084] In some embodiments, the Ligand Drug Conjugate has the following formula:

$$L - (A_a\text{-}W_w\text{-}Y_y\text{-}D)_p \qquad \textbf{(II)}$$

or a pharmaceutically acceptable salt thereof;

wherein:

L is the Ligand unit, i.e. DR5 binding agent; and

$-A_a-W_w-Y_y-$ is a Linker unit (LU), wherein:

-A- is a Stretcher unit,

a is 0 or 1,

each -W- is independently an Amino Acid unit,

w is an integer ranging from 0 to 12,

-Y- is a self-immolative spacer unit,

y is 0, 1 or 2;

-D is a drug unit having cytostatic or cytotoxic activity against the target cell; and

p is from 1 to 20.

[0085] In some embodiments, a is 0 or 1, w is 0 or 1, and y is 0, 1 or 2. In some embodiments, a is 0 or 1, w is 0 or 1, and y is 0 or 1. In some embodiments, p ranges from 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, or 1 to 2. In some embodiments, p ranges from 2 to 10, 2 to 9, 2 to 8, 2 to 7, 2 to 6, 2 to 5, 2 to 4 or 2 to 3. In other embodiments, p is 1, 2, 3, 4, 5 or 6. In some embodiments, when w is not zero, y is 1 or 2. In some embodiments, when w is 1 to 12, y is 1 or 2. In some embodiments, w is 2 to 12 and y is 1 or 2. In some embodiments, a is 1 and w and y are 0.

[0086] In compositions comprising a plurality of Ligand Drug Conjugates, p is the average number of Drug molecules per Ligand, also referred to as the average drug loading. Average drug loading may range from 1 to about 20 drugs (D) per Ligand. In some embodiments, the average p value (average drug loading per Ligand) is from about 2 to about 8. In some embodiments, the average p value (average drug loading per Ligand) is from about 3.5 to about 4.5. In some embodiments the average p value (average drug loading per Ligand) is about 1, about 2, about 3, about 4, about 5 or about 6. The average number of drugs per ligand in preparation of conjugation reactions may be characterized by conventional means such as mass spectroscopy, ELISA assay, and HPLC. The quantitative distribution of Ligand Drug Conjugates in terms of p may also be determined. In some instances, separation,

purification, and characterization of homogeneous Ligand Drug Conjugates where p is a certain value from Ligand Drug Conjugates with other drug loadings may be achieved by means such as reverse phase HPLC or electrophoresis.

[0087] The generation of Ligand Drug Conjugates can be accomplished by any technique known to the skilled artisan. Briefly, the Ligand Drug Conjugates comprise a DR5 binding agent as the Ligand unit, a drug, and optionally a linker that joins the drug and the binding agent. A number of different reactions are available for covalent attachment of drugs and/or linkers to binding agents. This is often accomplished by reaction of the amino acid residues of the binding agent, e.g., antibody molecule, including the amine groups of lysine, the free carboxylic acid groups of glutamic and aspartic acid, the sulfhydryl groups of cysteine and the various moieties of the aromatic amino acids. One of the most commonly used non-specific methods of covalent attachment is the carbodiimide reaction to link a carboxy (or amino) group of a compound to amino (or carboxy) groups of the antibody. Additionally, bifunctional agents such as dialdehydes or imidoesters have been used to link the amino group of a compound to amino groups of an antibody molecule. Also available for attachment of drugs to binding agents is the Schiff base reaction. This method involves the periodate oxidation of a drug that contains glycol or hydroxy groups, thus forming an aldehyde which is then reacted with the binding agent. Attachment occurs via formation of a Schiff base with amino groups of the binding agent. Isothiocyanates can also be used as coupling agents for covalently attaching drugs to binding agents. Other techniques are known to the skilled artisan and within the scope of the present invention.

[0088] In certain embodiments, an intermediate, which is the precursor of the linker, is reacted with the drug under appropriate conditions. In certain embodiments, reactive groups are used on the drug and/or the intermediate. The product of the reaction between the drug and the intermediate, or the derivatized drug, is subsequently reacted with the DR5 binding agent under appropriate conditions.

[0089] Each of the particular units of the Ligand Drug Conjugates is described in more detail herein. The synthesis and structure of exemplary linker units, stretcher units, amino acid units, self-immolative spacer unit, and drug units are also described in U.S. Patent Application Publication Nos. 2003-0083263, 2005-0238649, 2005-0009751, 2006-0074008, and 2009-0010945, each of which is incorporated herein by reference in its entirety and for all purposes.

LINKER UNITS

[0090] Typically, the Ligand Drug Conjugates comprise a linker region between the drug unit and the Ligand unit. In some embodiments, the linker is cleavable under intracellular conditions, such that cleavage of the linker releases the drug unit from the ligand in the intracellular environment. In yet other embodiments, the linker unit is not cleavable and the drug is released, for example, by antibody degradation.

[0091] In some embodiments, the linker is cleavable by a cleaving agent that is present in the intracellular environment (*e.g.*, within a lysosome or endosome or caveolea). The linker can be, *e.g.*, a peptidyl linker that is cleaved by an intracellular peptidase or protease enzyme, including, but not limited to, a lysosomal or endosomal protease. In some embodiments, the peptidyl linker is at least two amino acids long or at least three amino acids long. Cleaving agents can include cathepsins B and D and plasmin, all of which are known to hydrolyze dipeptide drug derivatives resulting in the release of active drug inside target cells (*see*, *e.g.*, Dubowchik and Walker, 1999, *Pharm. Therapeutics* 83:67-123). Most typical are peptidyl linkers that are cleavable by enzymes that are present in DR5-expressing cells. For example, a peptidyl linker that is cleavable by the thiol-dependent protease cathepsin-B, which is highly expressed in cancerous tissue, can be used (*e.g.*, a Phe-Leu or a Gly-Phe-Leu-Gly (SEQ ID NO:21) linker). Other examples of such linkers are described, *e.g.*, in U.S. Patent No. 6,214,345, incorporated herein by reference in its entirety and for all purposes. In a specific embodiment, the peptidyl linker cleavable by an intracellular protease is a Val-Cit linker or a Phe-Lys linker (*see, e.g.*, U.S. patent 6,214,345, which describes the synthesis of doxorubicin with the Val-Cit linker). One advantage of using intracellular proteolytic release of the therapeutic agent is that the agent is typically attenuated when conjugated and the serum stabilities of the conjugates are typically high.

[0092] In other embodiments, the cleavable linker is pH-sensitive, *i.e.*, sensitive to hydrolysis at certain pH values. Typically, the pH-sensitive linker is hydrolyzable under acidic conditions. For example, an acid-labile linker that is hydrolyzable in the lysosome (*e.g.*, a hydrazone, semicarbazone, thiosemicarbazone, cis-aconitic amide, orthoester, acetal, ketal, or the like) can be used. (*See, e.g.*, U.S. Patent Nos. 5,122,368; 5,824,805; 5,622,929; Dubowchik and Walker, 1999, *Pharm. Therapeutics* 83:67-123; Neville *et al.*, 1989, *Biol. Chem.* 264:14653-14661.) Such linkers are relatively stable under neutral pH conditions, such as those in the blood, but are unstable at below pH 5.5 or 5.0, the approximate pH of the lysosome. In certain embodiments, the hydrolyzable linker is a thioether linker (such as, *e.g.*,

a thioether attached to the therapeutic agent via an acylhydrazone bond (*see, e.g.*, U.S. Patent No. 5,622,929).

[0093] In yet other embodiments, the linker is cleavable under reducing conditions (*e.g.*, a disulfide linker). A variety of disulfide linkers are known in the art, including, for example, those that can be formed using SATA (N-succinimidyl-S-acetylthioacetate), SPDP (N-succinimidyl-3-(2-pyridyldithio)propionate), SPDB (N-succinimidyl-3-(2-pyridyldithio)butyrate) and SMPT (N-succinimidyl-oxycarbonyl-alpha-methyl-alpha-(2-pyridyl-dithio)toluene), SPDB and SMPT (*See, e.g.*, Thorpe *et al.*, 1987, *Cancer Res.* 47:5924-5931; Wawrzynczak *et al.*, In *Immunoconjugates: Antibody Conjugates in Radioimagery and Therapy of Cancer* (C. W. Vogel ed., Oxford U. Press, 1987. *See also* U.S. Patent No. 4,880,935.)

[0094] In yet other specific embodiments, the linker is a malonate linker (Johnson *et al.*, 1995, *Anticancer Res.* 15:1387-93), a maleimidobenzoyl linker (Lau *et al.*, 1995, *Bioorg-Med-Chem.* 3(10):1299-1304), a maleimidocaproyl ("mc") linker (Doronina *et al.*, 2006, *Bioconjug Chem.* 17:114-24), or a 3'-N-amide analog (Lau *et al.*, 1995, *Bioorg-Med-Chem.* 3(10):1305-12).

[0095] In yet other embodiments, the linker unit is not cleavable and the drug is released by antibody degradation. (See, for example, U.S. Publication No. 20050238649, incorporated by reference herein in its entirety and for all purposes).

[0096] Typically, the linker is not substantially sensitive to the extracellular environment. As used herein, "not substantially sensitive to the extracellular environment," in the context of a linker, means that no more than about 20%, typically no more than about 15%, more typically no more than about 10%, and even more typically no more than about 5%, no more than about 3%, or no more than about 1% of the linkers, in a sample of Ligand Drug Conjugate, are cleaved when the Ligand Drug Conjugate presents in an extracellular environment (*e.g.*, in plasma). Whether a linker is not substantially sensitive to the extracellular environment can be determined, for example, by incubating with plasma the Ligand Drug Conjugate for a predetermined time period (*e.g.*, 2, 4, 8, 16, or 24 hours) and then quantitating the amount of free drug present in the plasma.

[0097] In other, non-mutually exclusive embodiments, the linker promotes cellular internalization. In certain embodiments, the linker promotes cellular internalization when conjugated to the therapeutic agent (*i.e.*, in the milieu of the linker-therapeutic agent moiety of the Ligand Drug Conjugate as described herein). In yet other embodiments, the linker

promotes cellular internalization when conjugated to both the auristatin compound and the anti-DR5 antibody.

[0098] A variety of exemplary linkers that can be used with the present compositions and methods are described in WO 2004/010957, U.S. Publication No. 2006/0074008, U.S. Publication No. 2005/0238649, and U.S. Publication No. 2006/0024317 (each of which is incorporated by reference herein in its entirety and for all purposes).

[0099] A "Linker unit" (LU) is a bifunctional compound that can be used to link a Drug unit and a Ligand unit to form a Ligand Drug Conjugate. In some embodiments, the Linker unit has the formula:

$$-A_a-W_w-Y_y-$$

wherein:-A- is a Stretcher unit,
a is 0 or 1,
each -W- is independently an Amino Acid unit,
w is an integer ranging from 0 to 12,
-Y- is a self-immolative Spacer unit, and
y is 0, 1 or 2.

[0100] In some embodiments, a is 0 or 1, w is 0 or 1, and y is 0, 1 or 2. In some embodiments, a is 0 or 1, w is 0 or 1, and y is 0 or 1. In some embodiments, when w is 1 to 12, y is 1 or 2. In some embodiments, w is 2 to 12 and y is 1 or 2. In some embodiments, a is 1 and w and y are 0.

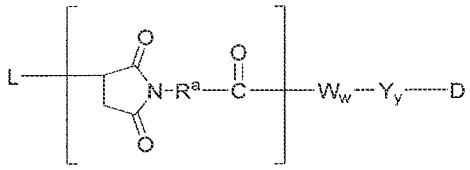
THE STRETCHER UNIT

[0101] The Stretcher unit (A), when present, is capable of linking a Ligand unit to an Amino Acid unit (-W-), if present; to a Spacer unit (-Y-), if present; or to a Drug unit (-D). Useful functional groups that can be present on a DR5 binding agent, either naturally or via chemical manipulation include, but are not limited to, sulfhydryl, amino, hydroxyl, the anomeric hydroxyl group of a carbohydrate, and carboxyl. Suitable functional groups are sulfhydryl and amino. In one example, sulfhydryl groups can be generated by reduction of the intramolecular disulfide bonds of an anti-DR5 antibody. In another embodiment, sulfhydryl groups can be generated by reaction of an amino group of a lysine moiety of an anti-DR5 antibody with 2-iminothiolane (Traut's reagent) or other sulfhydryl generating reagents. In certain embodiments, the anti-DR5 antibody is a recombinant antibody and is

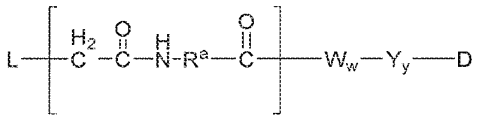
engineered to carry one or more lysines. In certain other embodiments, the recombinant anti-DR5 antibody is engineered to carry additional sulfhydryl groups, *e.g.*, additional cysteines.

[0102] In one embodiment, the Stretcher unit forms a bond with a sulfur atom of the Ligand unit. The sulfur atom can be derived from a sulfhydryl group of a Ligand. Representative Stretcher units of this embodiment are depicted within the square brackets of Formulas IIIa and IIIb, wherein L-, -W-, -Y-, -D, w and y are as defined above, and R^a is selected from -C_1-C_{10} alkylene-, -C_2-C_{10} alkenylene-, -C_2-C_{10} alkynylene-, -carbocyclo-, -O-(C_1-C_8 alkylene)-, O-(C_2-C_8 alkenylene)-, -O-(C_2-C_8 alkynylene)-, -arylene-, -C_1-C_{10} alkylene-arylene-, -C_2-C_{10} alkenylene-arylene, -C_2-C_{10} alkynylene-arylene, -arylene-C_1-C_{10} alkylene-, -arylene-C_2-C_{10} alkenylene-, -arylene-C_2-C_{10} alkynylene-, -C_1-C_{10} alkylene-(carbocyclo)-, -C_2-C_{10} alkenylene-(carbocyclo)-, -C_2-C_{10} alkynylene-(carbocyclo)-, -(carbocyclo)-C_1-C_{10} alkylene-, -(carbocyclo)-C_2-C_{10} alkenylene-, -(carbocyclo)-C_2-C_{10} alkynylene, heterocyclo-, -C_1-C_{10} alkylene-(heterocyclo)-, -C_2-C_{10} alkenylene-(heterocyclo)-, -C_2-C_{10} alkynylene-(heterocyclo)-, -(heterocyclo)-C_1-C_{10} alkylene-, -(heterocyclo)-C_2-C_{10} alkenylene-, -(heterocyclo)-C_2-C_{10} alkynylene-, -(CH_2CH_2O)$_r$-, or -(CH_2CH_2O)$_r$-CH_2-, and r is an integer ranging from 1-10, wherein said alkyl, alkenyl, alkynyl, alkylene, alkenylene, alkynylene, aryl, carbocyle, carbocyclo, heterocyclo, and arylene radicals, whether alone or as part of another group, are optionally substituted. In some embodiments, said alkyl, alkenyl, alkynyl, alkylene, alkenylene, alkynylene, aryl, carbocyle, carbocyclo, heterocyclo, and arylene radicals, whether alone or as part of another group, are unsubstituted. In some embodiments, R^a is selected from -C_1-C_{10} alkylene-, - carbocyclo-, -O-(C_1-C_8 alkylene)-, -arylene-, -C_1-C_{10} alkylene-arylene-, -arylene-C_1-C_{10} alkylene-, -C_1-C_{10} alkylene-(carbocyclo)-, -(carbocyclo)-C_1-C_{10} alkylene-, -C_3-C_8 heterocyclo-, -C_1-C_{10} alkylene-(heterocyclo)-, -(heterocyclo)-C_1-C_{10} alkylene-, -(CH_2CH_2O)$_r$-, and -(CH_2CH_2O)$_r$-CH_2-; and r is an integer ranging from 1-10, wherein said alkylene groups are unsubstituted and the remainder of the groups are optionally substituted.

[0103] It is to be understood from all the exemplary embodiments that even where not denoted expressly, from 1 to 20 drug moieties can be linked to a Ligand (p = 1-20). In some embodiments, 2, 4, 6, or 8 drug moieties are linked to a Ligand (p = 2, 4, 6, or 8).

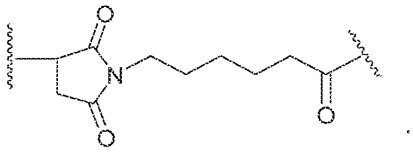


IIIa

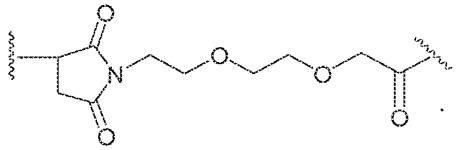


IIIb

[0104] An illustrative Stretcher unit is that of Formula IIIa wherein R^a is $-(CH_2)_5-$:

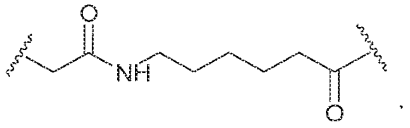


[0105] Another illustrative Stretcher unit is that of Formula IIIa wherein R^a is $-(CH_2CH_2O)_r-CH_2-$; and r is 2:

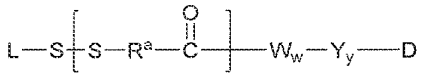


[0106] An illustrative Stretcher unit is that of Formula IIIa wherein R^a is -arylene- or arylene-C_1-C_{10} alkylene-. In some embodiments, the aryl group is an unsubstituted phenyl group.

[0107] Still another illustrative Stretcher unit is that of Formula IIIb wherein R^a is $-(CH_2)_5-$:

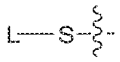


[0108] In certain embodiments, the Stretcher unit is linked to the Ligand unit via a disulfide bond between a sulfur atom of the Ligand unit and a sulfur atom of the Stretcher unit. A representative Stretcher unit of this embodiment is depicted within the square brackets of Formula IV, wherein R^a, L-, -W-, -Y-, -D, w and y are as defined above.

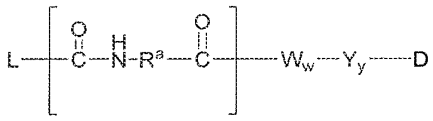


IV

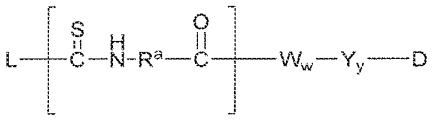
[0109] It should be noted that throughout this application, the S moiety in the formula below refers to a sulfur atom of the Ligand unit, unless otherwise indicated by context.



[0110] In yet other embodiments, the Stretcher, prior to attachment to L, contains a reactive site that can form a bond with a primary or secondary amino group of the Ligand. Examples of these reactive sites include, but are not limited to, activated esters such as succinimide esters, 4 nitrophenyl esters, pentafluorophenyl esters, tetrafluorophenyl esters, anhydrides, acid chlorides, sulfonyl chlorides, isocyanates and isothiocyanates. Representative Stretcher units of this embodiment are depicted within the square brackets of Formulas Va and Vb, wherein –Ra-, L-, -W-, -Y-, -D, w and y are as defined above;

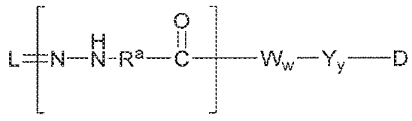


Va

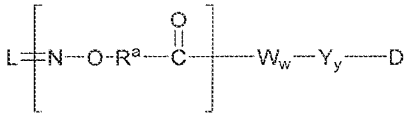


Vb

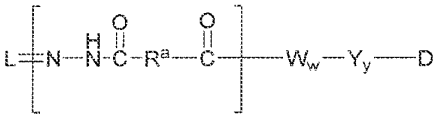
[0111] In some embodiments, the Stretcher contains a reactive site that is reactive to a modified carbohydrate's (-CHO) group that can be present on a Ligand. For example, a carbohydrate can be mildly oxidized using a reagent such as sodium periodate and the resulting (-CHO) unit of the oxidized carbohydrate can be condensed with a Stretcher that contains a functionality such as a hydrazide, an oxime, a primary or secondary amine, a hydrazine, a thiosemicarbazone, a hydrazine carboxylate, and an aryllhydrazide such as those described by Kaneko *et al.*, 1991, *Bioconjugate Chem.* 2:133-41. Representative Stretcher units of this embodiment are depicted within the square brackets of Formulas VIa, VIb, and VIc, wherein –R^a-, L-, -W-, -Y-, -D, w and y are as defined as above.



VIa



VIb

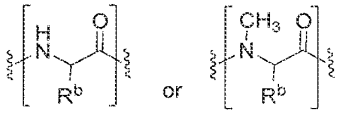


VIc

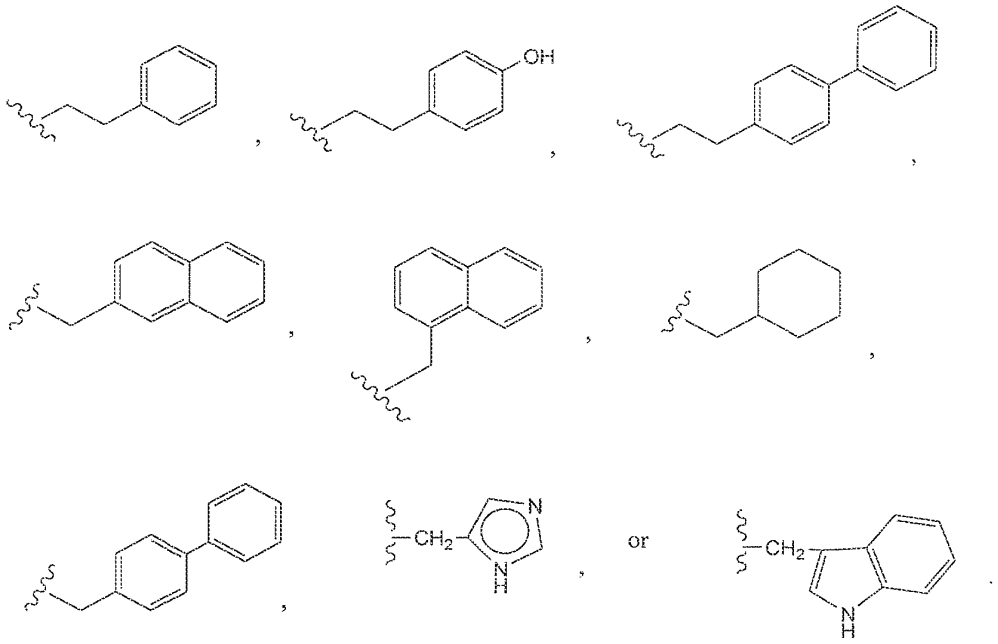
THE AMINO ACID UNIT

[0112] The Amino Acid unit (-W-), when present, links the Stretcher unit to the Spacer unit if the Spacer unit is present, links the Stretcher unit to the Drug moiety if the Spacer unit is absent, and links the Ligand unit to the Drug unit if the Stretcher unit and Spacer unit are absent.

[0113] W_w- can be, for example, a monopeptide, dipeptide, tripeptide, tetrapeptide, pentapeptide, hexapeptide, heptapeptide, octapeptide, nonapeptide, decapeptide, undecapeptide or dodecapeptide unit. Each -W- unit independently has the formula denoted below in the square brackets, and w is an integer ranging from 0 to 12:

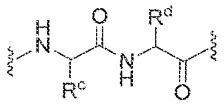


wherein R^b is hydrogen, methyl, isopropyl, isobutyl, *sec*-butyl, benzyl, *p*-hydroxybenzyl, -CH_2OH, -$CH(OH)CH_3$, -$CH_2CH_2SCH_3$, -CH_2CONH_2, -CH_2COOH, -$CH_2CH_2CONH_2$, -CH_2CH_2COOH, -$(CH_2)_3NHC(=NH)NH_2$, -$(CH_2)_3NH_2$, -$(CH_2)_3NHCOCH_3$, -$(CH_2)_3NHCHO$, -$(CH_2)_4NHC(=NH)NH_2$, -$(CH_2)_4NH_2$, -$(CH_2)_4NHCOCH_3$, -$(CH_2)_4NHCHO$, -$(CH_2)_3NHCONH_2$, -$(CH_2)_4NHCONH_2$, -$CH_2CH_2CH(OH)CH_2NH_2$, 2-pyridylmethyl-, 3-pyridylmethyl-, 4-pyridylmethyl-, phenyl, cyclohexyl,



[0114] In some embodiments, the Amino Acid unit can be enzymatically cleaved by one or more enzymes, including a cancer or tumor-associated protease, to liberate the Drug unit (-D), which in one embodiment is protonated *in vivo* upon release to provide a Drug (D).

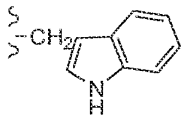
[0115] In certain embodiments, the Amino Acid unit can comprise natural amino acids. In other embodiments, the Amino Acid unit can comprise non-natural amino acids. Illustrative W_w units are represented by formulas (VII)-(IX):

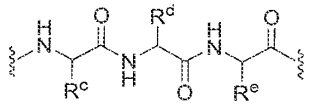


(VII)

wherein R^c and R^d are as follows:

R^c	R^d
Benzyl	$(CH_2)_4NH_2$;
methyl	$(CH_2)_4NH_2$;
isopropyl	$(CH_2)_4NH_2$;
isopropyl	$(CH_2)_3NHCONH_2$;
benzyl	$(CH_2)_3NHCONH_2$;
isobutyl	$(CH_2)_3NHCONH_2$;

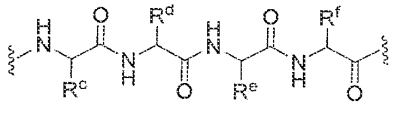
sec-butyl	$(CH_2)_3NHCONH_2$;
	$(CH_2)_3NHCONH_2$;
benzyl	methyl;
benzyl	$(CH_2)_3NHC(=NH)NH_2$;



(VIII)

wherein R^c, R^d and R^e are as follows:

R^c	R^d	R^e
benzyl	Benzyl	$(CH_2)_4NH_2$;
isopropyl	Benzyl	$(CH_2)_4NH_2$; and
H	Benzyl	$(CH_2)_4NH_2$;



(IX)

wherein R^c, R^d, R^e and R^f are as follows:

R^c	R^d	R^e	R^f
H	benzyl	isobutyl	H; and
methyl	isobutyl	methyl	isobutyl.

[0116] Exemplary Amino Acid units include, but are not limited to, units of formula VII where: R^c is benzyl and R^d is $-(CH_2)_4NH_2$; R^c is isopropyl and R^d is $-(CH_2)_4NH_2$; or R^c is isopropyl and R^d is $-(CH_2)_3NHCONH_2$. Another exemplary Amino Acid unit is a unit of formula VIII wherein R^c is benzyl, R^d is benzyl, and R^e is $-(CH_2)_4NH_2$.

[0117] Useful $-W_w-$ units can be designed and optimized in their selectivity for enzymatic cleavage by a particular enzyme, for example, a tumor-associated protease. In one

embodiment, a -W_w - unit is that whose cleavage is catalyzed by cathepsin B, C and D, or a plasmin protease.

[0118] In one embodiment, -W_w- is a dipeptide, tripeptide, tetrapeptide or pentapeptide. When R^b, R^c, R^d, R^e or R^f is other than hydrogen, the carbon atom to which R^b, R^c, R^d, R^e or R^f is attached is chiral.

[0119] Each carbon atom to which R^b, R^c, R^d, R^e or R^f is attached is independently in the (S) or (R) configuration.

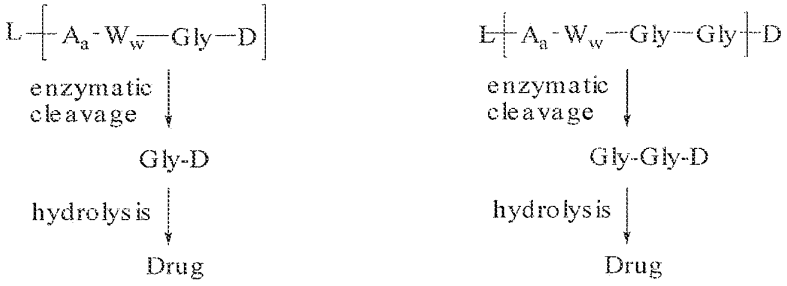
[0120] In one aspect of the Amino Acid unit, the Amino Acid unit is valine-citrulline (vc or val-cit). In another aspect, the Amino Acid unit is phenylalanine-lysine (i.e., fk). In yet another aspect of the Amino Acid unit, the Amino Acid unit is N-methylvaline-citrulline. In yet another aspect, the Amino Acid unit is 5-aminovaleric acid, homophenylalanine-lysine, tetraisoquinolinecarboxylate-lysine, cyclohexylalanine-lysine, isonepecotic acid-lysine, beta-alanine-lysine, or glycine-serine-valine-glutamine-isonepecotic acid.

THE SPACER UNIT

[0121] The Spacer unit (-Y-), when present, links an Amino Acid unit to the Drug unit when an Amino Acid unit is present. Alternately, the Spacer unit links the Stretcher unit to the Drug unit when the Amino Acid unit is absent. The Spacer unit also links the Drug unit to the Ligand unit when both the Amino Acid unit and Stretcher unit are absent.

[0122] Spacer units are of two general types: non self-immolative or self-immolative. A non self-immolative Spacer unit is one in which part or all of the Spacer unit remains bound to the Drug moiety after cleavage, particularly enzymatic, of an Amino Acid unit from the ligand- drug conjugate. Examples of a non self-immolative Spacer unit include, but are not limited to a (glycine-glycine) Spacer unit and a glycine Spacer unit (both depicted in **Scheme 1**) (infra). When a conjugate containing a glycine-glycine Spacer unit or a glycine Spacer unit undergoes enzymatic cleavage via an enzyme (*e.g.*, a tumor-cell associated-protease, a cancer-cell-associated protease or a lymphocyte-associated protease), a glycine-glycine-Drug moiety or a glycine-Drug moiety is cleaved from L-Aa-Ww-. In one embodiment, an independent hydrolysis reaction takes place within the target cell, cleaving the glycine-Drug moiety bond and liberating the Drug.

Scheme 1



[0123] In some embodiments, a non self-immolative Spacer unit (-Y-) is -Gly-. In some embodiments, a non self-immolative Spacer unit (-Y-) is -Gly-Gly-.

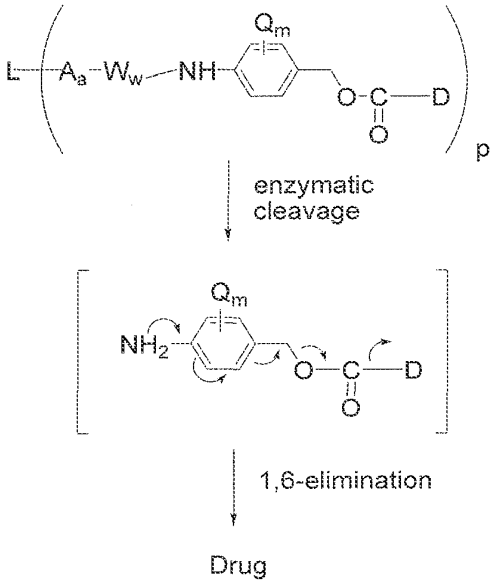
[0124] In one embodiment, a Drug-Linker conjugate is provided in which the Spacer unit is absent (y=0), or a pharmaceutically acceptable salt thereof.

[0125] Alternatively, a conjugate containing a self-immolative Spacer unit can release -D. As used herein, the term "self-immolative Spacer" refers to a bifunctional chemical moiety that is capable of covalently linking together two spaced chemical moieties into a stable tripartite molecule. It will spontaneously separate from the second chemical moiety if its bond to the first moiety is cleaved.

[0126] In some embodiments, $-Y_y-$ is a p-aminobenzyl alcohol (PAB) unit (see **Schemes 2** and **3**) whose phenylene portion is substituted with Q_m wherein Q is $-C_1-C_8$ alkyl, $-C_2-C_8$ alkenyl, $-C_2-C_8$ alkynyl, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -halogen, - nitro or -cyano; and m is an integer ranging from 0-4. The alkyl, alkenyl and alkynyl groups, whether alone or as part of another group, can be optionally substituted.

[0127] In some embodiments, -Y- is a PAB group that is linked to $-W_w-$ via the amino nitrogen atom of the PAB group, and connected directly to -D via a carbonate, carbamate or ether group. Without being bound by any particular theory or mechanism, **Scheme 2** depicts a possible mechanism of Drug release of a PAB group which is attached directly to -D via a carbamate or carbonate group as described by Toki *et al.*, 2002, *J. Org. Chem.* 67:1866-1872.

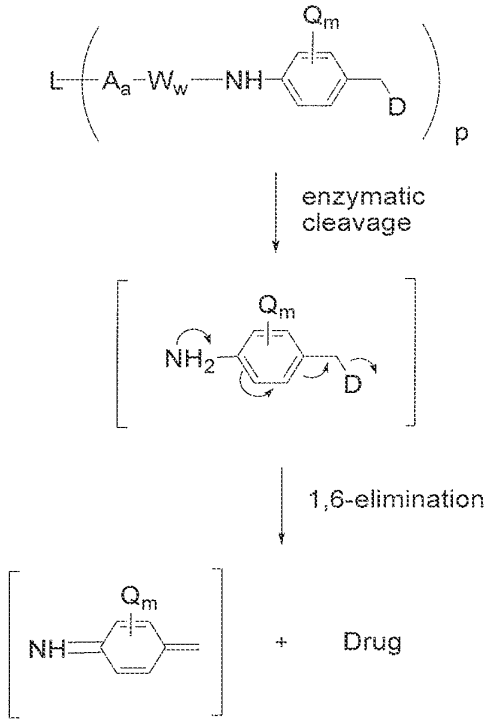
Scheme 2



[0128] In **Scheme 2**, Q is -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -halogen, -nitro or -cyano; m is an integer ranging from 0-4; and p ranges from 1 to about 20. The alkyl, alkenyl and alkynyl groups, whether alone or as part of another group, can be optionally substituted.

[0129] Without being bound by any particular theory or mechanism, **Scheme 3** depicts a possible mechanism of Drug release of a PAB group which is attached directly to -D via an ether or amine linkage, wherein D includes the oxygen or nitrogen group that is part of the Drug unit.

Scheme 3

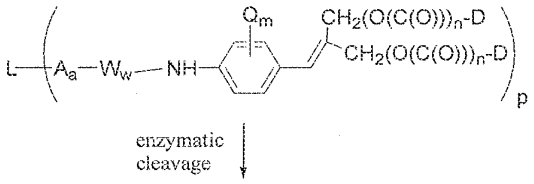


[0130] In **Scheme 3**, Q is -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -halogen, -nitro or -cyano; m is an integer ranging from 0-4; and p ranges from 1 to about 20. The alkyl, alkenyl and alkynyl groups, whether alone or as part of another group, can be optionally substituted.

[0131] Other examples of self-immolative spacers include, but are not limited to, aromatic compounds that are electronically similar to the PAB group such as 2-aminoimidazol-5-methanol derivatives (Hay *et al.*, 1999, *Bioorg. Med. Chem. Lett.* 9:2237) and ortho or para-aminobenzylacetals. Spacers can be used that undergo cyclization upon amide bond hydrolysis, such as substituted and unsubstituted 4-aminobutyric acid amides (Rodrigues *et al.*, 1995, *Chemistry Biology* 2:223), appropriately substituted bicyclo[2.2.1] and bicyclo[2.2.2] ring systems (Storm *et al.*, 1972, *J. Amer. Chem. Soc.* 94:5815) and 2-aminophenylpropionic acid amides (Amsberry *et al.*, 1990, *J. Org. Chem.* 55:5867). Elimination of amine-containing drugs that are substituted at the α-position of glycine (Kingsbury *et al.*, 1984, *J. Med. Chem.* 27:1447) are also examples of self-immolative spacers.

[0132] In one embodiment, the Spacer unit is a branched bis(hydroxymethyl)-styrene (BHMS) unit as depicted in **Scheme 4**, which can be used to incorporate and release multiple drugs.

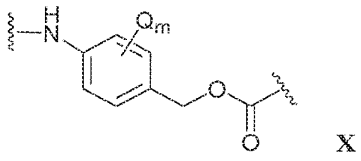
Scheme 4



[0133] In **Scheme 4**, Q is -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -halogen, -nitro or -cyano; m is an integer ranging from 0-4; n is 0 or 1; and p is an integer of from 1 to about 20. The alkyl, alkenyl and alkynyl groups, whether alone or as part of another group, can be optionally substituted.

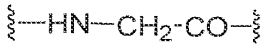
[0134] In some embodiments, the -D moieties are the same. In yet another embodiment, the -D moieties are different.

[0135] In one aspect, Spacer units (-Y_y-) are represented by Formulae (**X**)-(**XII**):



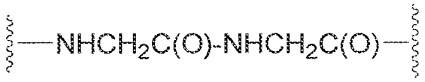
X

wherein Q is -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, -O-(C_1-C_8 alkyl), -O-(C_2-C_8 alkenyl), -O-(C_2-C_8 alkynyl), -halogen, -nitro or -cyano; and m is an integer ranging from 0-4. The alkyl, alkenyl and alkynyl groups, whether alone or as part of another group, can be optionally substituted.



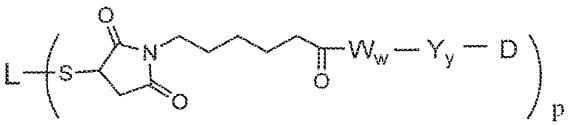
XI

and



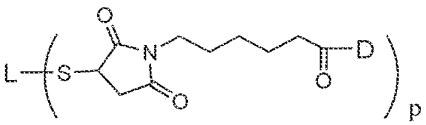
XII

[0136] In a group of selected embodiments, the conjugates of Formula I and II are:

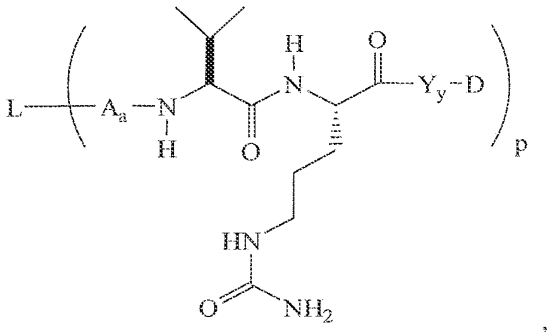


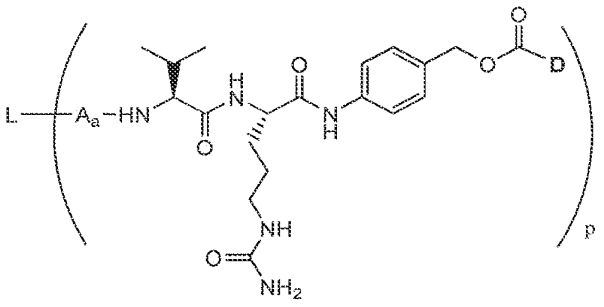
wherein w and y are each 0, 1 or 2,

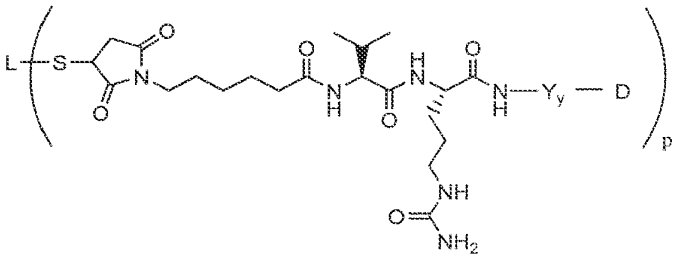
and,



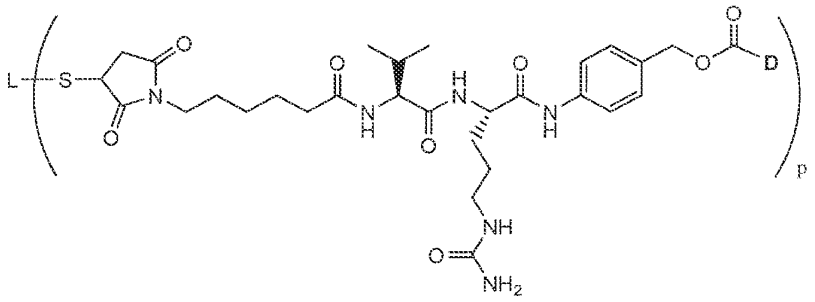
wherein w and y are each 0,







, and



wherein A_a, W_w, Y_y, D and L have the meanings provided above.

THE DRUG UNIT

[0137] The drug moiety (D) can be any cytotoxic, cytostatic or immunomodulatory (*e.g.*, immunosuppressive) agent or drug. D is a Drug unit (moiety) having an atom that can form a bond with the Spacer unit, with the Amino Acid unit, with the Stretcher unit or with the Ligand unit. In some embodiments, the Drug unit D has a nitrogen atom that can form a bond with the Spacer unit. As used herein, the terms "drug unit" and "drug moiety" are synonymous and used interchangeably.

[0138] Useful classes of cytotoxic or immunomodulatory agents include, for example, antitubulin agents, DNA minor groove binders, DNA replication inhibitors, and alkylating agents.

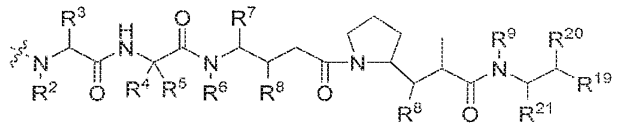
[0139] In some embodiments, the Drug is an auristatin, such as auristatin E (also known in the art as a derivative of dolastatin-10) or a derivative thereof. The auristatin can be, for example, an ester formed between auristatin E and a keto acid. For example, auristatin E can be reacted with paraacetyl benzoic acid or benzoylvaleric acid to produce AEB and AEVB, respectively. Other typical auristatins include AFP, MMAF, and MMAE. The synthesis and structure of exemplary auristatins are described in U.S. Patent Application Publication Nos. 2003-0083263, 2005-0238649 and 2005-0009751; International Patent Publication No. WO

04/010957, International Patent Publication No. WO 02/088172, and U.S. Patent Nos. 6,323,315; 6,239,104; 6,034,065; 5,780,588; 5,665,860; 5,663,149; 5,635,483; 5,599,902; 5,554,725; 5,530,097; 5,521,284; 5,504,191; 5,410,024; 5,138,036; 5,076,973; 4,986,988; 4,978,744; 4,879,278; 4,816,444; and 4,486,414, each of which is incorporated by reference herein in its entirety and for all purposes.

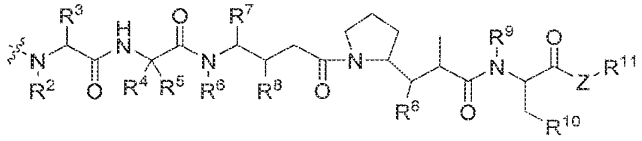
[0140] Auristatins have been shown to interfere with microtubule dynamics and nuclear and cellular division and have anticancer activity. Auristatins of the present invention bind tubulin and can exert a cytotoxic or cytostatic effect on a DR5 expressing cell line. There are a number of different assays, known in the art, that can be used for determining whether an auristatin or resultant antibody-drug conjugate exerts a cytostatic or cytotoxic effect on a desired cell line.

[0141] Methods for determining whether a compound binds tubulin are known in the art. See, for example, Muller et al., *Anal. Chem* 2006, 78, 4390-4397; Hamel et al., *Molecular Pharmacology,* 1995 47: 965-976; and Hamel et al., *The Journal of Biological Chemistry,* 1990 265:28, 17141-17149. For purposes of the present invention, the relative affinity of a compound to tubulin can be determined. Some preferred auristatins of the present invention bind tubulin with an affinity ranging from 10 fold lower (weaker affinity) than the binding affinity of MMAE to tubulin to 10 fold, 20 fold or even 100 fold higher (higher affinity) than the binding affinity of MMAE to tubulin.

[0142] In some embodiments, -D is an auristatin of the formula $\mathbf{D_E}$ or $\mathbf{D_F}$:



$\mathbf{D_E}$



$\mathbf{D_F}$

or a pharmaceutically acceptable salt form thereof; wherein, independently at each location:
the wavy line indicates a bond;
$\mathbf{R^2}$ is -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, or -C_2-C_{20} alkynyl;

R^3 is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, carbocycle, -C_1-C_{20} alkylene (carbocycle), -C_2-C_{20} alkenylene(carbocycle), -C_2-C_{20} alkynylene(carbocycle), -aryl, -C_1-C_{20} alkylene(aryl), -C_2-C_{20} alkenylene(aryl), -C_2-C_{20} alkynylene(aryl), -heterocycle, -C_1-C_{20} alkylene(heterocycle), -C_2-C_{20} alkenylene(heterocycle), or -C_2-C_{20} alkynylene(heterocycle);

R^4 is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, carbocycle, -C_1-C_{20} alkylene (carbocycle), -C_2-C_{20} alkenylene(carbocycle), -C_2-C_{20} alkynylene(carbocycle), -aryl, -C_1-C_{20} alkylene(aryl), -C_2-C_{20} alkenylene(aryl), -C_2-C_{20} alkynylene(aryl), -heterocycle, -C_1-C_{20} alkylene(heterocycle), -C_2-C_{20} alkenylene(heterocycle), or -C_2-C_{20} alkynylene(heterocycle);

R^5 is -H or -C_1-C_8 alkyl;

or R^4 **and** R^5 jointly form a carbocyclic ring and have the formula -$(CR^aR^b)_s$- wherein R^a and R^b are independently -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, or -carbocycle and s is 2, 3, 4, 5 or 6,

R^6 is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, or -C_2-C_{20} alkynyl;

R^7 is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, -carbocycle, -C_1-C_{20} alkylene (carbocycle), -C_2-C_{20} alkenylene(carbocycle), -C_2-C_{20} alkynylene(carbocycle), -aryl, -C_1-C_{20} alkylene(aryl), -C_2-C_{20} alkenylene(aryl), -C_2-C_{20} alkynylene(aryl), heterocycle, -C_1-C_{20} alkylene(heterocycle), -C_2-C_{20} alkenylene(heterocycle), or -C_2-C_{20} alkynylene(heterocycle);

each R^8 is independently -H, -OH, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, -O-(-C_1-C_{20} alkyl), -O-(-C_2-C_{20} alkenyl), -O-(-C_1-C_{20} alkynyl), or -carbocycle;

R^9 is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, or -C_2-C_{20} alkynyl;

R^{19} is -aryl, -heterocycle, or -carbocycle;

R^{20} is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, -carbocycle, -O-(-C_1-C_{20} alkyl), -O-(-C_2-C_{20} alkenyl), -O-(-C_2-C_{20} alkynyl), or OR^{18} wherein R^{18} is -H, a hydroxyl protecting group, or a direct bond where OR^{18} represents =O;

R^{21} is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, or -C_2-C_{20} alkynyl, -aryl, -heterocycle, or -carbocycle;

R^{10} is -aryl or -heterocycle;

Z is –O-, -S-, -NH-, or -NR^{12}-, wherein R^{12} is -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, or -C_2-C_{20} alkynyl; and

R^{11} is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, -aryl, -heterocycle, -$(R^{13}O)_m$-R^{14}, or -$(R^{13}O)_m$-$CH(R^{15})_2$, wherein

m is an integer ranging from 1-1000;

R^{13} is -C_2-C_{20} alkylene, -C_2-C_{20} alkenylene, or -C_2-C_{20} alkynylene;

R^{14} is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, or -C_2-C_{20} alkynyl;

each occurrence of R^{15} is independently -H, -COOH, $-(CH_2)_n$-$N(R^{16})_2$, $-(CH_2)_n$-SO_3H, $-(CH_2)_n$-SO_3-C_1-C_{20} alkyl, $-(CH_2)_n$-SO_3-C_2-C_{20} alkenyl, or $-(CH_2)_n$-SO_3-C_2-C_{20} alkynyl;

each occurrence of R^{16} is independently -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl or $-(CH_2)_n$-COOH; and

n is an integer ranging from 0 to 6; wherein said alkyl, alkenyl, alkynyl, alkylene, alkenylene, alkynyklene, aryl, carbocyle, and heterocycle radicals, whether alone or as part of another group, are optionally substituted.

[0143] Auristatins of the formula D_E include those wherein said alkyl, alkenyl, alkynyl, alkylene, alkenylene, alkynyklene, aryl, carbocyle, and heterocycle radicals are unsubstituted.

[0144] Auristatins of the formula D_E include those wherein the groups of R^2, R^3, R^4, R^5, R^6, R^7, R^8, and R^9 are unsubstituted and the groups of R^{19}, R^{20} and R^{21} are optionally substituted as described herein.

[0145] Auristatins of the formula D_E include those wherein

R^2 is -C_1-C_8 alkyl;

R^3, R^4 and R^7 are independently selected from -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, monocyclic C_3-C_6 carbocycle, -C_1-C_{20} alkylene(monocyclic C_3-C_6 carbocycle), -C_2-C_{20} alkenylene(monocyclic C_3-C_6 carbocycle), -C_2-C_{20} alkynylene(monocyclic C_3-C_6 carbocycle), -C_6-C_{10} aryl, -C_1-C_{20} alkylene(C_6-C_{10} aryl), -C_2-C_{20} alkenylene(C_6-C_{10} aryl), -C_2-C_{20} alkynylene(C_6-C_{10} aryl), -heterocycle, -C_1-C_{20} alkylene(heterocycle), -C_2-C_{20} alkenylene(heterocycle), or -C_2-C_{20} alkynylene(heterocycle); wherein said alkyl, alkenyl, alkynyl, alkylene, alkenylene, alkynylene, carbocycle, aryl, and heterocycle radicals are optionally substituted;

R^5 is -hydrogen;

R^6 is -C_1-C_8 alkyl;

each R^8 is independently selected from -OH, -O-(-C_1-C_{20} alkyl), -O-(-C_2-C_{20} alkenyl), or -O-(-C_2-C_{20} alkynyl) wherein said alkyl, alkenyl, and alkynyl radicals are optionally substituted;

R^9 is -hydrogen or -C_1-C_8 alkyl;

R^{19} is optionally substituted phenyl;

R^{20} is OR^{18}; wherein R^{18} is H, a hydroxyl protecting group, or a direct bond where OR^{18} represents =O;

R^{21} is selected from -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, or -carbocycle; wherein said alkyl, alkenyl, alkynyl, and carbocycle radicals are optionally substituted; or a pharmaceutically acceptable salt form thereof.

[0146] Auristatins of the formula D_E include those wherein

R^2 is methyl;

R^3 is -H, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, or -C_2-C_8 alkynyl, wherein said alkyl, alkenyl and alkynyl radicals are optionally optionally substituted;

R^4 is -H, -C_1-C_8 alkyl, -C_2-C_8 alkenyl, -C_2-C_8 alkynyl, monocyclic C_3-C_6 carbocycle, -C_6-C_{10} aryl, -C_1-C_8 alkylene(C_6-C_{10} aryl), -C_2-C_8 alkenylene(C_6-C_{10} aryl), -C_2-C_8 alkynylene(C_6-C_{10} aryl), -C_1-C_8 alkylene (monocyclic C_3-C_6 carbocycle), -C_2-C_8 alkenylene (monocyclic C_3-C_6 carbocycle), -C_2-C_8 alkynylene(monocyclic C_3-C_6 carbocycle); wherein said alkyl, alkenyl, alkynyl, alkylene, alkenylene, alkynylene, aryl, and carbocycle radicals whether alone or as part of another group are optionally substituted;

R^5 is H;

R^6 is methyl;

R^7 is -C_1-C_8 alkyl, -C_2-C_8 alkenyl or -C_2-C_8 alkynyl;

each R^8 is methoxy;

R^9 is -hydrogen or -C_1-C_8 alkyl;

R^{19} is phenyl;

R^{20} is OR^{18}; wherein R^{18} is -H, a hydroxyl protecting group, or a direct bond where OR^{18} represents =O;

R^{21} is methyl; or a pharmaceutically acceptable salt form thereof.

[0147] Auristatins of the formula D_E include those wherein

R^2 is methyl; R^3 is H or C_1-C_3 alkyl; R^4 is C_1-C_5 alkyl; R^5 is H; R^6 is methyl; R^7 is isopropyl or sec-butyl; R^8 is methoxy; R^9 is hydrogen or C_1-C_8 alkyl; R^{19} is phenyl; R^{20} is OR^{18}; wherein R^{18} is H, a hydroxyl protecting group, or a direct bond where OR^{18} represents =O; and R^{21} is methyl; or a pharmaceutically acceptable salt form thereof.

[0148] Auristatins of the formula D_E include those wherein

R^2 is methyl or or C_1-C_3 alkyl; R^3 is H or C_1-C_3 alkyl; R^4 is C_1-C_5 alkyl; R^5 is H; R^6 is C_1-C_3 alkyl; R^7 is C_1-C_5 alkyl; R^8 is C_1-C_3 alkoxy; R^9 is hydrogen or C_1-C_8 alkyl; R^{19} is phenyl; R^{20} is OR^{18}; wherein R^{18} is H, a hydroxyl protecting group, or a direct bond where OR^{18} represents =O; and R^{21} is C_1-C_3 alkyl; or a pharmaceutically acceptable salt form thereof.

[0149] Auristatins of the formula D_F include those wherein

R^2 is methyl;

R^3, R^4, and R^7 are independently selected from -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, monocyclic C_3-C_6 carbocycle, -C_1-C_{20} alkylene(monocyclic C_3-C_6 carbocycle), -C_2-C_{20} alkenylene(monocyclic C_3-C_6 carbocycle), -C_2-C_{20} alkynylene(monocyclic C_3-C_6 carbocycle), -C_6-C_{10} aryl, -C_1-C_{20} alkylene(C_6-C_{10} aryl), -C_2-C_{20} alkenylene(C_6-C_{10} aryl), -C_2-C_{20} alkynylene(C_6-C_{10} aryl), -heterocycle, -C_1-C_{20} alkylene(heterocycle), -C_2-C_{20} alkenylene(heterocycle), or -C_2-C_{20} alkynylene(heterocycle); wherein said alkyl, alkenyl, alkynyl, alkylene, alkenylene, alkynylene, carbocyle, aryl, and heteocycle radicals whether alone or as part of another group are optionally substituted;

R^5 is -H;

R^6 is methyl;

each R^8 is methoxy;

R^9 is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, or -C_2-C_{20} alkynyl; wherein said alkyl, alkenyl and alkynyl radical are optionally substituted;

R^{10} is optionally substituted aryl or optionally substituted heterocycle;

Z is –O-, -S-, -NH-, or -NR^{12}-, wherein R^{12} is -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, or -C_2-C_{20} alkynyl, each of which is optionally substituted; and

R^{11} is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl, -aryl, -heterocycle, -$(R^{13}O)_m$-R^{14}, or -$(R^{13}O)_m$-$CH(R^{15})_2$ wherein said alkyl, alkenyl, alkyny, aryl, and heterocycle radicals are optionally substituted; wherein

m is an integer ranging from 1-1000;

R^{13} is -C_2-C_{20} alkylene, -C_2-C_{20} alkenylene, or -C_2-C_{20} alkynylene, each of which is optionally substituted;

R^{14} is -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, or -C_2-C_{20} alkynyl wherein said alkyl, alkenyl and alkynyl radicals are optionally substituted;

each occurrence of R^{15} is independently -H, -COOH, –$(CH_2)_n$-$N(R^{16})_2$, –$(CH_2)_n$-SO_3H, –$(CH_2)_n$-SO_3-C_1-C_{20} alkyl, –$(CH_2)_n$-SO_3-C_2-C_{20} alkenyl, or –$(CH_2)_n$-SO_3-C_2-C_{20} alkynyl wherein said alkyl, alkenyl and alkynyl radicals are optionally substituted;

each occurrence of R^{16} is independently -H, -C_1-C_{20} alkyl, -C_2-C_{20} alkenyl, -C_2-C_{20} alkynyl or $-(CH_2)_n$-COOH wherein said alkyl, alkenyl and alkynyl radicals are optionally substituted; and

n is an integer ranging from 0 to 6; or a pharmaceutically acceptable salt form thereof.

[0150] In certain of these embodiments, R^{10} is optionally substituted phenyl.

[0151] Auristatins of the formula D_F include those wherein the groups of R^2, R^3, R^4, R^5, R^6, R^7, R^8, and R^9 are unsubstituted and the groups of R^{10} and R^{11} are as described herein.

[0152] Auristatins of the formula D_F include those wherein said alkyl, alkenyl, alkynyl, alkylene, alkenylene, alkynyklene, aryl, carbocyle, and heterocycle radicals are unsubstituted.

[0153] Auristatins of the formula D_F include those wherein

R^2 is C_1-C_3 alkyl; R^3 is H or C_1-C_3 alkyl; R^4 is C_1-C_5 alkyl; R^5 is H; R^6 is C_1-C_3 alkyl; R^7 is C_1-C_5 alkyl; R^8 is C_1-C_3 alkoxy; R^9 is hydrogen or C_1-C_8 alkyl; R^{10} is optionally substituted phenyl;Z is O, S, or NH; and R^{11} is as defined herein; or a pharmaceutically acceptable salt form thereof.

[0154] Auristatins of the formula D_F include those wherein

R^2 is methyl; R^3 is H or C_1-C_3 alkyl; R^4 is C_1-C_5 alkyl; R^5 is H; R^6 is methyl; R^7 is isopropyl or sec-butyl; R^8 is methoxy; R^9 is hydrogen or C_1-C_8 alkyl; R^{10} is optionally substituted phenyl;Z is O, S, or NH; and R^{11} is as defined herein; or a pharmaceutically acceptable salt form thereof.

[0155] Auristatins of the formula D_F include those wherein

R^2 is methyl; R^3 is H or C_1-C_3 alkyl; R^4 is C_1-C_5 alkyl; R^5 is H; R^6 is methyl; R^7 is isopropyl or sec-butyl; R^8 is methoxy; R^9 is hydrogen or C_1-C_8 alkyl; R^{10} is phenyl; and Z is O or NH and R^{11} is as defined herein, preferably hydrogen; or a pharmaceutically acceptable salt form thereof.

[0156] Auristatins of the formula D_F include those wherein

R^2 is C_1-C_3 alkyl; R^3 is H or C_1-C_3 alkyl; R^4 is C_1-C_5 alkyl; R^5 is H; R^6 is C_1-C_3 alkyl; R^7 is C_1-C_5 alkyl; R^8 is C_1-C_3 alkoxy; R^9 is hydrogen or C_1-C_8 alkyl; R^{10} is phenyl; and Z is O or NH and R^{11} is as defined herein, preferably hydrogen; or a pharmaceutically acceptable salt form thereof.

[0157] Auristatins of the formula D_E or D_F include those wherein R^3, R^4 and R^7 are independently isopropyl or sec-butyl and R^5 is -H. In an exemplary embodiment, R^3 and R^4

are each isopropyl, R^5 is H, and R^7 is sec-butyl. The remainder of the substituents are as defined herein.

[0158] Auristatins of the formula $\mathbf{D_E}$ or $\mathbf{D_F}$ include those wherein R^2 and R^6 are each methyl, and R^9 is H. The remainder of the substituents are as defined herein.

[0159] Auristatins of the formula $\mathbf{D_E}$ or $\mathbf{D_F}$ include those wherein each occurrence of R^8 is $-OCH_3$. The remainder of the substituents are as defined herein.

[0160] Auristatins of the formula $\mathbf{D_E}$ or $\mathbf{D_F}$ include those wherein R^3 and R^4 are each isopropyl, R^2 and R^6 are each methyl, R^5 is H, R^7 is sec-butyl, each occurrence of R^8 is $-OCH_3$, and R^9 is H. The remainder of the substituents are as defined herein.

[0161] Auristatins of the formula $\mathbf{D_F}$ include those wherein Z is -O- or -NH-. The remainder of the substituents are as defined herein.

[0162] Auristatins of the formula $\mathbf{D_F}$ include those wherein R^{10} is aryl. The remainder of the substituents are as defined herein.

[0163] Auristatins of the formula $\mathbf{D_F}$ include those where R^{10} is -phenyl. The remainder of the substituents are as defined herein.

[0164] Auristatins of the formula $\mathbf{D_F}$ include those wherein Z is -O-, and R^{11} is H, methyl or t-butyl. The remainder of the substituents are as defined herein.

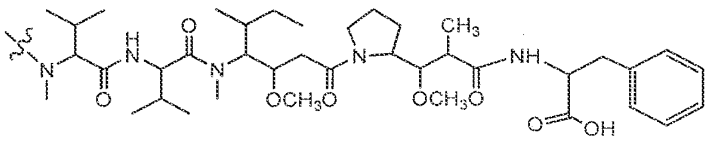
[0165] Auristatins of the formula $\mathbf{D_F}$ include those wherein, when Z is -NH, R^{11} is $-(R^{13}O)_m-CH(R^{15})_2$, wherein R^{15} is $-(CH_2)_n-N(R^{16})_2$, and R^{16} is $-C_1-C_8$ alkyl or $-(CH_2)_n-COOH$. The remainder of the substituents are as defined herein.

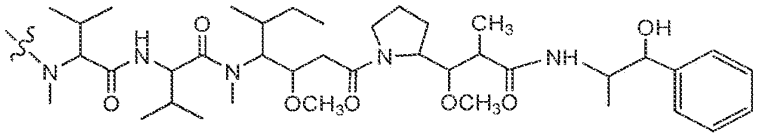
[0166] Auristatins of the formula $\mathbf{D_F}$ include those wherein when Z is -NH, R^{11} is $-(R^{13}O)_m-CH(R^{15})_2$, wherein R^{15} is $-(CH_2)_n-SO_3H$. The remainder of the substituents are as defined herein.

[0167] In preferred embodiments, when D is an auristatin of formula $\mathbf{D_E}$, w is an integer ranging from 1 to 12, preferably 2 to 12, y is 1 or 2, and a is preferably 1.

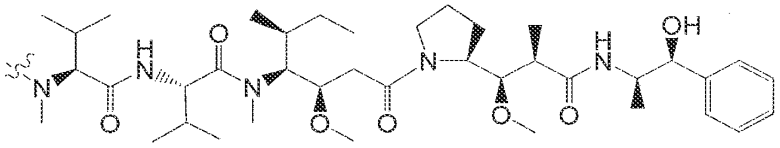
[0168] In some embodiments, wheren D is an auristatin of formula $\mathbf{D_F}$, a is 1 and w and y are 0.

[0169] Illustrative Drug units (-D) include the drug units having the following structures:

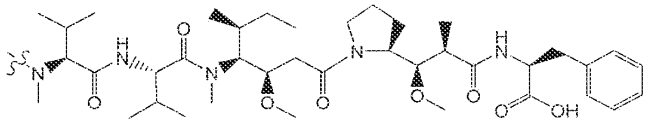




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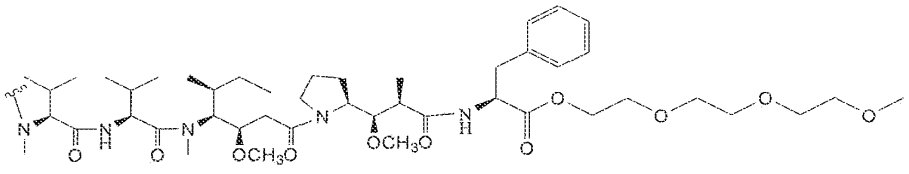


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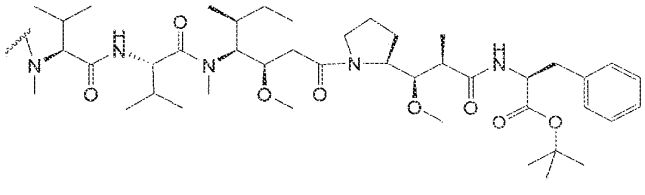


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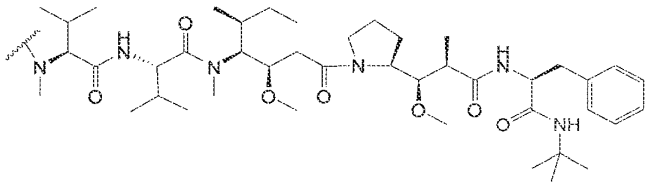
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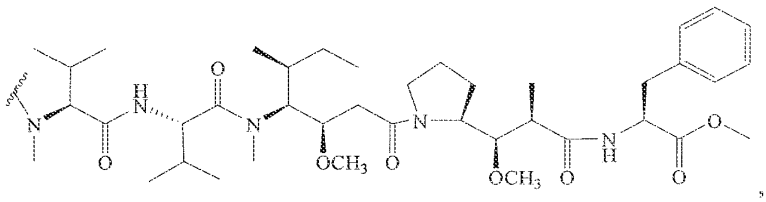
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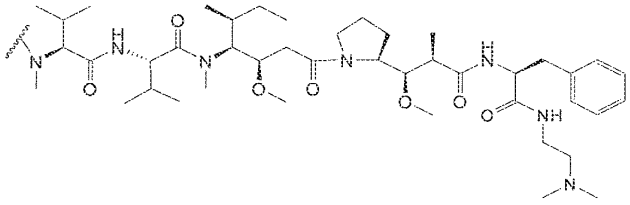
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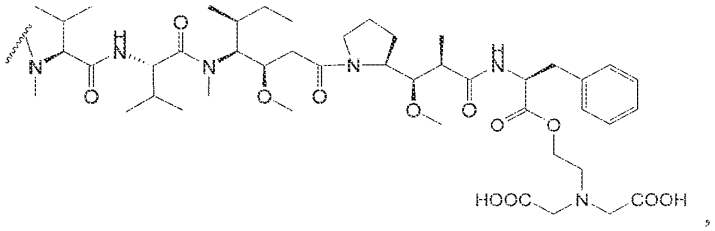
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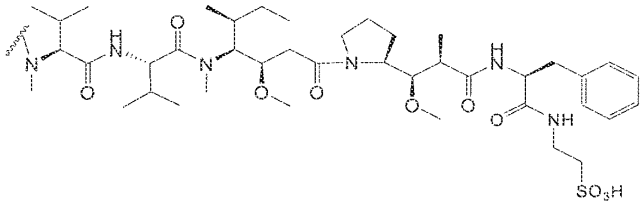
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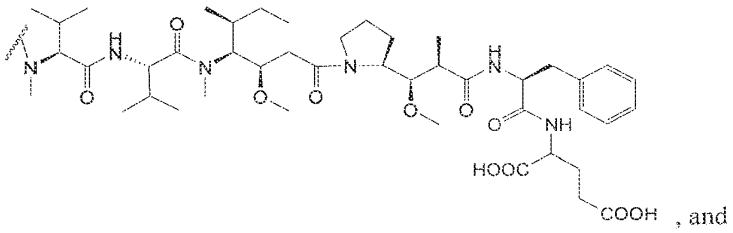
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HOOC
N
COOH

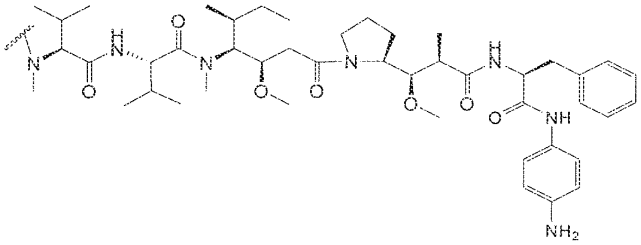
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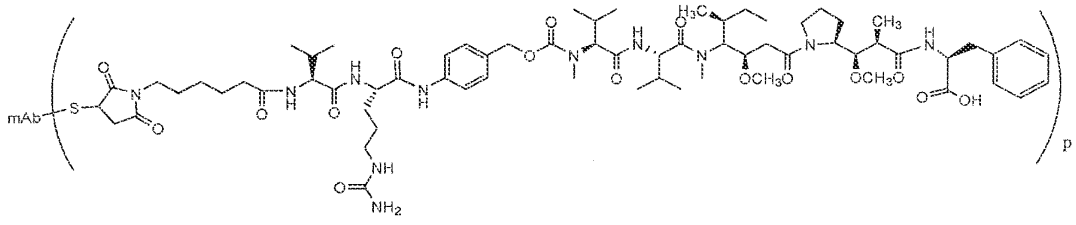


or pharmaceutically acceptable salts or solvates thereof.

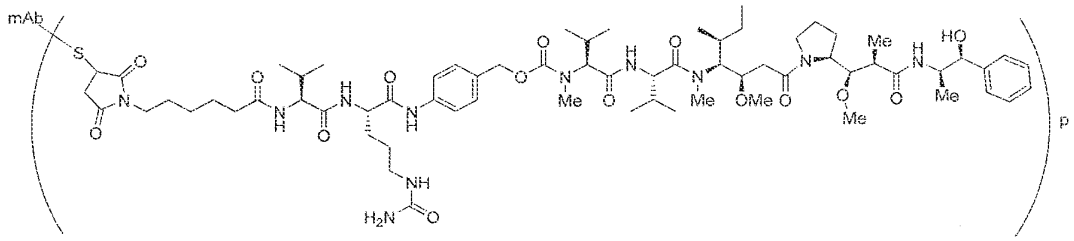
[0170] In one aspect, hydrophilic groups, such as but not limited to triethylene glycol esters (TEG) can be attached to the Drug Unit at R^{11}. Without being bound by theory, the hydrophilic groups assist in the internalization and non-agglomeration of the Drug unit.

[0171] In some embodiments, the Drug unit is not TZT-1027. In some embodiments, the Drug unit is not auristatin E, dolastatin 10, or auristatin PE.

[0172] Exemplary Ligand Drug Conjugates have the following structures wherein "mAb" represents an anti-DR5 antibody and S is a sulfur atom of the antibody. The subscript p is an integer of from 1 to about 20 and is preferably 1 to about 5.

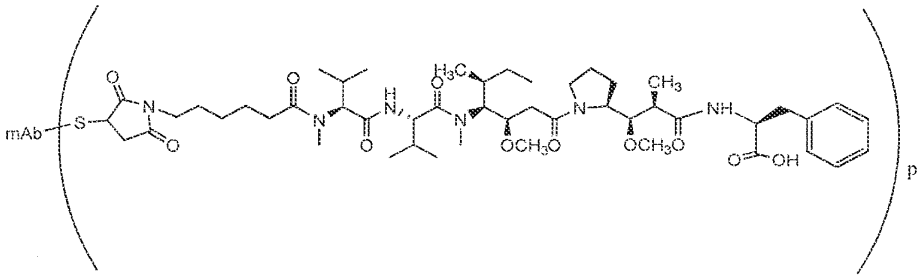


L-mc-vc-MMAF ,



L-mc-vc-MMAE ,

or



L-mc-MMAF

or pharmaceutically acceptable salt forms thereof.

[0173] In some embodiments, the Drug Unit is a calicheamicin, camptothecin, a maytansinoid, or an anthracycline. In some embodiments the drug is a taxane, a topoisomerase inhibitor, a vinca alkaloid, or the like.

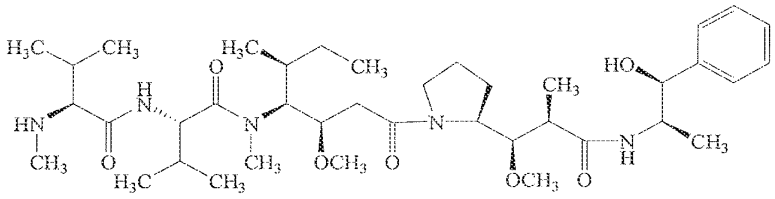
[0174] In some typical embodiments, suitable cytotoxic agents include, for example, DNA minor groove binders (*e.g.*, enediynes and lexitropsins, a CBI compound; see also U.S. Patent No. 6,130,237), duocarmycins, taxanes (*e.g.*, paclitaxel and docetaxel), puromycins, and vinca alkaloids. Other cytotoxic agents include, for example, CC-1065, SN-38, topotecan, morpholino-doxorubicin, rhizoxin, cyanomorpholino-doxorubicin, echinomycin, combretastatin, netropsin, epothilone A and B, estramustine, cryptophysins, cemadotin, maytansinoids, discodermolide, eleutherobin, and mitoxantrone.

[0175] In some embodiments, the Drug is an anti-tubulin agent. Examples of anti-tubulin agents include, auristatins, taxanes (*e.g.*, Taxol® (paclitaxel), Taxotere® (docetaxel)), T67 (Tularik) and vinca alkyloids (*e.g.*, vincristine, vinblastine, vindesine, and vinorelbine). Other antitubulin agents include, for example, baccatin derivatives, taxane analogs (*e.g.*,

epothilone A and B), nocodazole, colchicine and colcimid, estramustine, cryptophycins, cemadotin, maytansinoids, combretastatins, discodermolide, and eleutherobin.

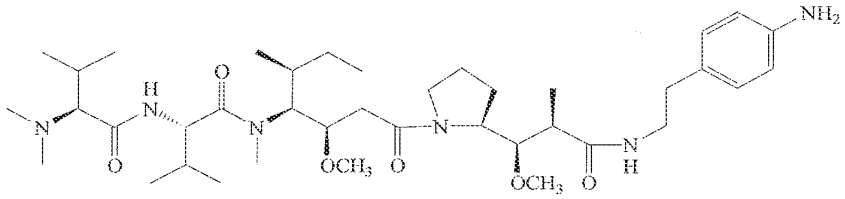
[0176] In certain embodiments, the cytotoxic agent is a maytansinoid, another group of anti-tubulin agents. For example, in specific embodiments, the maytansinoid is maytansine or DM-1 (ImmunoGen, Inc.; see also Chari *et al.*, 1992, *Cancer Res.* 52:127-131).

[0177] In certain embodiments, the cytotoxic or cytostatic agent is a dolastatin. In certain embodiments, the cytotoxic or cytostatic agent is of the auristatin class. Thus, in a specific embodiment, the cytotoxic or cytostatic agent is MMAE (Formula **XIII**). In another specific embodiment, the cytotoxic or cytostatic agent is AFP (Formula **XVIII**).

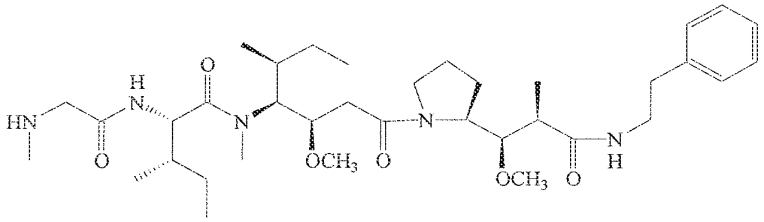


(XIII)

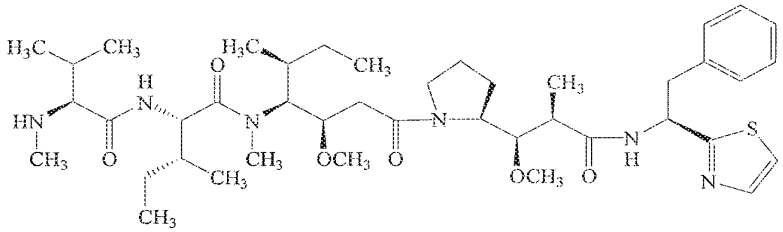
[0178] In certain embodiments, the cytotoxic or cytostatic agent is a compound of formulas **XIV-XXIII** or pharmaceutically acceptable salt form thereof:



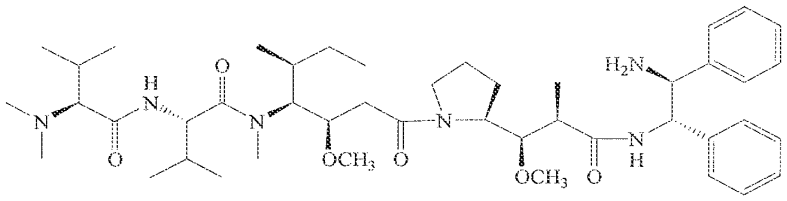
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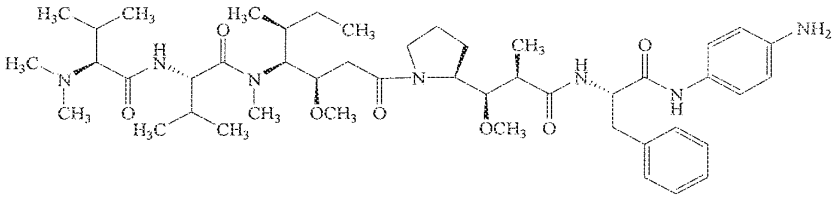
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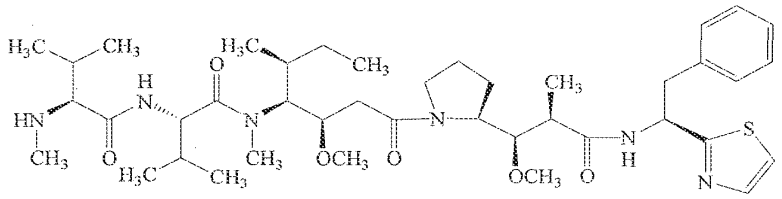
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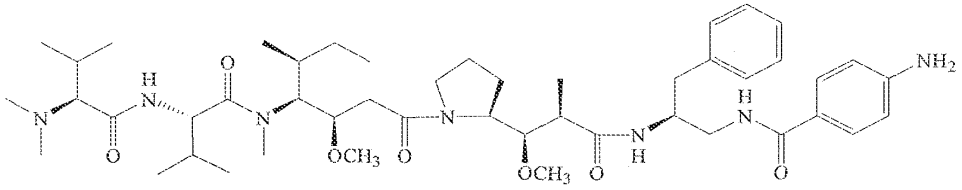
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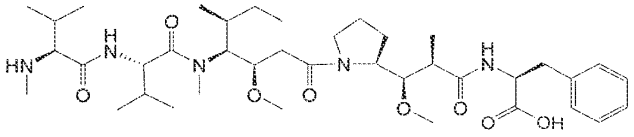
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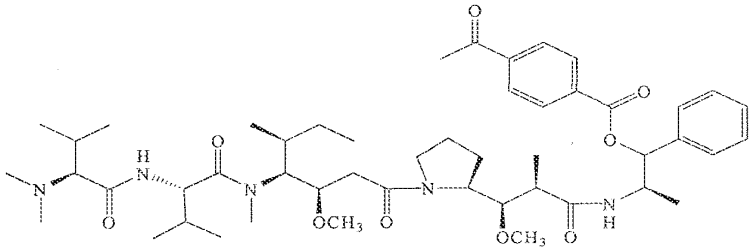
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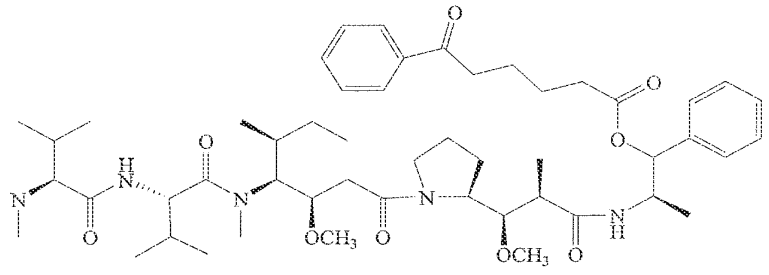
(XX)



(XXI)



(XXII)



(XXIII)

LIGAND UNIT

[0179] In the present invention, the Ligand unit (e.g., an antibody) in the Ligand Drug Conjugate specifically binds to DR5 and exhibits cytotoxic activity via internalization. The Ligand Drug Conjugate reaches cancer tissue expressing DR5 to which the Ligand unit (e.g., an antibody) specifically binds as its target. As a result, the Drug unit conjugated to the antibody can be allowed to selectively act on the target cells. Therefore, the efficacy of the antibody-drug conjugate can be more greatly enhanced than that of the antibody alone. Antibodies that bind to death domain-containing receptors, especially an anti-DR5 antibody, can be selected as an antibody that can be contained in the antibody-drug conjugate according to the present invention.

Antibodies binding to DR5

(1) DR5 gene

[0180] The nucleotide sequence and amino acid sequence of the human death receptor 5 (DR5) gene has been registered as GI:22547118 (accession no. NM_147187) in GenBank. A nucleotide sequence coding an amino acid sequence with one or more amino acids replaced, deleted, or added in the amino acid sequence of DR5 and having bioactivity comparable to that of DR5 is also included in the nucleotide sequence of the DR5 gene. In addition, a protein that consists of an amino acid sequence with one or more amino acids replaced, deleted, or added in the amino acid sequence of DR5 and that has bioactivity comparable to that of DR5 is also included in DR5.

(2) Antibody against DR5

[0181] The antibody against DR5 according to the present invention can be obtained in the usual way by immunizing an animal with DR5 or any polypeptide selected from the amino

acid sequence of DR5. Such antibody produced in the living body can be collected and purified.

[0182] In addition, a monoclonal antibody can also be obtained from a hybridoma established by fusing an antibody-producing cell that produces an antibody against DR5 with a myeloma cell according to a known method (for example, Kohler and Milstein, *Nature* (1975) 256, p.495-497; Kennet, R. ed., *Monoclonal Antibody*, p.365-367, Prenum Press, N.Y. (1980)).

[0183] DR5 as the antigen can be obtained from genetically engineered host cells expressing the DR5 gene.

[0184] More specifically, DR5 can be obtained by preparing a vector that can express the DR5 gene, introducing the vector into host cells to express the gene, and purifying the expressed DR5.

[0185] In addition, after an artificial gene fusing the extracellular region of DR5 with the constant region of an antibody is constructed, a protein prepared in an appropriate expression system of the gene can also be used as an immunogen.

(3) Other antibodies

[0186] In addition to the monoclonal antibody against the above DR5, the antibodies according to the present invention include recombinant antibodies artificially altered to reduce heterologous antigenicity against humans, such as chimeric antibodies, humanized antibodies, and human antibodies. These antibodies can be produced by means of known methods.

[0187] Such chimeric antibodies include an antibody whose variable region and constant region are heterologous to each other, and an example thereof is a chimeric antibody created by joining the variable region genes of a mouse-derived antibody to human constant region genes (*Proc. Natl. Acad. Sci. U.S.A.*, 81, 6851-6855 (1984)).

[0188] Examples of such humanized antibodies include an antibody in which only the complementarity-determining regions (CDRs) are transferred into a human antibody (*Nature* (1986) 321, p.522-525) and an antibody in which CDR sequences and amino acid residues in part of the framework are grafted into a human antibody by CDR grafting (International Publication No. WO90/07861).

[0189] In addition, there are human antibodies. The term "human anti DR5 antibody" refers to a human antibody that only has gene sequences of a human chromosome-derived antibody. The anti-human DR5 antibody can be obtained by a method that uses a human antibody-producing mouse having a chromosome fragment containing H- and L- chain genes for a human antibody (Tomizuka, K. *et al.*, *Nature Genetics* (1997) 16, p.133-143; Kuroiwa, Y . et.al., *Nuc. Acids Res.* (1998) 26, p.3447-3448; Yoshida, H. *et.al.*, *Animal Cell Technology: Basic and Applied Aspects* vol. 10, p.69-73 (Kitagawa, Y., Matuda, T. and Iijima, S. eds.), Kluwer Academic Publishers, 1999; Tomizuka, K. et.al., *Proc. Natl. Acad. Sci. USA* (2000) 97, p.722-727).

[0190] Such a transgenic animal, or more specifically, a genetically modified animal in which the gene loci for endogenous immunoglobulin heavy and light chains in a nonhuman mammal are destroyed and instead the gene loci for human immunoglobulin heavy and light chains are introduced into this knockout animal via a yeast artificial chromosome (YAC) vector or the like, can be produced by preparing a knockout animal and a transgenic animal as mentioned above and crossbreeding these animals.

[0191] The antibody can also be obtained from culture supernatant produced by transforming eukaryotic cells with cDNA, preferably a vector containing the cDNA coding for each of the humanized antibody heavy and light chains by recombinant DNA technology and culturing the transformed cells producing a recombinant human monoclonal antibody.

[0192] Here, examples of cells that can be used as a host include eukaryotic cells, preferably mammalian cells such as CHO cells, lymphocytes, and myeloma.

[0193] In addition, a method of obtaining a phage display-derived human antibody screened from a human antibody library (Wormstone, I. M. *et.al*, *Investigative Ophthalmology & Visual Science* (2002) 43 (7), p.2301-2308; Carmen, S. *et.al.*, *Briefings in Functional Genomics and Proteomics* (2002), 1 (2), p.189-203; Siriwardena, D. *et.al.*, *Opthalmology* (2002) 109 (3), p.427-431) is also known.

[0194] For example, a human antibody heavy and light variable regions are displayed on a phage surface as a single-chain antibody (scFv) and then an antigen-binding phage is selected (*Nature Biotechnology* (2005), 23, (9), p.1105-1116).

[0195] The DNA sequence coding an antigen-binding human antibody variable region can be determined by analyzing the genes of the phage selected by antigen binding.

[0196] Once the DNA sequence of the antigen-binding scFv becomes clear, a human antibody can be obtained by preparing an expression vector having the sequence and introducing the vector into an appropriate host for expression (See, for example, WO92/01047, WO92/20791, WO93/06213, WO93/11236, WO93/19172, WO95/01438, WO95/15388; *Annu. Rev. Immunol* (1994) 12, p.433-455; *Nature Biotechnology* (2005) 23 (9), p.1105-1116).

[0197] When the antibody genes have been isolated and are introduced into an appropriate host to prepare the antibody, an appropriate combination of a host and an expression vector can be used.

[0198] When eukaryotic cells are used as a host, animal cells, plant cells, or eukaryotic microorganisms can be used.

[0199] Examples of such animal cells include simian COS cells (Gluzman, Y., *Cell* (1981) 23, p.175-182, ATCC CRL-1650), murine fibroblasts NIH3T3 (ATCC No. CRL-1658), and dihydrofolate reductase-deficient strains of Chinese hamster ovary cells (CHO cells, ATCC CCL-61) (Urlaub, G. and Chasin, L. A., *Proc. Natl. Acad. Sci. U.S.A.* (1980) 77, p.4216-4220).

[0200] Examples of prokaryotic cells that can be used include *Escherichia coli* and *Bacillus subtilis*.

[0201] The antibody can be obtained by introducing the antibody genes of interest into these cells by transformation and culturing the transformed cells in vitro.

[0202] The isotype of the antibody according to the present invention can be any isotype. Examples thereof include IgG (IgG1, IgG2, IgG3, and IgG4), IgM, IgA (IgA1 and IgA2), IgD, and IgE, but IgG and IgM are preferable.

[0203] In addition, the antibody according to the present invention may be a fragment of an antibody having an antigen-binding site of the antibody or a modified version thereof if it maintains antigen binding.

[0204] Examples of such antibody functional fragments include Fab, $F(ab')_2$, a monovalent variable region fragment Fab' obtained by reducing $F(ab')_2$, Fv, single-chain Fv (scFv) obtained by linking heavy-chain and light-chain Fv by an appropriate linker, diabody (diabodies), linear antibodies, and polyspecific antibodies formed of antibody fragments, but the fragments are not limited to the above fragments if they maintain antigen binding. The above antibody fragments can be obtained by processing full-length antibody molecules with

an enzyme such as papain or pepsin. The above antibody fragments can also be obtained by using nucleic acid sequences coding the heavy chain and light chain of the above antibody fragments to allow an appropriate gene expression system to produce the corresponding proteins.

[0205] These antibody fragments can be produced by obtaining and expressing the genes in the same way as above to allow a host to produce the corresponding proteins.

[0206] The antibody according to the present invention may be a polyclonal antibody, a mixture of several anti-DR5 antibodies having different amino acid sequences. An example of such a polyclonal antibody is a mixture of several antibodies having different CDRs. As such a polyclonal antibody, an antibody obtained by culturing a mixture of cells producing different antibodies and purifying the culture can be used (WO2004/061104).

[0207] The antibody obtained can be uniformly purified. The separation and purification of the antibody may be conducted by means of the separation and purification methods used for normal proteins.

[0208] For example, the antibody can be separated and purified by appropriately selecting and combining chromatography columns, filters, ultrafiltration, salting-out, dialysis, preparative polyacrylamide gel electrophoresis, isoelectric focusing, and the like (Strategies for Protein Purification and Charcterization: A Laboratoy Course Manual, Daniel R. Marshak et al. Cold Spring Harbor Laboratory Press (1996); Antibodies: A Laboratory Manual. Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988)), but the separation and purification methods are not limited to these.

[0209] Examples of chromatography include affinity chromatography, ion exchange chromatography, hydrophobic chromatography, gel filtration, reversed phase chromatography, and adsorption chromatography. These types of chromatography can be performed by using liquid-phase chromatography such as HPLC and FPLC.

[0210] Examples of the columns used in affinity chromatography include protein A columns and protein G columns.

[0211] Examples of the protein A columns include Hyper D, POROS, and Sepharose F. F. (Pharmacia).

[0212] In addition, the antibody can also be purified by its binding to the antigen immobilized on a carrier.

(4) **Examples of anti-DR5 antibodies**

[0213] For example, the anti-DR5 antibodies inducing apoptosis in DR5-expressing cells described in International Publication Nos. WO98/51793, WO2001/83560, WO2002/94880, WO2003/54216, WO2004/50895, WO2006/83971, WO2007/22157, and WO2011/038159 can be used as components of the antibody-drug conjugate according to the present invention. In addition, anti-DR5 antibodies called Lexatumumab, HGS-TR2J, Apomab, drozitumab, Conatumumab, and LBY135 and variants thereof can also be used as components of the antibody-drug conjugate according to the present invention. However, the antibodies that can be used as such components are not limited to the above examples if such antibodies have a capacity to bind to DR5 protein.

[0214] The Ligand unit of the present invention is typically a DR5 binding agent. In one group of embodiments, the Ligand unit comprises a heavy chain amino acid sequence corresponding to humanized B273 (SEQ ID NO:9). Humanized B273 is abbreviated as hB273 in the specification. Methods of producing hB273 are described in PCT Application No. PCT/JP2011/074866, titled "A new anti-DR5 antibody" and filed October 27, 2011 (published as WO 2012/057288), incorporated herein by reference in its entirety and for all purposes. In another group of embodiments, the Ligand unit comprises a heavy chain amino acid sequence corresponding to humanized B273 which lacks the carboxy terminal lysine residue (*i.e.,* a heavy chain having the amino acid sequence of amino acid residues 1-451 of SEQ ID NO:9). Post-translational processing of proteins to remove lysine or arginine residues from the carboxy terminus has been reported, for example, by Harris, *J. Chromatography* (1995), 705:129-134. In another group of embodiments, the Ligand unit comprises a light chain amino acid sequence corresponding to humanized B273 (SEQ ID NO:10). In yet another embodiment, the Ligand unit comprises both a heavy and light chain amino acid sequence of SEQ ID NOs:9 and 10. In still another embodiment, the Ligand unit comprises both a heavy chain amino acid sequence of amino acid residues 1-451 of SEQ ID NO:9 and a light chain amino acid sequence of SEQ ID NO:10. In still another embodiment, the Ligand unit comprises (a) a heavy chain immunoglobulin having the CDR1 consisting of amino residues 1-5 of SEQ ID NO:11, the CDR2 consisting of amino acid residues 1-17 of SEQ ID NO:12, and the CDR3 consisting of amino acid residues 1-13 of SEQ ID NO:13; and (b) a light chain immunoglobulin having the CDR1 consisting of amino residues 1-16 of SEQ ID NO:14, the CDR2 consisting of amino acid residues 1-7 of SEQ ID NO:15, and the CDR3 consisting of amino acid residues 1-9 of SEQ ID NO:16. In another embodiment, the Ligand unit comprises the heavy chain variable region of hB273 comprising amino acid residues 1-

122 of SEQ ID NO:9 and the light chain variable region of hB273 comprising amino acid residues 1-114 of SEQ ID NO:10.

[0215] Additionally, the Ligand unit (L) has at least one functional group that can form a bond with a functional group of a Linker unit. Useful functional groups that can be present on a Ligand unit, either naturally, via chemical manipulation or via engineering, include, but are not limited to, sulfhydryl (-SH), amino, hydroxyl, carboxy, the anomeric hydroxyl group of a carbohydrate, and carboxyl. In some embodiments, a Ligand unit functional group is a sulfhydryl group. The sulfhydryl group is typically a solvent accessible sulfhydryl group, such as a solvent accessible sulfhydryl group on a cysteine residue. Sulfhydryl groups can be generated by reduction of an intramolecular or intermolecular disulfide bond of a Ligand. Sulfhydryl groups also can be generated by reaction of an amino group of a lysine moiety of a Ligand using 2-iminothiolane (Traut's reagent) or another sulfhydryl generating reagent.

[0216] In some embodiments, one or more sulfhydryl groups are engineered into a Ligand unit, such as by amino acid substitution. For example, a sulfhydryl group can be introduced into a Ligand unit. In some embodiments, a sulfhydryl group is introduced by an amino acid substitution of serine or threonine to a cysteine residue, and/or by addition of a cysteine residue into a Ligand unit (an engineered cysteine residue). In some embodiments, the cysteine residue is an internal cysteine residue, *i.e.*, not located at the N-terminus or C-terminus of the Ligand moiety.

[0217] In an exemplary embodiment, a cysteine residue can be engineered into an antibody heavy or light variable region (*e.g.*, of an antibody fragment, such as a diabody) by amino acid substitution. The amino acid substitution is typically introduced into the framework region and is located distal to the epitope-binding face of the variable region. For example, the amino acid substitution can be at least 10 angstroms, at least 20 angstroms or at least 25 angstroms from the epitope-binding face or the CDRs. Suitable positions for substitution of a cysteine residue can be determined based on the known or predicted three dimensional structures of antibody variable regions. (See generally Holliger and Hudson, 2005, *Nature BioTechnology* 23(9):1126-1136.) In exemplary embodiments, a serine to cysteine amino acid substitution is introduced at amino acid position 84 of the V_H region and/or position 14 of the V_L region (according to the numbering system of Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th edition, (Bethesda, MD, NIH) 1991).

[0218] To control the number of Drug or Linker unit-Drug units attached to a Ligand unit, one or more cysteine residues can be eliminated by amino acid substitution. For example, the

number of solvent accessible cysteine residues in an immunoglobulin hinge region can be reduced by amino acid substitution of cysteine to serine residues.

[0219] In some embodiments, a Ligand unit contains 1, 2, 3, 4, 5, 6, 7 or 8 solvent-accessible cysteine residues. In some embodiments, a Ligand unit contains 2 or 4 solvent-accessible cysteine residues.

ASSAY

[0220] Methods of determining whether a Drug or Ligand Drug Conjugate exerts a cytostatic and/or cytotoxic effect on a cell are known. Generally, the cytotoxic or cytostatic activity of a Ligand Drug Conjugate can be measured by: exposing mammalian cells expressing a target protein of the Ligand Drug Conjugate in a cell culture medium; culturing the cells for a period from about 6 hours to about 5 days; and measuring cell viability. Cell-based *in vitro* assays can be used to measure viability (proliferation), cytotoxicity, and induction of apoptosis (caspase activation) of the Ligand Drug Conjugate.

[0221] For determining whether a Ligand Drug Conjugate exerts a cytostatic effect, a thymidine incorporation assay may be used. For example, cancer cells expressing a target antigen at a density of 5,000 cells/well of a 96-well plated can be cultured for a 72-hour period and exposed to 0.5 μCi of ^{3}H-thymidine during the final 8 hours of the 72-hour period. The incorporation of ^{3}H-thymidine into cells of the culture is measured in the presence and absence of the Ligand Drug Conjugate.

[0222] For determining cytotoxicity, necrosis or apoptosis (programmed cell death) can be measured. Necrosis is typically accompanied by increased permeability of the plasma membrane; swelling of the cell, and rupture of the plasma membrane. Apoptosis is typically characterized by membrane blebbing, condensation of cytoplasm, and the activation of endogenous endonucleases. Determination of any of these effects on cancer cells indicates that a Ligand Drug Conjugate is useful in the treatment of cancers.

[0223] Cell viability can be measured by determining in a cell the uptake of a dye such as neutral red, trypan blue, or ALAMAR™ blue (*see, e.g.*, Page *et al.*, 1993, *Intl. J. Oncology* 3:473-476). In such an assay, the cells are incubated in media containing the dye, the cells are washed, and the remaining dye, reflecting cellular uptake of the dye, is measured spectrophotometrically. The protein-binding dye sulforhodamine B (SRB) can also be used to measure cytoxicity (Skehan *et al.*, 1990, *J. Natl. Cancer Inst.* 82:1107-12).

[0224] Alternatively, a tetrazolium salt, such as MTT, is used in a quantitative colorimetric assay for mammalian cell survival and proliferation by detecting living, but not dead, cells (*see, e.g.*, Mosmann, 1983, *J. Immunol. Methods* 65:55-63).

[0225] Apoptosis can be quantitated by measuring, for example, DNA fragmentation. Commercial photometric methods for the quantitative *in vitro* determination of DNA fragmentation are available. Examples of such assays, including TUNEL (which detects incorporation of labeled nucleotides in fragmented DNA) and ELISA-based assays, are described in *Biochemica*, 1999, no. 2, pp. 34-37 (Roche Molecular Biochemicals).

[0226] Apoptosis can also be determined by measuring morphological changes in a cell. For example, as with necrosis, loss of plasma membrane integrity can be determined by measuring uptake of certain dyes (*e.g.*, a fluorescent dye such as, for example, acridine orange or ethidium bromide). A method for measuring apoptotic cell number has been described by Duke and Cohen, Current Protocols in Immunology (Coligan *et al.* eds., 1992, pp. 3.17.1-3.17.16). Cells also can be labeled with a DNA dye (*e.g.*, acridine orange, ethidium bromide, or propidium iodide) and the cells observed for chromatin condensation and margination along the inner nuclear membrane. Other morphological changes that can be measured to determine apoptosis include, *e.g.*, cytoplasmic condensation, increased membrane blebbing, and cellular shrinkage.

[0227] The presence of apoptotic cells can be measured in both the attached and "floating" compartments of the cultures. For example, both compartments can be collected by removing the supernatant, trypsinizing the attached cells, combining the preparations following a centrifugation wash step (*e.g.*, 10 minutes at 2000 rpm), and detecting apoptosis (*e.g.*, by measuring DNA fragmentation). (*See, e.g.*, Piazza *et al.*, 1995, *Cancer Research* 55:3110-16).

[0228] The effects of Ligand Drug Conjugates can be tested or validated in animal models. A number of established animal models of cancers are known to the skilled artisan, any of which can be used to assay the efficacy of a Ligand Drug Conjugate. Non-limiting examples of such models are described *infra*. Moreover, small animal models to examine the *in vivo* efficacies of Ligand Drug Conjugates can be created by implanting human tumor cell lines into appropriate immunodeficient rodent strains, *e.g.*, athymic nude mice or SCID mice.

COMPOSITIONS AND METHODS OF ADMINISTRATION

[0229] Various delivery systems are known and can be used to administer the ligand- drug conjugates. Methods of introduction include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, and subcutaneous routes. Administration can be, for example, by infusion or bolus injection. In certain preferred embodiments, administration of the Ligand Drug Conjugate is by infusion. Parenteral administration is the preferred route of administration.

[0230] The Ligand Drug Conjugates can be administered as pharmaceutical compositions comprising one or more pharmaceutically compatible ingredients. For example, the pharmaceutical composition typically includes one or more pharmaceutical carriers (*e.g.*, sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like). Water is a more typical carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients are known in the art. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. The formulations correspond to the mode of administration.

[0231] In typical embodiments, the pharmaceutical composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the pharmaceutical can also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the pharmaceutical is to be administered by infusion, it can be dispensed, for example, with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the pharmaceutical is administered by injection, an ampoule of sterile water for injection or saline can, for example, be provided so that the ingredients can be mixed prior to administration.

[0232] The amount of the compound that is effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays can optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the compositions will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances.

[0233] The compositions comprise an effective amount of a compound such that a suitable dosage will be obtained. Typically, this amount is at least about 0.01% of a compound by weight of the composition.

[0234] For intravenous administration, the composition can comprise from about 0.01 to about 100 mg of a compound per kg of the animal's body weight. In one aspect, the composition can include from about 1 to about 100 mg of a compound per kg of the animal's body weight. In another aspect, the amount administered will be in the range from about 0.1 to about 25 mg/kg of body weight of a compound.

[0235] Generally, the dosage of a compound administered to a patient is typically about 0.01 mg/kg to about 100 mg/kg of the subject's body weight. In some embodiments, the dosage administered to a patient is between about 0.01 mg/kg to about 15 mg/kg of the subject's body weight. In some embodiments, the dosage administered to a patient is between about 0.1 mg/kg and about 15 mg/kg of the subject's body weight. In some embodiments, the dosage administered to a patient is between about 0.1 mg/kg and about 20 mg/kg of the subject's body weight. In some embodiments, the dosage administered is between about 0.1 mg/kg to about 5 mg/kg or about 0.1 mg/kg to about 10 mg/kg of the subject's body weight. In some embodiments, the dosage administered is between about 1 mg/kg to about 15 mg/kg of the subject's body weight. In some embodiments, the dosage administered is between about 1 mg/kg to about 10 mg/kg of the subject's body weight.

[0236] The pharmaceutical compositions are generally formulated as sterile, substantially isotonic and in full compliance with all Good Manufacturing Practice (GMP) regulations of the U.S. Food and Drug Administration.

THERAPEUTIC METHODS USING LIGAND DRUG CONJUGATES

[0237] The Ligand Drug Conjugates are useful for inhibiting the multiplication of a tumor cell or cancer cell, or for treating cancer in an animal. The Ligand Drug Conjugates can be used accordingly in a variety of settings for the treatment of animal cancers.

[0238] Particular types of cancers that can be treated with the Ligand Drug Conjugates include, but are not limited to: (1) solid tumors, including but not limited to fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon cancer, colorectal cancer, kidney cancer, pancreatic cancer, bone cancer, breast cancer, ovarian cancer, prostate cancer, esophogeal cancer, stomach cancer, oral cancer, nasal cancer, throat cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, uterine cancer, testicular cancer, small cell lung carcinoma, bladder carcinoma, lung cancer, epithelial carcinoma, glioma, glioblastoma, multiforme astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, skin cancer, melanoma, neuroblastoma, and retinoblastoma; (2) blood-borne cancers, including but not limited to acute lymphoblastic leukemia "ALL", acute lymphoblastic B-cell leukemia, acute lymphoblastic T-cell leukemia, acute myeloblastic leukemia "AML", acute promyelocytic leukemia "APL", acute monoblastic leukemia, acute erythroleukemic leukemia, acute megakaryoblastic leukemia, acute myelomonocytic leukemia, acute nonlymphocyctic leukemia, acute undifferentiated leukemia, chronic myelocytic leukemia "CML", chronic lymphocytic leukemia "CLL", hairy cell leukemia, multiple myeloma, acute and chronic leukemias, e.g., lymphoblastic myelogenous and lymphocytic myelocytic leukemias; and (3) lymphomas such as Hodgkin's disease, non-Hodgkin's Lymphoma, Multiple myeloma, Waldenstrom's macroglobulinemia, Heavy chain disease, and Polycythemia vera. In some embodiments, the cancer is a DR5-positive or DR5-expressing cancer.

[0239] In some embodiments, the invention provides methods of treating cancer, comprising administering to a subject in need thereof an effective amount of a Ligand Drug Conjugate or a pharmaceutical composition thereof, comprising a DR5 binding agent covalently attached to a cytotoxic agent. In some embodiments, the Ligand Drug Conjugate

comprises formula I as provided above. An effective amount of a Ligand Drug Conjugate will be dependent on the subject being treated, the severity of the affliction, and the manner of administration. Determination of an effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. Generally, an efficacious or effective amount of a Ligand Drug Conjugate is determined by first administering a low dose or small amount, and then incrementally increasing the administered dose or dosages until a desired therapeutic effect is observed in the treated subject, with minimal or no toxic side effects. Applicable methods for determining an appropriate dose and dosing schedule for administration of the present invention are described, for example, in *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 11th Ed., Brunton, Lazo and Parker, Eds., McGraw-Hill (2006), and in *Remington: The Science and Practice of Pharmacy*, 21st Ed., Gennaro, Ed., Lippencott Williams & Wilkins (2003), both of which are hereby incorporated herein by reference.

EXAMPLES

Conjugation of DR5 antibody drug conjugates

[0240] hB273 antibody drug conjugates were prepared as follows. A hB273 antibody comprising a heavy chain corresponding to the amino acid sequence of SEQ ID NO:9 or to an amino acid sequence of SEQ ID NO:9 that lacks the carboxy terminal lysine residue (*i.e.*, the amino acid sequence of amino acid residues 1-451 of SEQ ID NO:9) and comprising a light chain corresponding to the amino acid sequence of SEQ ID NO:10 was used as the Ligand unit. A solution of hB273 antibody at a concentration ranging between 5 and 25 mg/mL was pre-equilibrated at 37°C, and then 5-15% (v/v) 1 M potassium phosphate (pH 7.4) was added to raise the pH to 7.5-8.0. DTPA was also added at a final concentration of 1 mM. The antibody was partially reduced by adding between 2 and 3 molar equivalents of TCEP per mole of antibody. The amount of TCEP needed to yield an antibody with an average of 4 free thiols was empirically determined by several small scale reductions and conjugation reactions prior to gram scale reduction and conjugation. After a reduction period of 1 to 2 hours, the antibody solution was cooled to 22°C then treated with 4 to 6 molar equivalentsof drug linker (e.g., mc-vc-MMAF or mc-vc-MMAE or mc-MMAF) from a 10 to 20 mM stock solution in DMSO. The drug-linker stock was added over a 15 minute period. Additional DMSO was introduced to bring the mixture to 15% total DMSO by volume. The reaction mixture was stirred for an additional 30 minutes after the drug-linker addition was complete

before treatment with a 5-fold molar excess of N-acetyl cysteine per mole of drug-linker added. After quenching, residual free drug and other process impurities were removed by constant volume diafiltration using 10 diavolumes of PBS. The following antibody drug conjugates were generated: an antibody drug conjugate of hB273 conjugated with mc-vc-MMAE ("hB273-vc-MMAE") and an antibody drug conjugate of hB273 conjugated with mc-MMAF ("hB273-mc-MMAF"). The resulting antibody drug conjugates had an average drug loading of about 3.5 to 4.5 drug-linker units per antibody (*i.e.*, had an average p value of about 3.5 to 4.5). The average drug loading, or average p value, can be measured according to methods known in the art. As a non-limiting example, p values can be measured according to the methods described in Hamblett *et al., Clinical Cancer Research* 10:7063-7070 (2004), incorporated by reference herein. In one method, hydrophobic interaction chromatography—high-performance liquid chromatography is performed on a sample comprising antibody drug conjugates, *e.g.*, using an Ether-5PW column (Tosoh Bioscience, Montgomeryville, PA). Because the antibody and drug have distinct absorbance maxima, it is possible to identify and quantify the number of drug units per antibody by overlaying peak spectra, thereby allowing the determination of average drug loading. In another method, p values are measured by ultraviolet and visible absorption spectroscopy (UV-VIS) if the antibody and drug have distinct absorbance maxima (λ_{max}). By using the measured total absorbance (absorbance of the drug and the antibody) and the extinction coefficients of the antibody and drug, the concentrations of the antibody and the drug can be determined, from which the molar ratio of drug to antibody can be calculated.

[0241] The above protocol can also be used to generate an antibody drug conjugate of hB273 conjugated with mc-vc-MMAF ("hB273-vc-MMAF"). To prepare antibody drug conjugates with an average drug loading of about 2 drug-linker units per antibody (*i.e.*, having an average p value of about 2), the protocol above is modified by reducing the amount of TCEP by 50%. The amount of drug linker is also reduced by 50%. The corresponding antibody drug conjugate is abbreviated as hB273-vc-MMAE(2), hB273-mc-MMAF(2), or hB273-vc-MMAF(2).

[0242] An antibody drug conjugate of hTRA-8 conjugated with mc-MMAF ("hTRA-8-mc-MMAF") was prepared as follows. A hTRA-8 antibody comprising a heavy chain corresponding to the amino acid sequence of SEQ ID NO:1 or to an amino acid sequence of SEQ ID NO:1 that lacks the carboxy terminal lysine residue (*i.e.*, the amino acid sequence of amino acid residues 1-448 of SEQ ID NO:1) and comprising a light chain corresponding to the amino acid sequence of SEQ ID NO:2 was used as the Ligand unit. This hTRA-8

antibody is referred to as Tigatuzumab. A solution of hTRA-8 antibody at 7.6 mg/mL is pre-equilibrated at 37°C, and then a 15% volume of 500 mM sodium borate, pH 8.0 is added to raise the pH to 7.5-8.0. The solution also contains 1 mM DTPA. The antibody is partially reduced by adding 2.6 equivalents of TCEP per mole of antibody and stirring at 37°C. After 28 minutes, the solution of reduced antibody is placed on ice, then treated immediately with 4.8-4.9 molar equivalents (relative to antibody) of drug linker (e.g., mc-vc-MMAF or mc-vc-MMAE or mc-MMAF) as a 20.5 mM solution in DMSO. Additional DMSO is introduced to bring the mixture to 10% DMSO by volume. The reaction mixture is stirred on ice for ~90 minutes before treatment with a 5-fold molar excess of N-acetyl cysteine (relative to mc-vc-MMAF). The conjugate is isolated by tangential flow filtration, first being concentrated to ~10 mg/mL, then diafiltered with ~10 diavolumes of PBS. The resulting antibody drug conjugate had an average drug loading of about four drug-linker units per antibody.

In vitro cytotoxic activity of hB273 ADCs against several tumor cell lines

[0243] hB273, hB273-vcMMAE, hB273-mcMMAF and hTRA-8-mcMMAF were diluted in culture medium to obtain 2000 ng/mL, in which 4000 ng/mL of goat anti-human IgG Fc antibody (MP Biomedicals) was involved. These solutions were serially diluted with the culture medium, and added to a 96-well microplate by 50 μL/well in duplicate. The cell lines used *in vitro* study were A375 (human melanoma), NCI-H2122 (human lung adenocarcinoma), HCT 15 (human colorectal adenocarcinoma), SK-OV-3 (human ovarian adenocarcinoma), LoVo (human colorectal adenocarcinoma), A2780 (human ovarian carcinoma), A549 (human lung adenocarcinoma), Caki-2 (human renal cell carcinoma), HCT 116 (human colorectal adenocarcinoma), NCI-H1975 (human lung adenocarcinoma), MDA-MB-231 (human breast adenocarcinoma) and U-87 MG (human glioblastoma). The cell suspension was adjusted to 4.0×10^4 viable cells/mL with the culture medium and added to the wells by 50 μL/well ($= 2 \times 10^3$ viable cells/well). The cells were not seeded in the blank wells. After 72 hr of incubation in a CO_2 incubator, the amount of ATP was detected by CellTiter Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. The luminescence was measured by a microplate reader (Mithras LB940, Berthold Technologies). The cell viability of each well was calculated according to the following equation:

$$\text{Viability (\%)} = 100 \times (T\text{-}B)/(C\text{-}B)$$

T: luminescence of a test well

C: average luminescence of wells with untreated cells

B: average luminescence of blank wells

[0244] **Figures 1-12** provide the results for 12 cell lines evaluated with hB273 Ligand Drug Conjugates of the present invention. As the Figures illustrate, hB273-vcMMAE and hB273-mcMMAF showed more potent cytotoxic activity than hB273 against several human tumor cell lines such as LoVo (**Figure 5**), A549 (**Figure 7**) and Caki-2 (**Figure 8**). In addition, hB273-vcMMAE and hB273-mcMMAF showed more potent cytotoxic activity than hTRA-8-mcMMAF against all tumor cell lines used for this assay.

Determination of binding affinity of anti-DR5 ADCs by BIACore

[0245] **Production of DR5 extracellular domain protein expression vector:** In order to construct a vector expressing a region of DR5 (hereinafter referred to as "sDR5") consisting of amino acid residues 1-130 of the human DR5 sequence of SEQ ID NO:17, a PCR reaction was performed using a primer set for amplifying sDR5:

DR5 Ndefw: 5'-gtggcatatggctctgatcacccaacaa-3' (SEQ ID NO:18) and

DR5 Xhorv: 5'-cgcctcgagtgattctttgtggacaca-3' (SEQ ID NO:19),

using a cDNA encoding a human DR5 extracellular domain as a template. The resulting PCR product was cleaved with NdeI and XhoI and cloned into the NdeI/XhoI site of pET21b(+) (manufactured by Novagen, Inc.) (hereinafter abbreviated as "pET21b(+)-sDR5"). The recombinant DR5 protein expressed by "pET21b(+)-sDR5" is referred to herein as "rsDR5" (SEQ ID NO:20).

[0246] **Production of DR5 extracellular domain protein (rsDR5):** *Escherichia coli* Origami B (DE3) (manufactured by Novagen, Inc.) was transformed with the expression plasmid pET21b(+)-sDR5 and cultured in 2-YT medium supplemented with 100 μg/ml ampicillin (manufactured by Sigma Co., Ltd.) and 15 μg/ml kanamycin (manufactured by Wako Pure Chemical Industries, Ltd.), and the expression of a partial protein of DR5 was induced by the addition of 0.5 mM IPTG. The cells were collected by centrifugation at 6000 rpm for 20 minutes and suspended in a binding buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl), followed by ultrasonic homogenization on ice. The resulting homogenate was centrifuged at 25,000 rpm for 20 minutes. The supernatant was recovered and applied to Ni-NTA (manufactured by Invitrogen, Inc.). After washing the sample with the binding buffer,

elution was performed with an elution buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl and 300 mM imidazole). The eluted sample was dialyzed with a dialysis buffer (50 mM Tris-HCl pH 8.0, 20 mM NaCl) and applied to MONO Q, and gradient elution was performed with an elution buffer (50 mM Tris-HCl pH 8.0, 1 M NaCl). The eluted sample was further purified by gel filtration column chromatography (Superdex 75 16/60, manufactured by GE Healthcare Bio-Sciences Co., Ltd.) using PBS as a solvent. The concentration of the recombinant protein obtained by this method was measured at UV 280 nm (molar absorbance constant: 14855).

[0247] Measurement of binding activity using Biacore: The kinetic constants k_{on} and k_{off} were determined with serial dilutions of the recombinant soluble DR5 protein (rsDR5) binding to captured hB273, hB273-vc-MMAE, or hB273-mc-MMAF using the BIAcore 3000 instrument (Biacore). Using the Human Antibody Capture Kit (GE Healthcare Bio-Sciences AB), hB273, hB273-vc-MMAE, or hB273-mc-MMAF was captured by anti-human IgG (Fc) antibody covalently immobilized on a CM5 sensor chip (Biacore) by standard EDC-NHS amine coupling chemistry. For the direct coupling of anti-human IgG (Fc), CM5 senor chips and the reference flow cells were coated with ~1500-2000 RU in HBS-EP+ (10 mM HEPES buffer, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.05% Surfactant P20). The hB273, hB273-vc-MMAE, or hB273-mc-MMAF (0.5~1 μg/ml) was loaded at flow rate of 10 μl/min for 1 min, and then kinetic measurements were performed at a flow rate of 30 μl/min for 5 min using rsDR5 concentration range from 0.3-85 nM, followed by 5 min dissociation phase. For regeneration, 3 M $MgCl_2$ was loaded at flow rate of 10 μl/min for 30 sec. All sensograms were globally fitted using BIAevaluation software 3.1 (Biacore) to determine the dissociation constant KD ($KD = k_{off}/k_{on}$). The results are shown in Table 1 below:

Table 1. Antibody affinities

Name	k_{on} (1/Ms)	k_{off} (1/d)	kD (nM)
hB273	2.6E+06	6.7E-03	2.6
hB273-mc-MMAF	2.5E+06	6.2E-03	2.5
hB273-vc-MMAE	2.5E+06	7.8E-03	3.2

In vivo Anti-Tumor Efficacy – Methods

[0248] Specific pathogen-free female and male CAnN.Cg-*Foxn1*nu/CrlCrlj mice (nude mice), aged 5 to 6 weeks, were purchased from Charles River Laboratories Japan Inc., and

were used when they reached 6 to 8 weeks of age. Four to six mice were housed together in sterilized cages and maintained under specific pathogen-free conditions. In the experimental room, the environmental conditions were set at a temperature of 23°C and 55% humidity with artificial illumination of 12 h (8:00 - 20:00). The mice were fed an FR-2 diet (Funabashi Farm Co., Ltd.) and provided with water with chlorine (5 -15 ppm) *ad libitum*.

[0249] In all the studies, tumor-bearing mice were selected and divided into experimental groups based on the tumor volume. After the establishment of the tumors on the nude mice, tumor length and width (mm) in all the tumor-bearing mice were measured with a digital caliper (CD15-CX, Mitutoyo Corp.) to two decimal places. The data was automatically recorded in the Sankyo management system for animal experimental data (SMAD, JMACSOFT Corp.). The tumor volume of each mouse was automatically calculated in SMAD according to the following equation.

$$\text{Tumor volume } (mm^3) = 1/2 \times \text{length} \times \text{width}^2$$

[0250] The tumor volumes were rounded to integers and the rounded tumor volumes were used for further analyses. Based on the tumor volume, a certain number of tumor-bearing mice was selected by eliminating the mice with the largest Studentized residual in absolute value. The selected mice were divided into experimental groups utilizing SMAD by the randomized block method using the tumor volume. After grouping, the tumor length and width in each mouse were measured with a digital caliper once or twice a week. The mean tumor volume and the standard error (SE) of each group were calculated and rounded to integers in SMAD using the rounded tumor volume of each mouse. The tumor growth inhibition (TGI, %) on each measured day was also calculated in SMAD using the rounded tumor volume according to the following equation, and rounded to an integer.

$$TGI\ (\%) = (1-T/C) \times 100$$

T: mean tumor volume (mm^3) of the drug-treated groups

C: mean tumor volume (mm^3) of the untreated group

[0251] hB273, hB273-vcMMAE, hB273-mcMMAF and hTRA-8-mcMMAF were diluted in saline and administered to tumor-bearing nude mice at the volume of 10 mL/kg of mouse body weight.

[0252] The detailed procedure of each human tumor xenograft study is as follows:

A375

[0253] Human melanoma cell line A375 was purchased from American Type Cell Collection (ATCC). 5×10^6 cells were subcutaneously inoculated into the right flank of female nude mice on Day 0. On Day 14, the tumor-bearing nude mice were divided into experimental groups (n = 6). 3 mg/kg of hB273 and 1 mg/kg of hB273-vcMMAE, hB273-mcMMAF and hTRA-8-mcMMAF were intravenously administered to the mice on Days 14, 21 and 28.

A2780

[0254] Human ovarian carcinoma cell line A2780 was purchased from American Type Cell Collection (ATCC). 5×10^6 cells were subcutaneously inoculated into the right flank of female nude mice on Day 0. On Day 11, the tumor-bearing nude mice were divided into experimental groups (n = 6). hB273, hB273-vcMMAE, hB273-mcMMAF and hTRA-8-mcMMAF were intravenously administered to the mice at the dose of 3 mg/kg on Days 11, 18 and 25.

Caki-2

[0255] Human renal cell carcinoma cell line Caki-2 was purchased from American Type Cell Collection (ATCC). Solid tumor pieces ($5 \times 5 \times 5$ mm^3) were subcutaneously inoculated into the right flank of male nude mice on Day 0. On Day 18, the tumor-bearing nude mice were divided into experimental groups (n = 8). hB273, hB273-vcMMAE, hB273-mcMMAF and hTRA-8-mcMMAF were intravenously administered to the mice at the dose of 3 mg/kg on Days 18, 25 and 32.

HCT-116

[0256] Human colorectal adenocarcinoma cell line HCT-116 was purchased from American Type Cell Collection (ATCC). 6×10^6 cells were subcutaneously inoculated into the right flank of female nude mice on Day 0. On Day 11, the tumor-bearing nude mice were divided into experimental groups (n = 6). hB273, hB273-vcMMAE, hB273-mcMMAF and hTRA-8-mcMMAF were intravenously administered to the mice at the dose of 3 mg/kg on Days 11, 18 and 25.

HCT-15

[0257] Human colorectal adenocarcinoma cell line HCT-15 was purchased from American Type Cell Collection (ATCC). 6×10^6 cells were subcutaneously inoculated into the right flank of female nude mice on Day 0. On Day 9, the tumor-bearing nude mice were divided into experimental groups (n = 6). hB273, hB273-vcMMAE, hB273-mcMMAF and hTRA-8-

mcMMAF were intravenously administered to the mice at the dose of 3 mg/kg on Days 9, 16 and 23.

LoVo

[0258] Human colorectal adenocarcinoma cell line LoVo was purchased from American Type Cell Collection (ATCC). Solid tumor pieces ($5 \times 5 \times 5$ mm^3) were subcutaneously inoculated into the right flank of female nude mice on Day 0. On Day 10, the tumor-bearing nude mice were divided into experimental groups (n = 8). hB273, hB273-vcMMAE, hB273-mcMMAF and hTRA-8-mcMMAF were intravenously administered to the mice at the dose of 3 mg/kg on Days 10, 17 and 24.

NCI-H1299

[0259] Human lung carcinoma cell line NCI-H1299 was purchased from American Type Cell Collection (ATCC). 4×10^6 cells were subcutaneously inoculated into the right flank of female nude mice on Day 0. On Day 14, the tumor-bearing nude mice were divided into experimental groups (n = 6). hB273, hB273-vcMMAE, hB273-mcMMAF and hTRA-8-mcMMAF were intravenously administered to the mice at the dose of 3 mg/kg on Days 14, 21 and 28.

NCI-H1975

[0260] Human lung adenocarcinoma cell line NCI-H975 was purchased from American Type Cell Collection (ATCC). 3×10^6 cells were subcutaneously inoculated into the right flank of female nude mice on Day 0. On Day 13, the tumor-bearing nude mice were divided into experimental groups (n = 6). hB273, hB273-vcMMAE, hB273-mcMMAF and hTRA-8-mcMMAF were intravenously administered to the mice at the dose of 3 mg/kg on Days 13, 20 and 27.

NCI-H2122

[0261] Human lung adenocarcinoma cell line NCI-H2122 was purchased from American Type Cell Collection (ATCC). 3×10^6 cells were subcutaneously inoculated into the right flank of female nude mice on Day 0. On Day 15, the tumor-bearing nude mice were divided into experimental groups (n = 6). hB273, hB273-vcMMAE, hB273-mcMMAF and hTRA-8-mcMMAF were intravenously administered to the mice at the dose of 1 mg/kg on Days 15, 22 and 29.

SK-OV-3

[0262] Human ovarian adenocarcinoma cell line SK-OV-3 was purchased from American Type Cell Collection (ATCC). Solid tumor pieces ($5 \times 5 \times 5$ mm^3) were subcutaneously inoculated into the right flank of female nude mice on Day 0. On Day 14, the tumor-bearing nude mice were divided into experimental groups (n = 10). hB273, hB273-vcMMAE, hB273-mcMMAF and hTRA-8-mcMMAF were intravenously administered to the mice at the dose of 3 mg/kg on Days 14, 21 and 28.

U-87 MG

[0263] Human glioblastoma cell line U-87 MG was purchased from American Type Cell Collection (ATCC). 5×10^6 cells were subcutaneously inoculated into the right flank of female nude mice on Day 0. On Day 7, the tumor-bearing nude mice were divided into experimental groups (n = 6 or 7). hB273, hB273-vcMMAE, hB273-mcMMAF and hTRA-8-mcMMAF were intravenously administered to the mice at the dose of 3 mg/kg on Days 7, 14 and 21.

In vivo Anti-Tumor Efficacy – Results

[0264] In the Caki-2 xenograft model (**Figure 15**), none of the antibodies (hB273, hB273-vcMMAE, hB273-mcMMAF and hTRA-8-mcMMAF) showed any anti-tumor activities. Both hB273-vcMMAE and hB273-mcMMAF showed more potent anti-tumor efficacy than hB273 in the xenograft models of A375 (**Figure 13**), A2780 (**Figure 14**), HCT 116 (**Figure 16**), LoVo (**Figure 18**), NCI-H1299 (**Figure 19**), NCI-H1975 (**Figure 20**), SK-OV-3 (**Figure 22**) and U-87 MG (**Figure 23**). The anti-tumor activity of hB273-mcMMAF was as effective as that of hB273 in the HCT-15 (**Figure 17**) and NCI-H2122 (**Figure 21**) xenograft models, whereas the anti-tumor activity of hB273-vcMMAE was less effective than that of hB273 in these models. These results demonstrate that hB273 Ligand Drug Conjugates have more potent anti-tumor efficacy than hB273.

[0265] Both hB273-vcMMAE and hB273-mcMMAF showed more potent anti-tumor efficacy than hTRA-8-mcMMAF in the xenograft models of A2780 (**Figure 14**), HCT 116 (**Figure 16**), LoVo (**Figure 18**), NCI-H1299 (**Figure 19**), NCI-H1975 (**Figure 20**), NCI-H2122 (**Figure 21**), SK-OV-3 (**Figure 22**) and U-87 MG (**Figure 23**). hB273-mcMMAF was more effective than hTRA-8-mcMMAF in the A375 (**Figure 13**) and HCT-15 (**Figure 17**) xenograft models. In the A375 xenograft model (**Figure 13**), 1 out of 6 mice treated with hTRA-8-mcMMAF had no palpable tumor on Day 48, whereas all the mice (n = 6) treated with hB273-mcMMAF had no palpable tumor on Day 48. These results demonstrate that

hB273 Ligand Drug Conjugates have more potent anti-tumor efficacy than hTRA-8 Ligand Drug Conjugates.

***

[0266] The present invention is not limited in scope by the specific embodiments described herein. Various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims. Unless otherwise apparent from the context any step, element, embodiment, feature, or aspect of the invention can be used in combination with any other. All patent filings, scientific publications, accession numbers and the like referred to in this application are hereby incorporated by reference in their entirety for all purposes to the same extent as if so individually denoted.

WHAT IS CLAIMED IS:

1. A ligand drug conjugate having the formula:

$$L-(LU\text{-}D)_p \qquad \textbf{(I)}$$

or a pharmaceutically acceptable salt thereof, having specificity for target cells expressing DR5, wherein:

L is a Ligand unit which is an anti-DR5 antibody comprising (a) a heavy chain immunoglobulin having the CDR1 consisting of amino residues 1-5 of SEQ ID NO:11, the CDR2 consisting of amino acid residues 1-17 of SEQ ID NO:12, and the CDR3 consisting of amino acid residues 1-13 of SEQ ID NO:13; and (b) a light chain immunoglobulin having the CDR1 consisting of amino residues 1-16 of SEQ ID NO:14, the CDR2 consisting of amino acid residues 1-7 of SEQ ID NO:15, and the CDR3 consisting of amino acid residues 1-9 of SEQ ID NO:16; and

(LU-D) is a Linker unit-Drug unit moiety, wherein

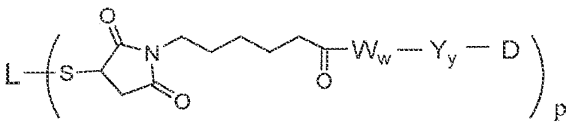
LU is a Linker unit, and

D is a Drug unit, wherein the Drug unit is an auristatin; and

the subscript p is an integer of from 1 to 20.

2. The ligand drug conjugate of claim **1**, wherein the auristatin is auristatin E, AEB, AEVB, AFP, MMAF, or MMAE.

3. The ligand drug conjugate of claim **1**, having the formula:



or a pharmaceutically acceptable salt form thereof, wherein:

S is a sulfur atom of the Ligand unit;

each W is independently an Amino Acid unit;

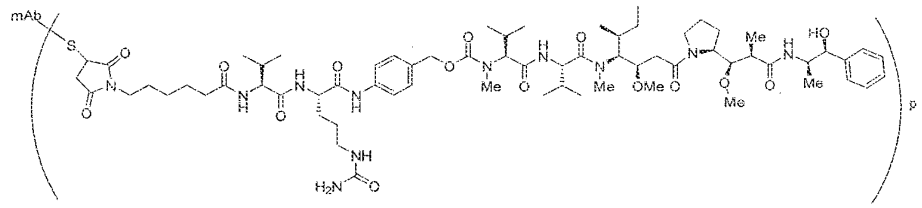
the subscript w is an integer of from 0 to 12;

Y is a Spacer unit; and

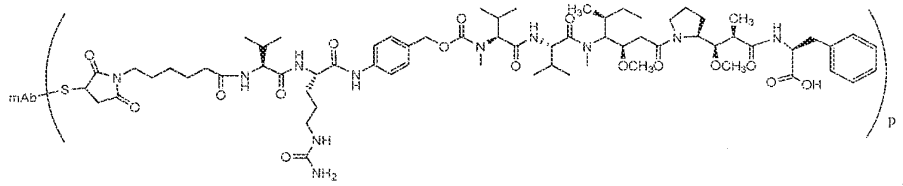
the subscript y is 0, 1, or 2.

4. The ligand drug conjugate of claim **3**, wherein W_w is valine-citrulline.

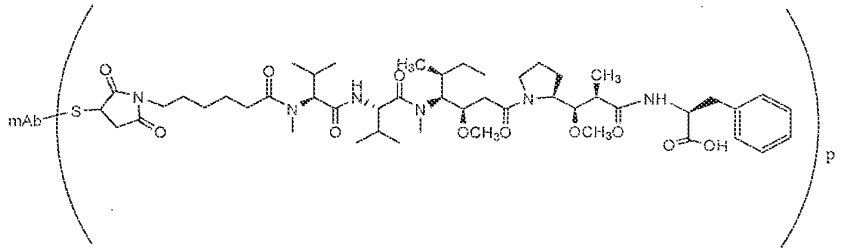
5. The ligand drug conjugate of claim **1**, having the formula:



,



, or



or a pharmaceutically acceptable salt form thereof, wherein:

mAb is the anti-DR5 antibody;

S is a sulfur atom of the antibody; and

p is an integer of from 1 to 8.

6. The ligand drug conjugate of claim 1, wherein the anti-DR5 antibody comprises a heavy chain variable region comprising amino acid residues 1-122 of SEQ ID NO:9 and a light chain variable region comprising amino acid residues 1-114 of SEQ ID NO:10.

7. The ligand drug conjugate of claim 1, wherein the anti-DR5 antibody comprises a heavy chain consisting of amino residues 1-452 of SEQ ID NO:9 and a light chain consisting of amino acid residues 1-219 of SEQ ID NO:10.

8. The ligand drug conjugate of claim 1, wherein the anti-DR5 antibody comprises a heavy chain consisting of amino residues 1-451 of SEQ ID NO:9 and a light chain consisting of amino acid residues 1-219 of SEQ ID NO:10.

9. The ligand drug conjugate of claim 1, wherein p is from 1 to 8.

10. A pharmaceutical composition comprising the ligand drug conjugate of any of claims **1-9**, in admixture with a pharmaceutically acceptable excipient.

11. A composition comprising a mixture of ligand drug conjugates of any of claims **1-9**, wherein the average p value is between 1 and 20.

12. The composition of claim **11**, wherein the average p value is from about 3.5 to about 4.5.

13. An anti-tumor agent comprising the ligand drug conjugate of any of claims **1-9**, as an effective ingredient.

14. A method of treating cancer that expresses a DR5 protein comprising administering to a subject in need thereof an effective amount of a ligand drug conjugate of any of claims **1-9**.

15. A method in accordance with claim **14**, wherein said cancer that expresses a DR5 protein is selected from the group consisting of melanoma, colorectal cancer, non-small cell lung carcinoma, uterine cancer, pancreatic cancer, prostate cancer, breast cancer, ovarian cancer, and hematological cancer.

16. A method in accordance with claim **15**, wherein said cancer that expresses a DR5 protein is pancreatic cancer.

17. A method in accordance with claim **15**, wherein said cancer that expresses a DR5 protein is melanoma.

18. A method in accordance with claim **15**, wherein said cancer that expresses a DR5 protein is breast cancer.

19. A method in accordance with claim **15**, wherein said cancer that expresses a DR5 protein is ovarian cancer.

20. A method in accordance with claim **15**, wherein said cancer that expresses a DR5 protein is colorectal cancer.

21. A method in accordance with claim **15**, wherein said cancer that expresses a DR5 protein is renal cancer.

22. A method in accordance with claim 15, wherein said cancer that expresses a DR5 protein is glioblastoma.

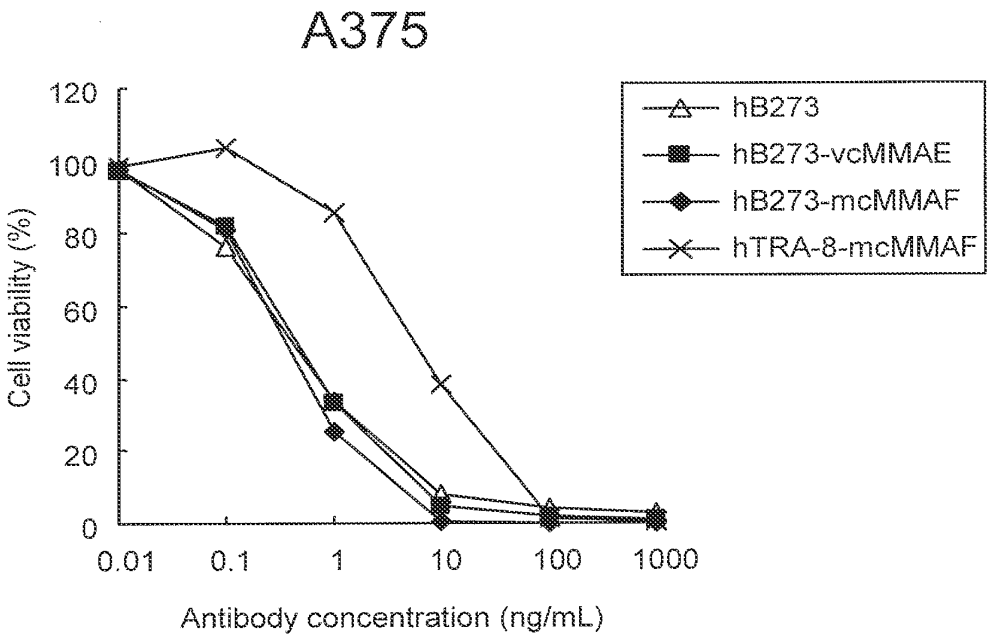

A375
120
100
80
60
40
20
0
Cell viability (%)
0.01
0.1
1
10
100
1000
Antibody concentration (ng/mL)
hB273
hB273-vcMMAE
hB273-mcMMAF
hTRA-8-mcMMAF

FIG. 1

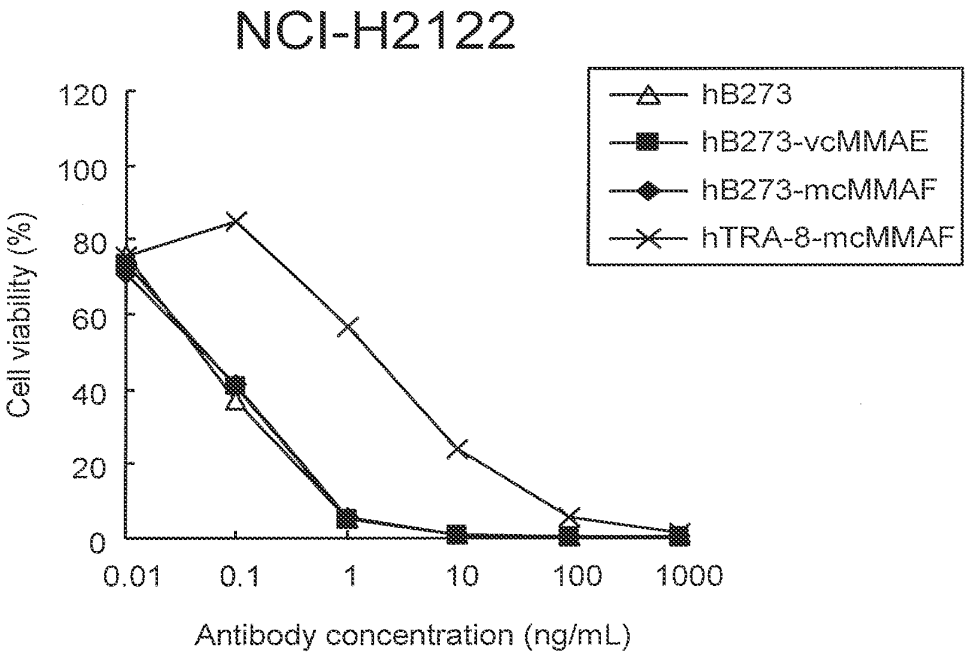

NCI-H2122
hB273
hB273-vcMMAE
hB273-mcMMAF
hTRA-8-mcMMAF
Cell viability (%)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Antibody concentration (ng/mL)

FIG. 2

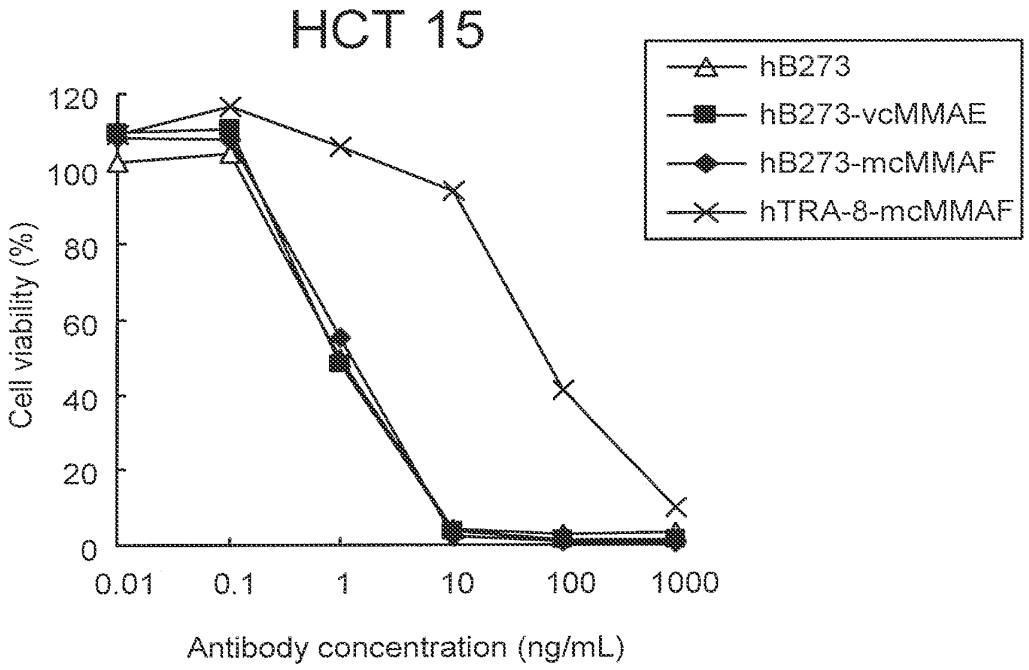

HCT 15
hB273
hB273-vcMMAE
hB273-mcMMAF
hTRA-8-mcMMAF
Cell viability (%)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Antibody concentration (ng/mL)

FIG. 3

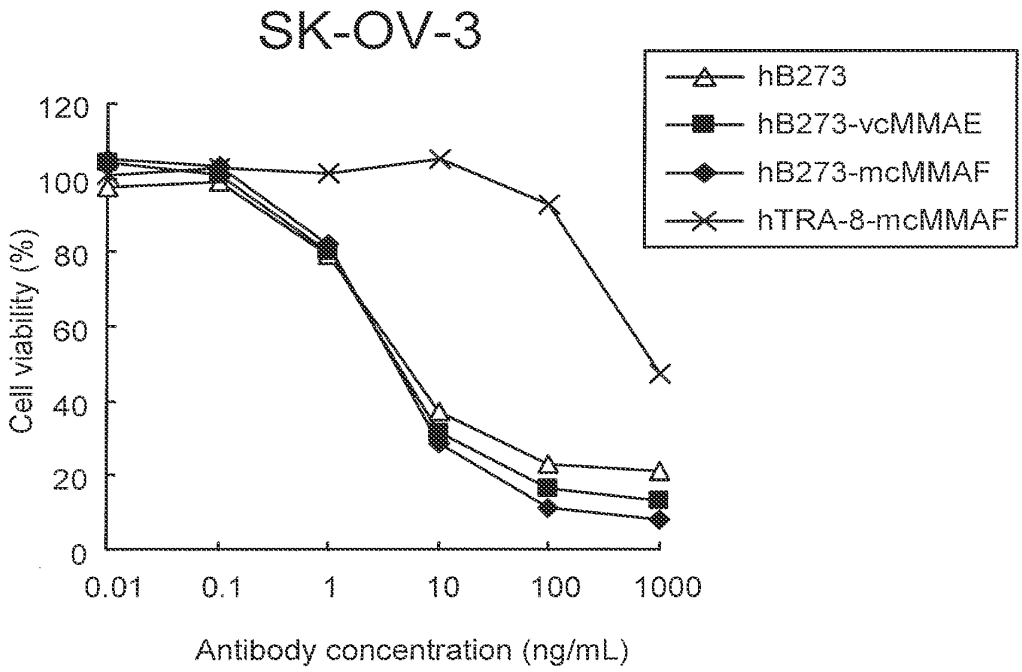

SK-OV-3
hB273
hB273-vcMMAE
hB273-mcMMAF
hTRA-8-mcMMAF
Cell viability (%)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Antibody concentration (ng/mL)

FIG. 4

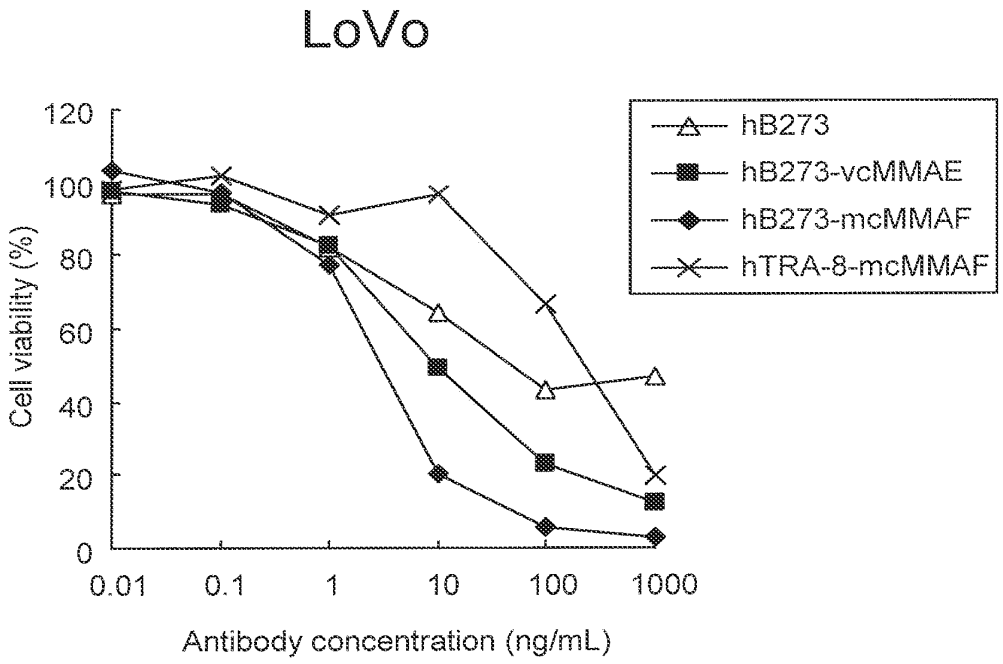

LoVo
120
100
80
60
40
20
0
Cell viability (%)
0.01
0.1
1
10
100
1000
Antibody concentration (ng/mL)
hB273
hB273-vcMMAE
hB273-mcMMAF
hTRA-8-mcMMAF

FIG. 5

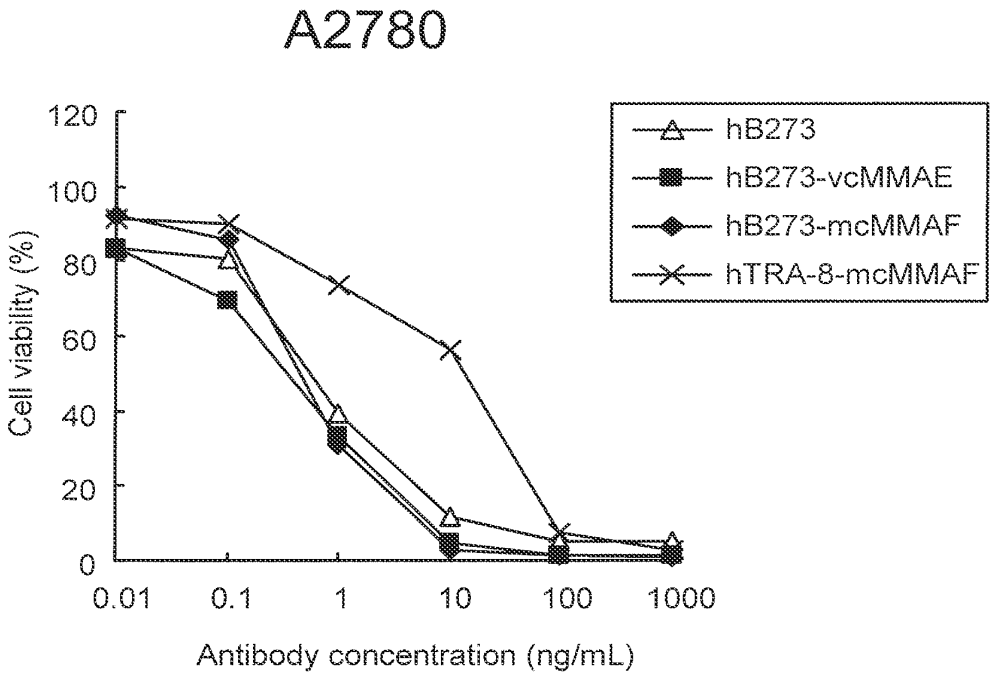

A2780
120
100
80
60
40
20
0
Cell viability (%)
0.01
0.1
1
10
100
1000
Antibody concentration (ng/mL)
hB273
hB273-vcMMAE
hB273-mcMMAF
hTRA-8-mcMMAF

FIG. 6

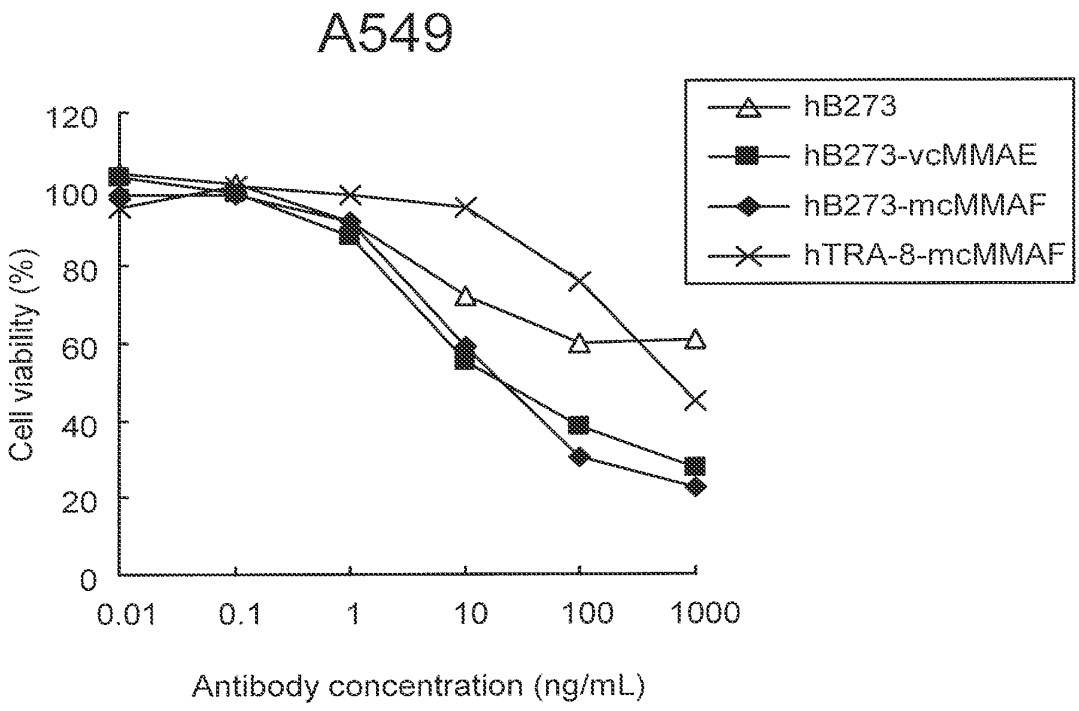

A549
hB273
hB273-vcMMAE
hB273-mcMMAF
hTRA-8-mcMMAF
Cell viability (%)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Antibody concentration (ng/mL)

FIG. 7

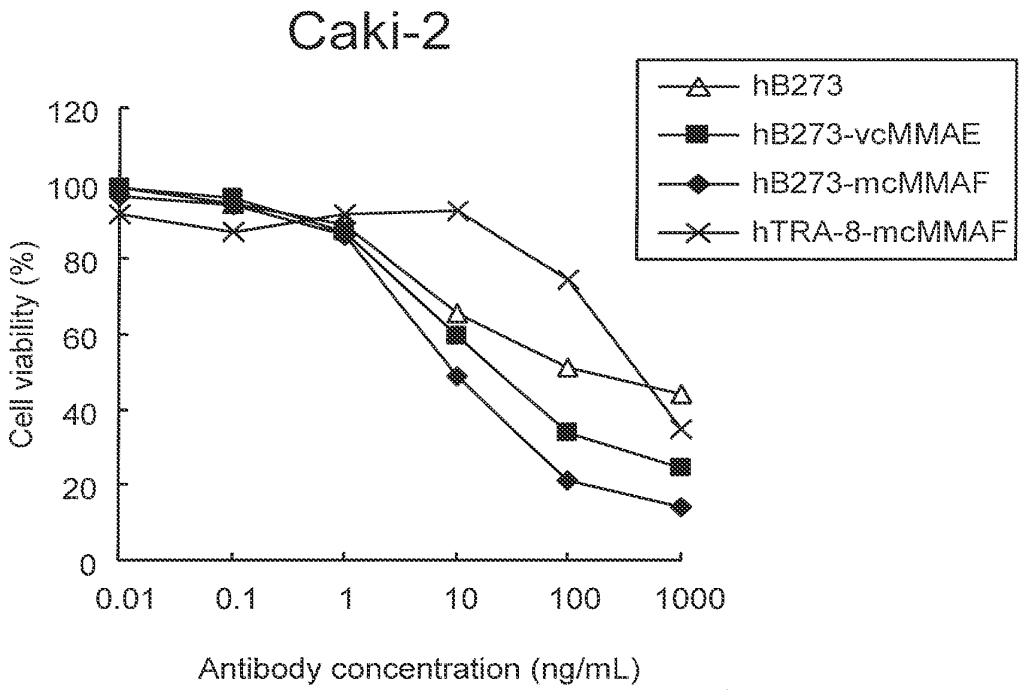

Caki-2
hB273
hB273-vcMMAE
hB273-mcMMAF
hTRA-8-mcMMAF
Cell viability (%)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Antibody concentration (ng/mL)

FIG. 8

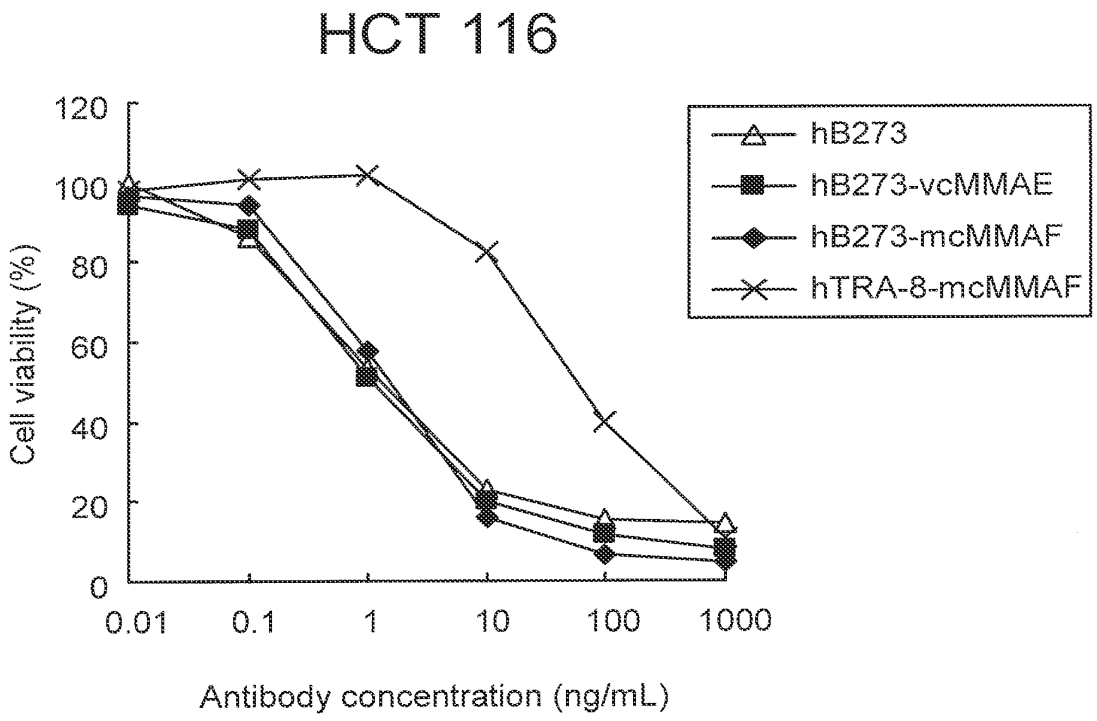

HCT 116
120
100
80
60
40
20
0
Cell viability (%)
0.01
0.1
1
10
100
1000
Antibody concentration (ng/mL)
hB273
hB273-vcMMAE
hB273-mcMMAF
hTRA-8-mcMMAF

FIG. 9

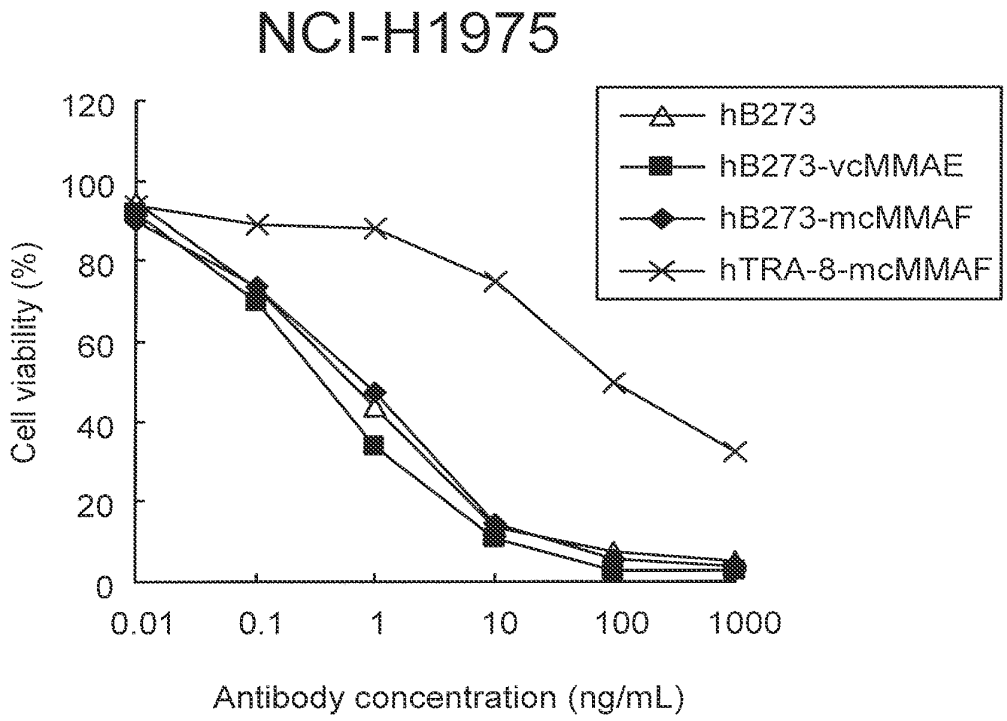

NCI-H1975
Cell viability (%)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Antibody concentration (ng/mL)
hB273
hB273-vcMMAE
hB273-mcMMAF
hTRA-8-mcMMAF

FIG. 10

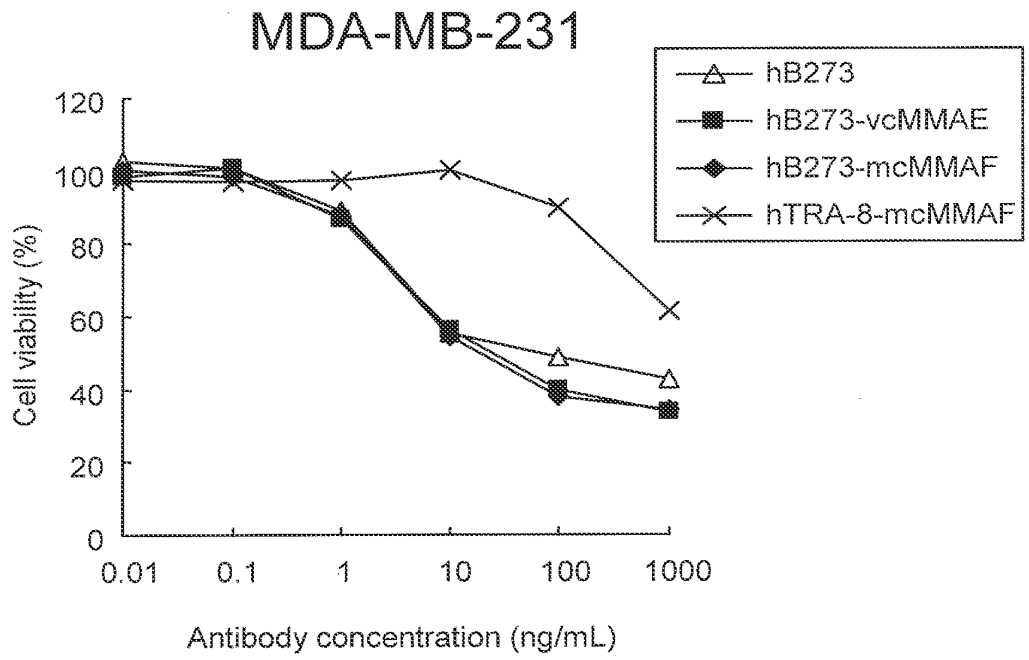

MDA-MB-231
hB273
hB273-vcMMAE
hB273-mcMMAF
hTRA-8-mcMMAF
Cell viability (%)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Antibody concentration (ng/mL)

FIG. 11

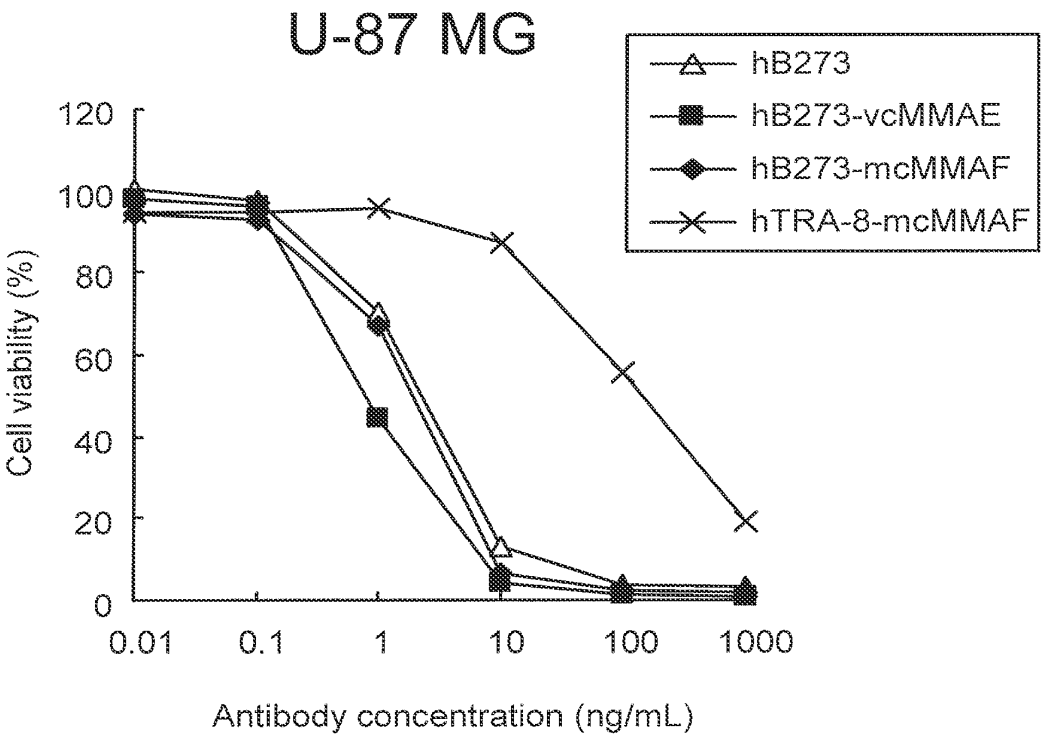

U-87 MG
hB273
hB273-vcMMAE
hB273-mcMMAF
hTRA-8-mcMMAF
Cell viability (%)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Antibody concentration (ng/mL)

FIG. 12

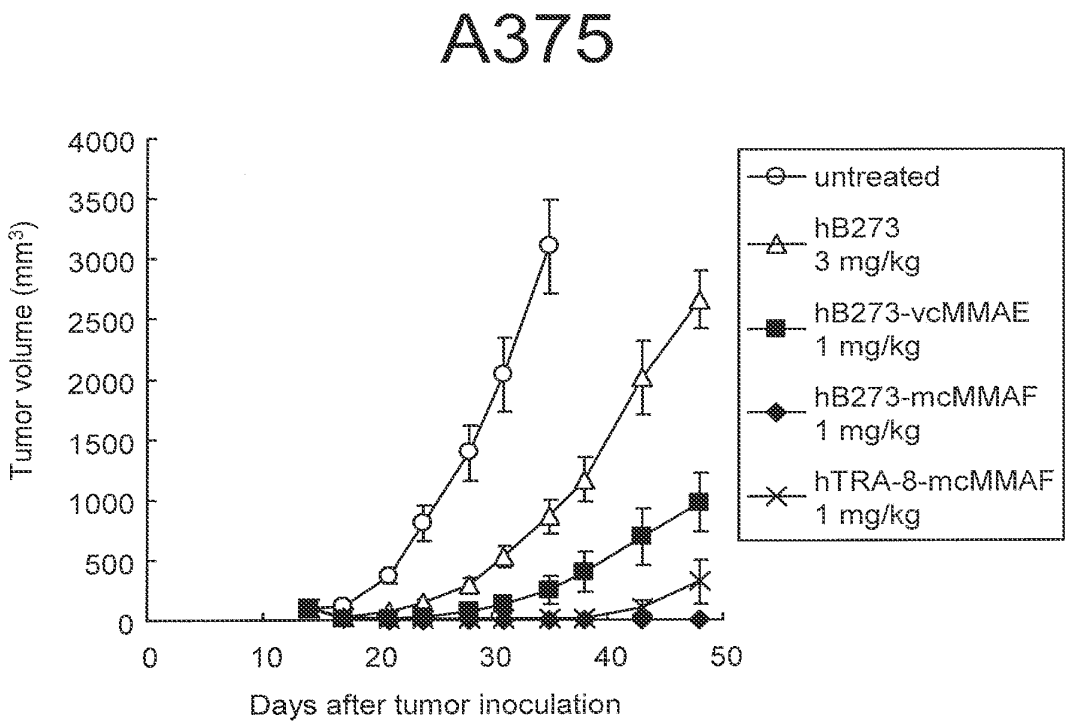

A375
Tumor volume (mm3)
4000
3500
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
Days after tumor inoculation
untreated
hB273
3 mg/kg
hB273-vcMMAE
1 mg/kg
hB273-mcMMAF
1 mg/kg
hTRA-8-mcMMAF
1 mg/kg

FIG. 13

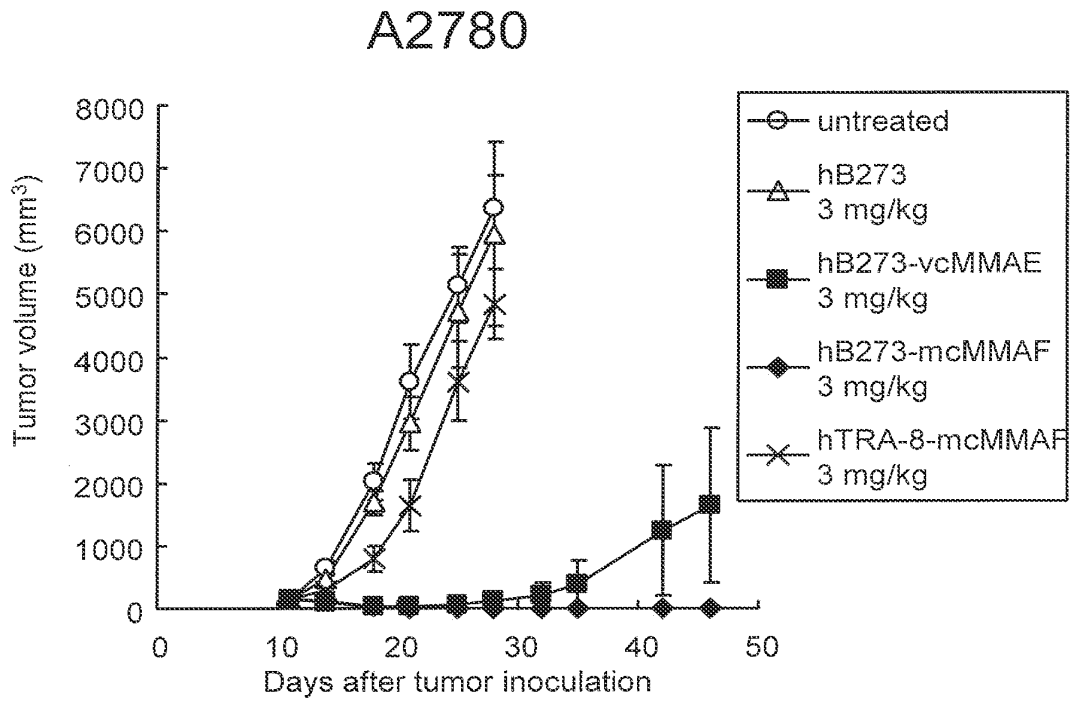

A2780
Tumor volume (mm3)
8000
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
Days after tumor inoculation
untreated
hB273
3 mg/kg
hB273-vcMMAE
3 mg/kg
hB273-mcMMAF
3 mg/kg
hTRA-8-mcMMAF
3 mg/kg

FIG. 14

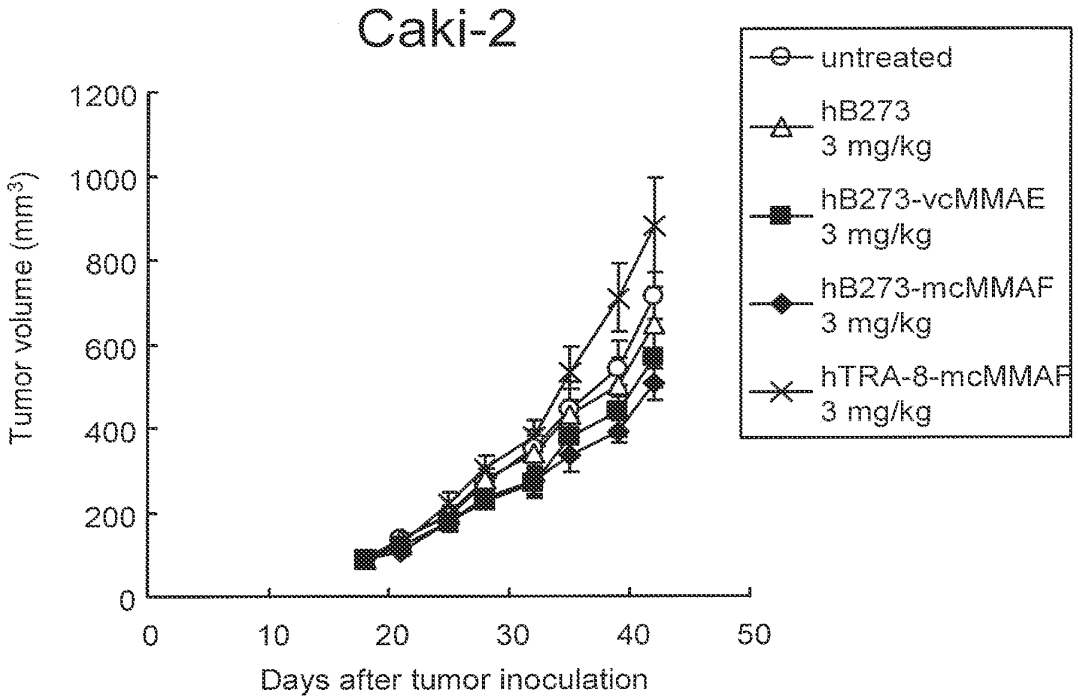

Caki-2
Tumor volume (mm3)
1200
1000
800
600
400
200
0
0
10
20
30
40
50
Days after tumor inoculation
untreated
hB273
3 mg/kg
hB273-vcMMAE
3 mg/kg
hB273-mcMMAF
3 mg/kg
hTRA-8-mcMMAF
3 mg/kg

FIG. 15

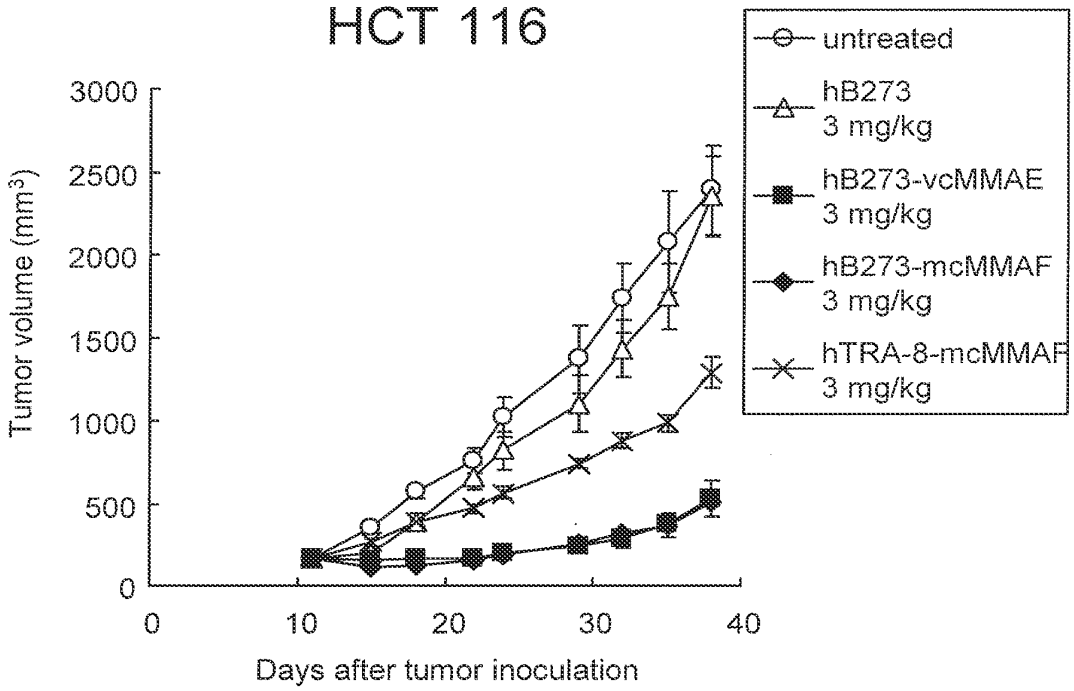

HCT 116
Tumor volume (mm3)
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
Days after tumor inoculation
untreated
hB273
3 mg/kg
hB273-vcMMAE
3 mg/kg
hB273-mcMMAF
3 mg/kg
hTRA-8-mcMMAF
3 mg/kg

FIG. 16

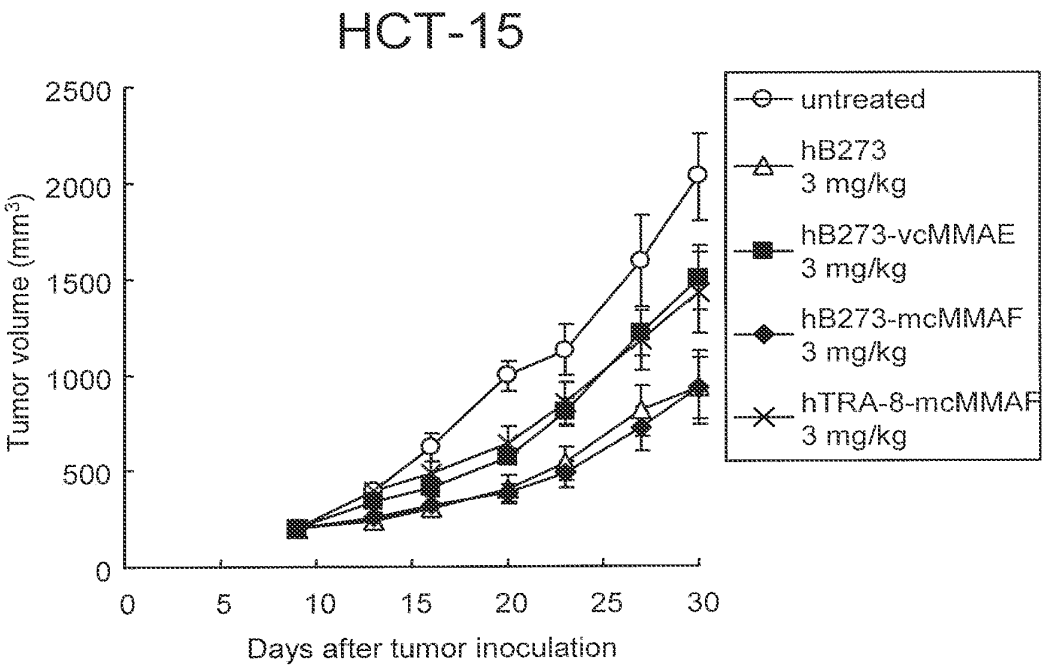

HCT-15
Tumor volume (mm3)
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
Days after tumor inoculation
untreated
hB273
3 mg/kg
hB273-vcMMAE
3 mg/kg
hB273-mcMMAF
3 mg/kg
hTRA-8-mcMMAF
3 mg/kg

FIG. 17

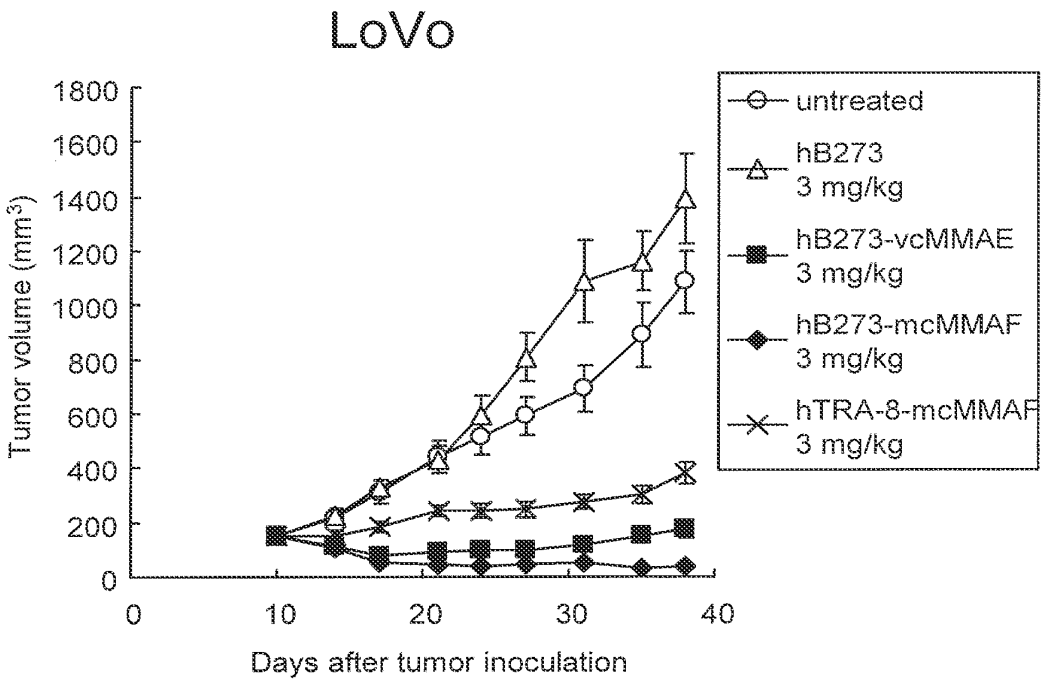

LoVo
Tumor volume (mm3)
1800
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
Days after tumor inoculation
untreated
hB273
3 mg/kg
hB273-vcMMAE
3 mg/kg
hB273-mcMMAF
3 mg/kg
hTRA-8-mcMMAF
3 mg/kg

FIG. 18

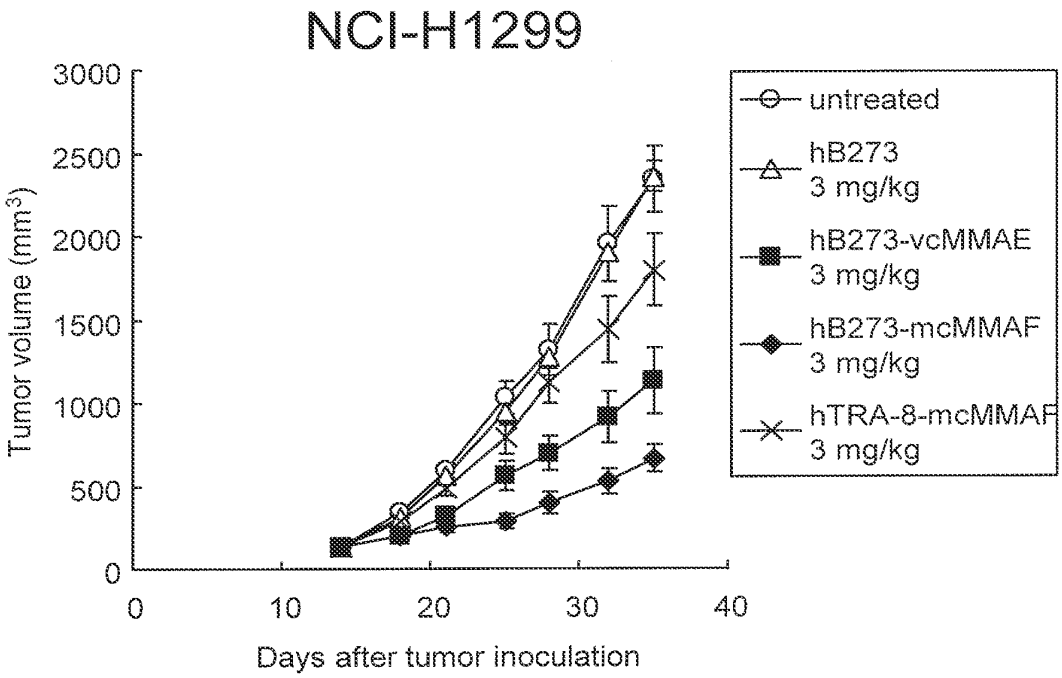

NCI-H1299
Tumor volume (mm3)
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
Days after tumor inoculation
untreated
hB273
3 mg/kg
hB273-vcMMAE
3 mg/kg
hB273-mcMMAF
3 mg/kg
hTRA-8-mcMMAF
3 mg/kg

FIG. 19

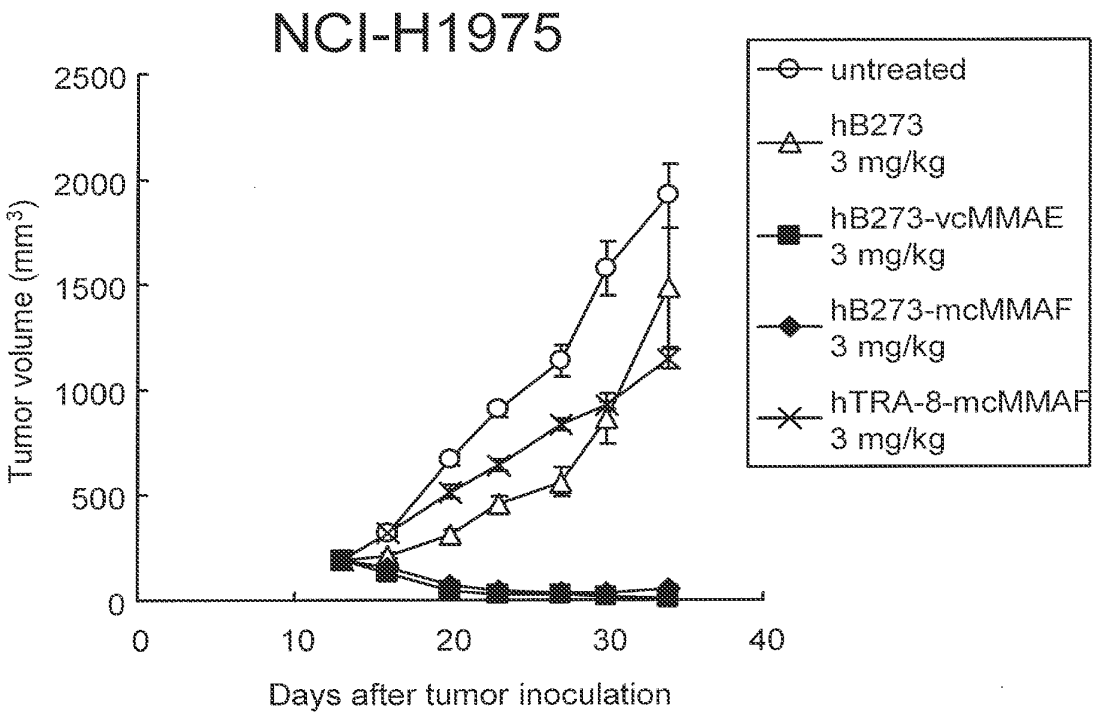

NCI-H1975
Tumor volume (mm3)
2500
2000
1500
1000
500
0
0
10
20
30
40
Days after tumor inoculation
untreated
hB273
3 mg/kg
hB273-vcMMAE
3 mg/kg
hB273-mcMMAF
3 mg/kg
hTRA-8-mcMMAF
3 mg/kg

FIG. 20

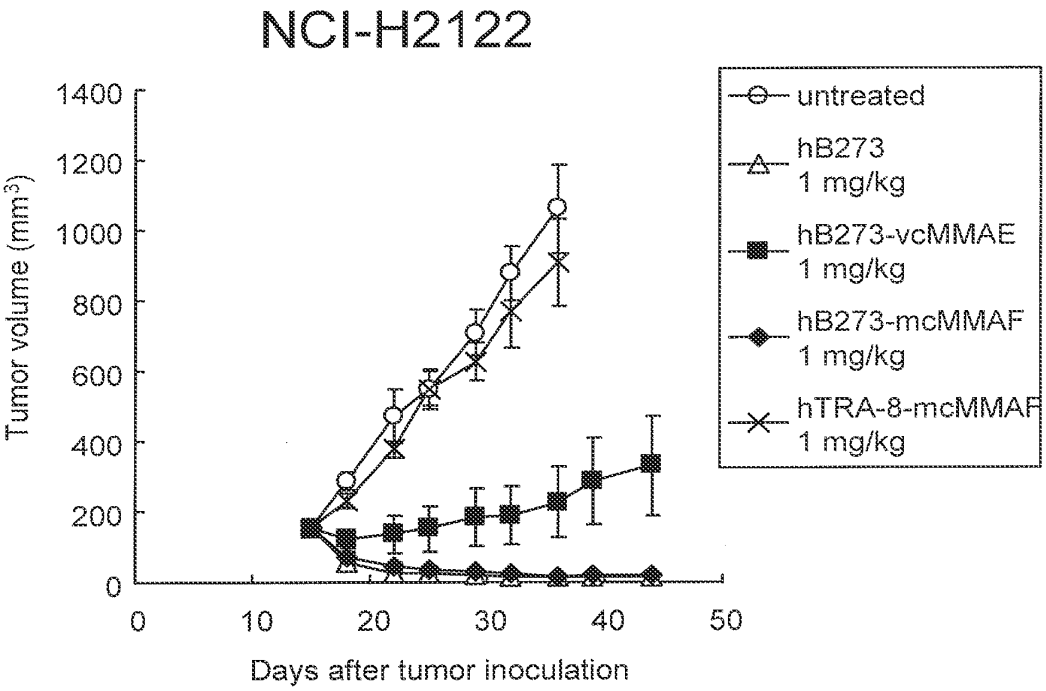

NCI-H2122
Tumor volume (mm3)
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
Days after tumor inoculation
untreated
hB273
1 mg/kg
hB273-vcMMAE
1 mg/kg
hB273-mcMMAF
1 mg/kg
hTRA-8-mcMMAF
1 mg/kg

FIG. 21

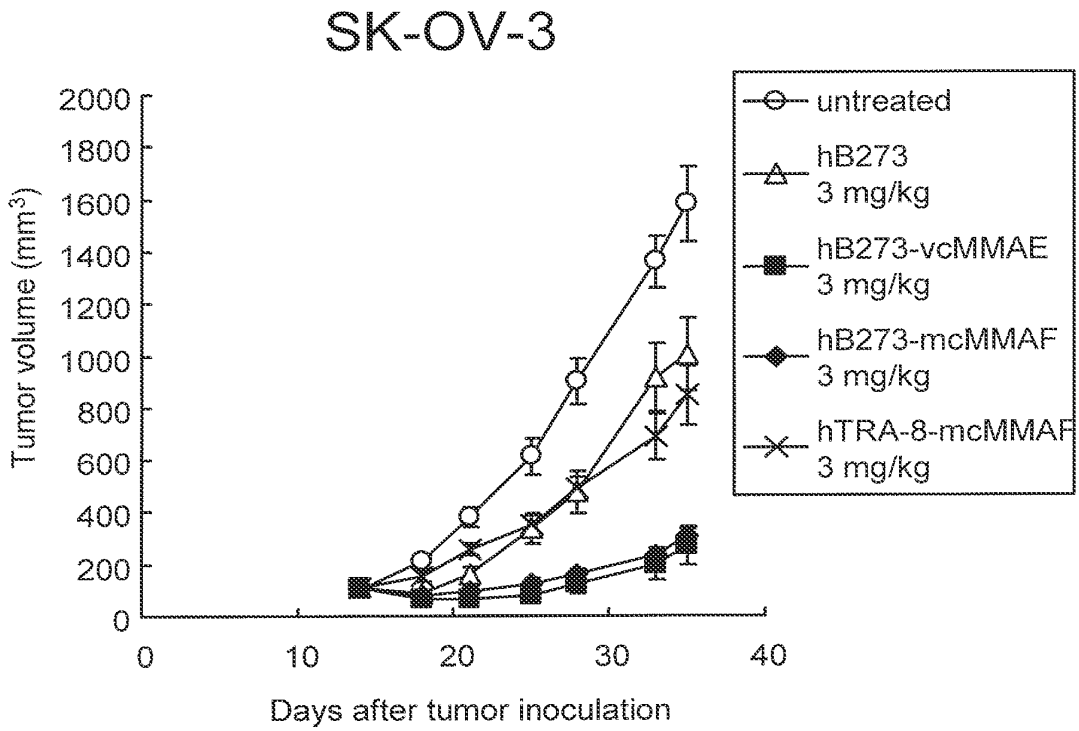

SK-OV-3
Tumor volume (mm3)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
Days after tumor inoculation
untreated
hB273
3 mg/kg
hB273-vcMMAE
3 mg/kg
hB273-mcMMAF
3 mg/kg
hTRA-8-mcMMAF
3 mg/kg

FIG. 22

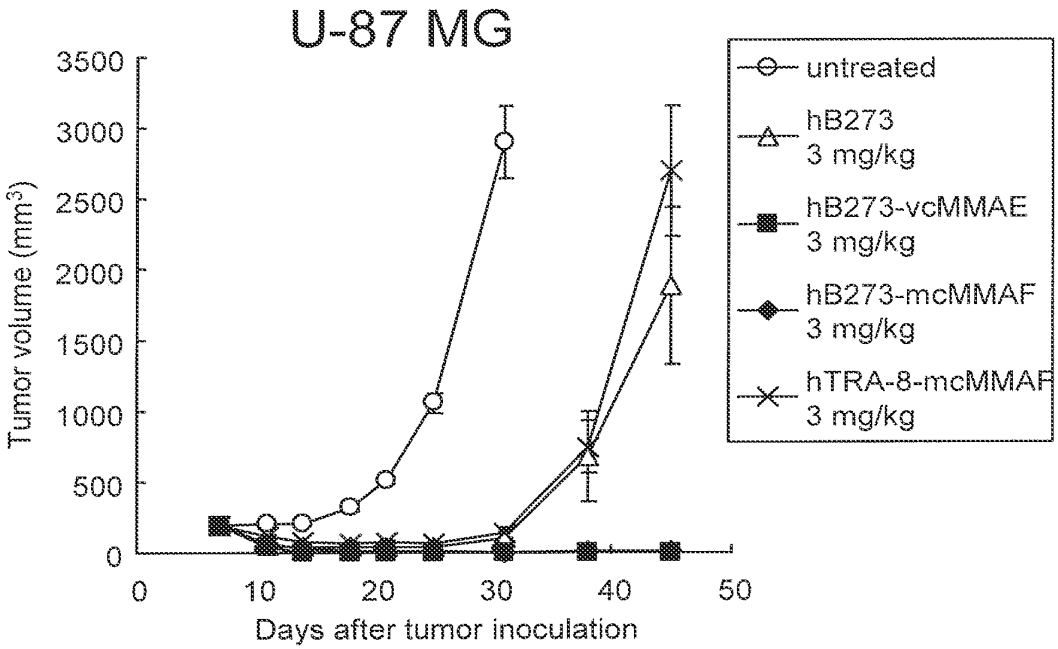

U-87 MG
Tumor volume (mm3)
3500
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
Days after tumor inoculation
untreated
hB273
3 mg/kg
hB273-vcMMAE
3 mg/kg
hB273-mcMMAF
3 mg/kg
hTRA-8-mcMMAF
3 mg/kg

FIG. 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/037861

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/395 (2013.01)
USPC - 424/179.1
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)
IPC(8) - A61K 39/395; A61P 35/00; C07K 7/02, 16/00, 16/28 (2013.01)
USPC - 424/178.1, 179.1; 530/323, 391.7, 391.9

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - A61K 38/00, 47/48715 (2013.01)

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2012/0003247 A1 (DORONINA et al) 05 January 2012 (05.01.2012) entire document	1-22
A	US 2010/0111852 A1 (YOSHIDA) 06 May 2010 (06.05.2010) entire document	1-22
A	US 2003/0166011 A1 (TAMATANI et al) 04 September 2003 (04.09.2003) entire document	1-22
A	US 2007/0190599 A1 (NAKANO et al) 16 August 2007 (16.08.2007) entire document	1-22
A	US 2002/0098527 A1 (SHITARA et al) 25 July 2002 (25.07.2002) entire document	1-22
A	US 2011/0070248 A1 (ICHIKAWA et al) 24 March 2011 (24.03.2011) entire document	1-22
A	US 2009/0047296 A1 (DORONINA et al) 19 February 2009 (19.02.2009) entire document	1-22
A	US 2008/0248053 A1 (DORONINA et al) 09 October 2008 (09.10.2008) entire document	1-22

☐ Further documents are listed in the continuation of Box C. ☐

* 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
20 August 2013	13 SEP 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/037861

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 filed or furnished:

   a. (means)
   - ☐ on paper
   - ☒ in electronic form

   b. (time)
   - ☒ in the international application as filed
   - ☐ together with the international application in electronic form
   - ☐ subsequently to this Authority for the purposes of search

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 in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Specifically, SEQ ID NOs: 9-16 were searched.

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(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 December 2013 (19.12.2013)

WIPO | PCT

(10) International Publication Number
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A61K 47/48 (2006.01) *C07K 16/30* (2006.01)

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14 June 2013 (14.06.2013)

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(71) Applicant: **AMBRX, INC.** [US/US]; 10975 North Torrey Pines Road, Suite 100, La Jolla, CA 92037 (US).

(72) Inventors: **SUN, Ying**; 14972 Avenida Venusto #65, San Diego, CA 92128 (US). **ZOU, Ning**; 9829 Fieldthorn Street, San Diego, CA 92127 (US). **HEWET, Amha**; 1170 La Vida Court, Chula Vista, CA 91915 (US). **PINKSTAFF, Jason, K.**; 2095 Wandering Road, Encinitas, CA 92024 (US). **SRINAGESH, Shailaja**; 3877 Pell Place, Unit 104, San Diego, CA 92130 (US). **BARNETT, Richard, S.**; 306 Belmont Court, San Marcos, CA 92069 (US). **TIAN, Feng**; 8374 Orange Haven Place, San Diego, CA 92129 (US). **PUTNAM, Anna-Maria, A. Hays**; 11522 Cesped Drive, San Diego, CA 92124 (US). **GYMNOPOULOS, Marco**; 3620 3rd Avenue, San Diego, CA 92103 (US). **KNUDSEN, Nick**; 1916 Rohn Road, Escondido, CA 92025 (US). **BECK, Andrew**; 4732 Kansas Street, Apt. 2, San Diego, CA 92116 (US).

(74) Agent: **WALLEN, III, John W.**; 10975 North Torrey Pines Road, Suite 100, La Jolla, California 92037 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, 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, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, 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, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2013/188740 A1

(54) Title: ANTI-PSMA ANTIBODIES CONJUGATED TO NUCLEAR RECEPTOR LIGAND POLYPEPTIDES

(57) Abstract: This invention relates to anti-prostate-specific membrane antigen antibodies (αPSMA) and αPSMA antibody - nuclear receptor ligand (NRL) conjugates comprising at least one non-naturally-encoded amino acid.

Anti-PSMA Antibodies Conjugated to Nuclear Receptor Ligand Polypeptides

FIELD OF THE INVENTION

[01] This invention relates to anti-prostate-specific membrane antigen antibodies (αPSMA) and αPSMA antibody - nuclear receptor ligand (NRL) conjugates comprising at least one non-naturally-encoded amino acid.

BACKGROUND OF THE INVENTION

[02] Prostate cancer is the most commonly diagnosed non-skin related malignancy in males in developed countries. It is estimated that one in six males will be diagnosed with prostate cancer. The diagnosis of prostate cancer has greatly improved following the use of serum-based markers such as the prostate-specific antigen (PSA). In addition, prostate tumor-associated antigens offer targets for tumor imaging, diagnosis, and targeted therapies. The prostate specific membrane antigen (PSMA), a prostate tumor associated marker, is such a target.

[03] PSMA is a glycoprotein highly restricted to prostate secretory epithelial cell membranes. Its expression level has been correlated with tumor aggressiveness. Various immunohistological studies have demonstrated increased PSMA levels in virtually all cases of prostatic carcinoma compared to those levels in benign prostate epithelial cells. Intense PSMA staining is found in all stages of the disease, including prostatic intraepithelial neoplasia, late stage androgen-independent prostate cancer and secondary prostate tumors localized to lymph nodes, bone, soft tissue, and lungs.

[04] PSMA forms a noncovalent homodimer that possesses glutamate carboxypeptidase activity based on its ability to process the neuropeptide N-acetylaspartylglutamate and glutamate-conjugated folate derivatives. Although the precise biological role played by PSMA in disease pathogenesis remains unknown, its overexpression in prostate tumors is well known. It has been suggested that PSMA performs multiple physiological functions related to cell survival and migration.

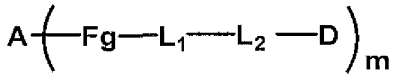
[05] Antibody-based therapeutics have emerged as important components of therapies for an increasing number of human malignancies in such fields as oncology, inflammatory and infectious diseases. In most cases, the basis of the therapeutic function is the high degree of specificity and affinity the antibody-based drug has for its target antigen. Arming monoclonal antibodies with drugs, toxins, or radionuclides is yet another strategy by which

mAbs may induce therapeutic effect. By combining the exquisite targeting specificity of antibody with the tumor killing power of toxic effector molecules, immunoconjugates permit sensitive discrimination between target and normal tissue thereby resulting in fewer side effects than most conventional chemotherapeutic drugs.

[06] Given the physical properties of PSMA and its expression pattern in relation to prostate cancer progression PSMA is an excellent target in the development of antibody-drug conjugates for imaging, diagnostic and therapeutic uses. The first PSMA-specific MAb reported, 7E11, was subsequently developed and commercialized as a diagnostic agent for tumor imaging (ProstaScint, Cytogen, Princeton, N.J.). However, this antibody recognizes an intracellular epitope of PSMA which limits its usefulness as an imaging agent for the detection of PSMA. More recently, MAbs such as J591 that recognize the extracellular portion of PSMA have been identified. Anti-PSMA antibody conjugates that can be utilized for imaging, diagnostic and/or therapeutic uses are therefore needed. The present invention provides such antibody conjugates for use in prostate cancer.

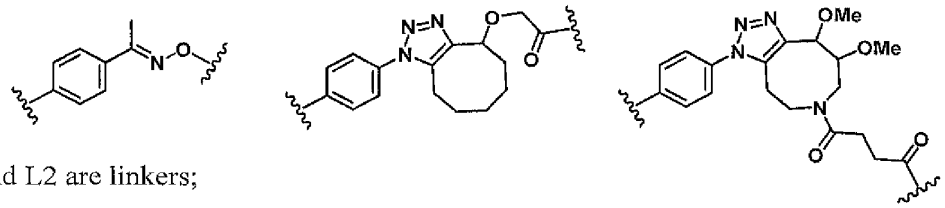
SUMMARY OF THE INVENTION

[07] Provided herein are targeting moiety peptides conjugated to glucocorticoids and glucocorticoid analogs via a linker. In some embodiments, the targeting moiety is an anti-prostate-specific membrane antigen antibody. In some embodiments, the glucocorticoids and glucocorticoid analogs (also referred to as nuclear receptor ligands or NRLs) may include, but are not limited to, FK506, rapamycin, cyclosporine A, dasatinib, dexamethasone, and analolgs. By way of non-limiting example, the present invention includes:



wherein A is an αPSMA antibody;

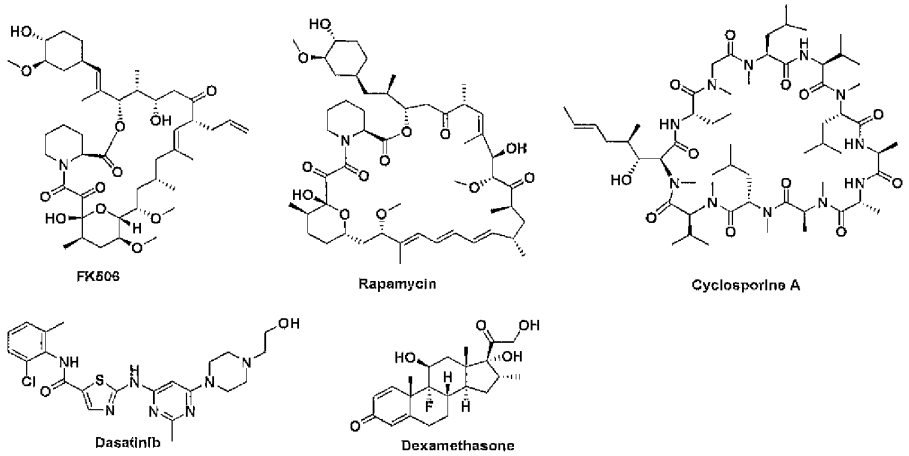
Fg is functional group connecting antibody and linker, which is selected from:



L1 and L2 are linkers;

D is selected from: glucocorticoids; fluorinated 4-azasteroids; fluorinated 4-azasteroids derivatives; antiandrogens; alpha-substituted steroids; carbonylamino-benzimidazole; 17-

hydroxy 4-aza androstan-3-ones; antiandrogenic biphenyls; goserelin; nilutamid; decursin; flutamide; p,p'-DDE; vinclozolin; cyproterone acetate; linuron; kinase inhibitors; staurosporine, saracatinib, fingolimod, and dexamethasone:

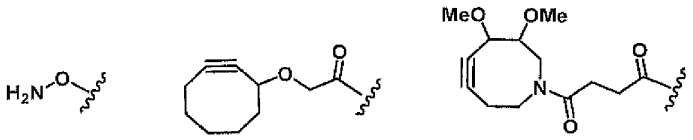


m= 1-4

[08] In some of the embodiments of the present invention,



wherein G is functional group for conjugation to connect antibody and linker, which is selected from:



L1 is selected from –J-W-, -NH-J-W-,

J is selected from: $-C_1$-C_{30} alkylene-, $-C_2$-C_{30} alkenylene- containing 0 to 20 heteroatoms selected from O, S or N; substituted $-C_1$-C_{30} alkylene, substituted $-C_2$-C_{30} alkenylene containing 0 to 20 heteroatoms selected from O, S or N;

W is selected from none, -CO-, -NHCO-,-OCO-

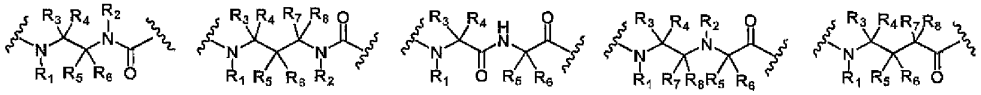
L2 is selected from $-(E-Q)_k-$,

E is an enzyme cleavage substrate: a dipeptide up to hexapeptide with or without para aminobenzyl alcohol, selected from:

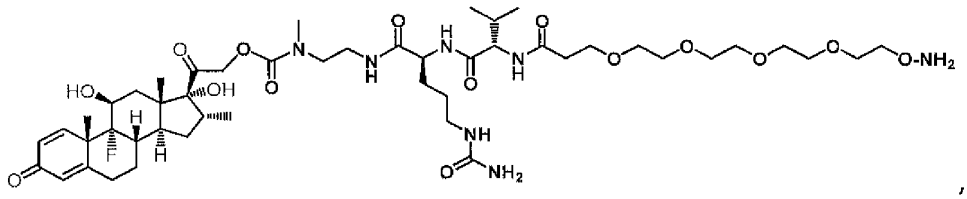
-ValCit-(p-amino-benzylalcohol-CO)k-, -ValLys-(p-amino-benzylalcohol-CO)k-, -ValArg-(p-amino-benzylalcohol-CO)k-, -PheLys-(p-amino-benzylalcohol-CO)k-, -PheArg-(p-amino-benzylalcohol-CO)k-,

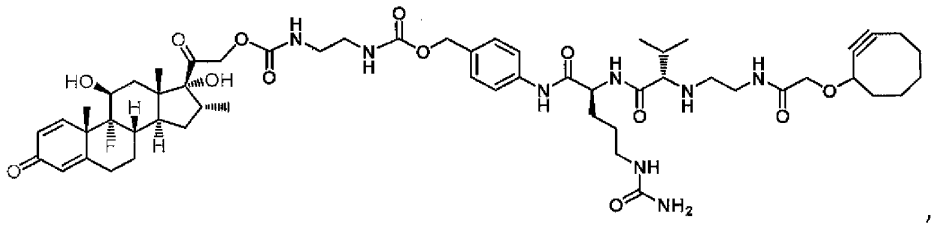
k=0,1;

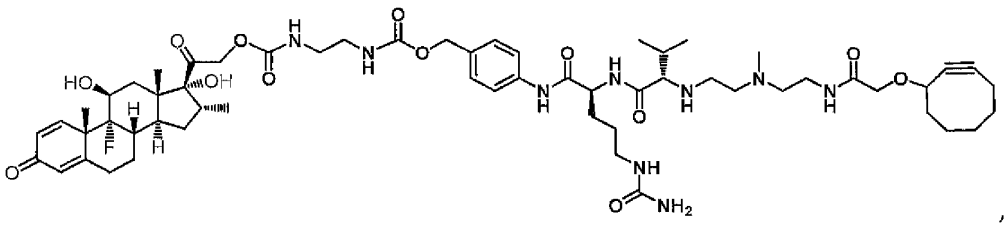
Q is a spacer, selected from:



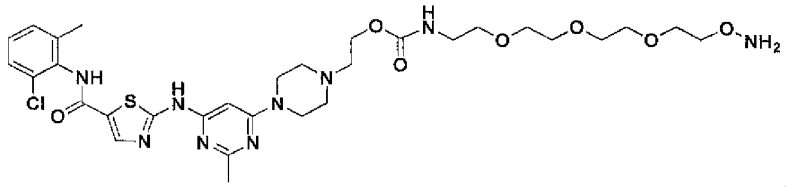
and R1, R2, R3, R4, R5, R6, R7, R8 is independently selected from H, CH3, (C1-C6) alkyl





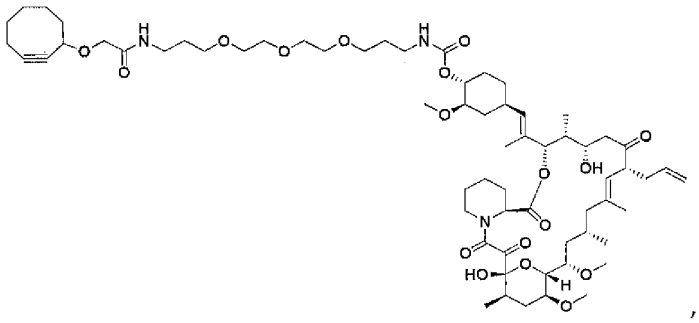


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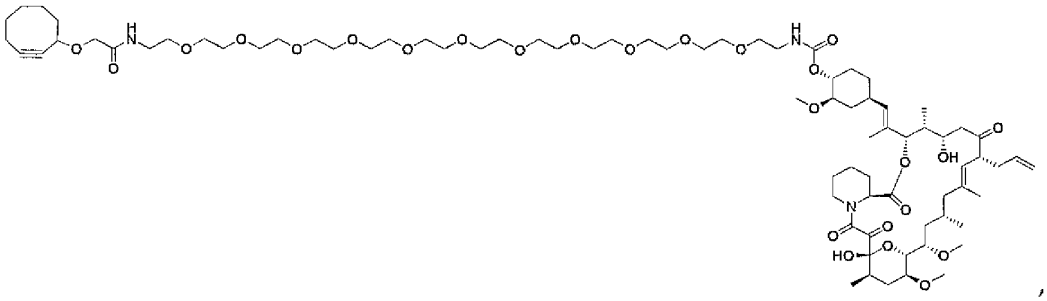


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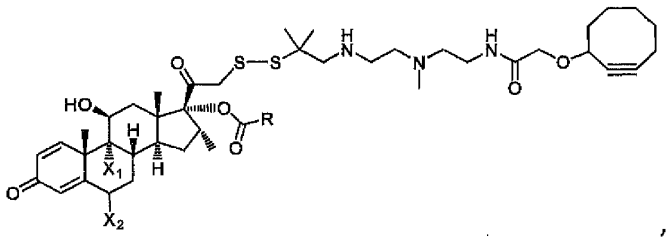


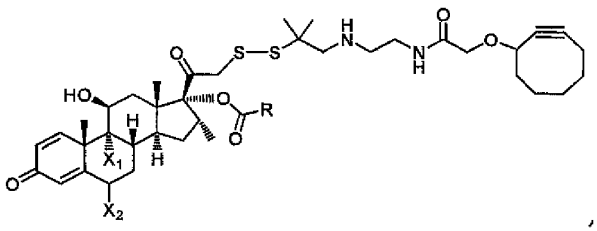
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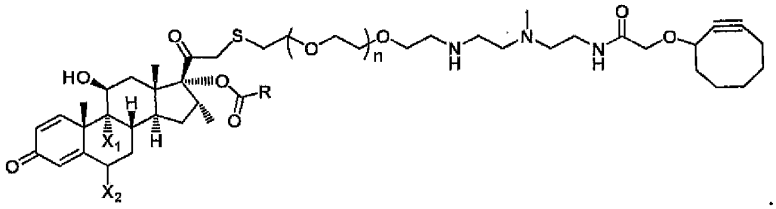


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[09] These conjugates with plural activities are useful for the treatment of a variety of diseases.

[10] The nuclear receptor ligand conjugates of the invention can also be represented by the following formula:

Ab-L-Y

wherein Ab is a targeting moiety peptide, in comes embodiments an αPSMA antibody; Y is a nuclear receptor ligand (NRL); and L is a linking group or a bond.

[11] In some embodiments, Ab is a polypeptide. In specific embodiments, the polypeptide is an antibody. In certain specific embodiments, the antibody is αPSMA. The activity of the antibody at the receptor can be in accordance with any of the teachings set forth herein.

[12] The nuclear receptor ligand (Y) is wholly or partly non-peptidic and acts at a nuclear receptor or nuclear hormone receptor with an activity in accordance with any of the teachings set forth herein. In some embodiments the NRL has an EC50 or IC50 of about 1 mM or less, or 100 μM or less, or 10 μM or less, or 1 μM or less. In some embodiments, the NRL has a molecular weight of up to about 5000 daltons, or up to about 2000 daltons, or up to about 1000 daltons, or up to about 500 daltons. The NRL may act at any of the nuclear hormone receptors described herein or have any of the structures described herein.

[13] In some embodiments, the antibody has an EC50 (or IC50) at the receptor within about 100-fold, or within about 75-fold, or within about 50-fold, or within about 40-, 30-, 25-, 20-, 15-, 10- or 5- fold of the EC50 or IC50 of the NRL at its nuclear receptor. In some embodiments , the antibody has an EC50 (or IC50) at its receptor within about 100-fold, or within about 75-fold, or within about 50-fold, or within about 40-, 30-, 25-, 20-, 15-, 10- or 5-fold of the EC50 or IC50 of the NRL at its nuclear receptor. In some embodiments , the antibody has an EC50 (or IC50) at the receptor within about 100-fold, or within about 75-fold, or within about 50-fold, or within about 40-, 30-, 25-, 20-, 15-, 10- or 5- fold of the EC50 or IC50 of the NRL at its nuclear receptor.

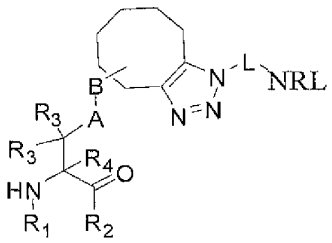
[14] In some aspects of the invention, prodrugs of Ab-L-Y are provided wherein the prodrug comprises a dipeptide prodrug element (A-B) covalently linked to an active site of Ab via an amide linkage. Subsequent removal of the dipeptide under physiological conditions and in the absence of enzymatic activity restores full activity to the Ab-L-Y conjugate.

[15] In some aspects of the invention, pharmaceutical compositions comprising the Ab-L-Y conjugate and a pharmaceutically acceptable carrier are also provided.

[16] In other aspects of the invention, methods are provided for administering a therapeutically effective amount of a Ab-L-Y conjugate described herein for treating a disease or medical condition in a patient. In some embodiments, the disease or medical condition is selected from the group consisting of metabolic syndrome, diabetes, obesity, liver steatosis, and a neurodegenerative disease.

[17] Disclosed herein are embodiments of the present invention for use in the treatment of conditions related to immunology. In some embodiments of the present invention, glucocorticoids with one or more linker(s) are linked to non-natural amino acids, and methods for making such non-natural amino acids and polypeptides.

[18] In some embodiments, a compound is described comprising Formula (XXXI-A):



(XXXI-A)

wherein:

NRL is any nuclear receptor ligand;

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

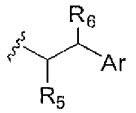
B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, $-S(O)_kN(R')-$, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;

R_1 is H, an amino protecting group, resin, at least one amino acid, polypeptide, or polynucleotide;

R_2 is OH, an ester protecting group, resin, at least one amino acid, polypeptide, or polynucleotide;

R_3 and R_4 are each independently H, halogen, lower alkyl, or substituted lower alkyl, or R_3 and R_4 or two R_3 groups optionally form a cycloalkyl or a heterocycloalkyl;

Z has the structure of:



;

R_5 is H, CO_2H, C_1-C_6alkyl, or thiazole;

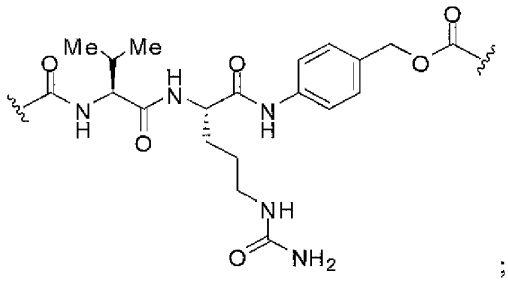
R_6 is OH or H;

Ar is phenyl or pyridine;

R_7 is C_1-C_6alkyl or hydrogen;

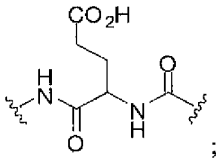
L is a linker selected from the group consisting of –alkylene–, –alkylene–C(O)–, –(alkylene–O)$_n$–alkylene–, –(alkylene–O)$_n$–alkylene–C(O)–, –(alkylene–O)$_n$–$(CH_2)_{n'}$–NHC(O)–$(CH_2)_{n''}$–$C(Me)_2$–S–S–$(CH_2)_{n'''}$–NHC(O)–(alkylene–O)$_{n''''}$–alkylene–, –(alkylene–O)$_n$–alkylene–W–, –alkylene–C(O)–W–, –(alkylene–O)$_n$–alkylene–U–alkylene–C(O)–, and –(alkylene–O)$_n$–alkylene–U–alkylene–;

W has the structure of:



;

U has the structure of:



; and

each n, n', n'', n''' and n'''' are independently integers greater than or equal to one;

or an active metabolite, or a pharmaceutically acceptable prodrug or solvate thereof.

[19] In certain embodiments, a pharmaceutical composition is provided comprising any of the compounds described and a pharmaceutically acceptable carrier, excipient, or binder.

[20] In further or alternative embodiments are methods for detecting the presence of a polypeptide in a patient, the method comprising administering a polypeptide comprising at least one heterocycle-containing non-natural amino acid and the resulting heterocycle-containing non-natural amino acid polypeptide modulates the immunogenicity of the polypeptide relative to the homologous naturally-occurring amino acid polypeptide.

[21] It is to be understood that the methods and compositions described herein are not limited to the particular methodology, protocols, cell lines, constructs, and reagents described herein and as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the

scope of the methods and compositions described herein, which will be limited only by the appended claims.

[22] As used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly indicates otherwise.

[23] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the inventions described herein belong. Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the inventions described herein, the preferred methods, devices and materials are now described.

[24] All publications and patents mentioned herein are incorporated herein by reference in their entirety for the purpose of describing and disclosing, for example, the constructs and methodologies that are described in the publications, which might be used in connection with the presently described inventions. The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors described herein are not entitled to antedate such disclosure by virtue of prior invention or for any other reason.

[25] The term "targeting moiety" as used herein, refers to any molecule or agent that specifically recognizes and binds to a cell-surface receptor, such that the targeting moiety directs the delivery of the conjugate of the present disclosures to a population of cells on which surface the receptor (e.g. PSMA, CD45, CD70, CD74, CD22) is expressed. Targeting moieties include, but are not limited to, antibodies, αPSMA antibodies, or fragments thereof, peptides, hormones, growth factors, cytokines, and any other natural or non-natural ligands, which bind to cell surface receptors (e.g., Epithelial Growth Factor Receptor (EGFR), T-cell receptor (TCR), B-cell receptor (BCR), CD28, Platelet-derived Growth Factor Receptor (PDGF), nicotinic acetylcholine receptor (nAChR), etc.).

[26] As used herein a "linker" is a bond, molecule or group of molecules that binds two separate entities to one another. Linkers may provide for optimal spacing of the two entities or may further supply a labile linkage that allows the two entities to be separated from each other. Labile linkages include photocleavable groups, acid-labile moieties, base-labile moieties, hydrolyzable groups, and enzyme-cleavable groups. The term "linker" in some embodiments refers to any agent or molecule that bridges the conjugate of the present disclosures to the targeting moiety. One of ordinary skill in the art recognizes that sites on the conjugate of the present disclosures, which are not necessary for the function of the conjugate

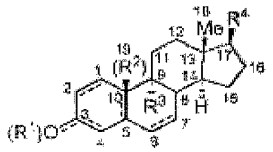
of the present disclosures, are ideal sites for attaching a linker and/or a targeting moiety, provided that the linker and/or targeting moiety, once attached to the conjugate of the present disclosures, do(es) not interfere with the function of the conjugate of the present disclosures, i.e., the ability to stimulate cAMP secretion from cells, to treat diabetes or obesity.

[27] As used herein, "nuclear receptors" (NRs) refers to ligand-activated proteins that regulate gene expression within the cell nucleus, sometimes in concert with other co-activators and co-repressors. Nuclear receptors are a class of proteins found within cells that are responsible for sensing, as a non-limiting example, steroid and thyroid hormones and certain other molecules. In response, these receptors work with other proteins to regulate the expression of specific genes, thereby controlling the development, homeostasis, and metabolism of the organism. Nuclear receptors have the ability to directly bind to DNA and regulate the expression of adjacent genes, hence these receptors are classified as transcription factors. The regulation of gene expression by nuclear receptors generally only happens when a ligand — a molecule that affects the receptor's behavior — is present. More specifically, ligand binding to a nuclear receptor results in a conformational change in the receptor, which, in turn, activates the receptor, resulting in modulation, up-regulation or down-regulation, of gene expression. A unique property of nuclear receptors that differentiates them from other classes of receptors is their ability to directly interact with and control the expression of genomic DNA. As a consequence, nuclear receptors play key roles in both embryonic development and adult homeostasis. Some nuclear receptors may be classified according to either mechanism or homology.

[28] As used herein, "NR ligand", "nuclear receptor ligand", and "NRL" refers to a molecule that interacts with a nuclear receptor, and may comprise a hydrophobic or lipophilic moiety and that has biological activity (either agonist or antagonist) at one or more nuclear receptor (NR). The NRL may be wholly or partly non-peptidic. In some embodiments, the NRL is an agonist that binds to and activates the NR. In other embodiments, the NRL is an antagonist. In some embodiments, the NRL is an antagonist that acts by competing with or blocking binding of native or non-native ligand to the active site. In some embodiments, the NRL is an antiandrogenic compound. In certain embodiments, the antiandrogenic NRL is selected from the group consisting of antiandrogens; alpha-substituted steroids; carbonylamino-benzimidazole; 17-hydroxy 4-aza androstan-3-ones; antiandrogenic biphenyls; goserelin; nilutamid; decursin; flutamide; p,p'-DDE; vinclozolin; cyproterone acetate; linuron. In certain embodiments, the antiandrogenic NRL is selected from the group

consisting of fluorinated 4-azasteroids; fluorinated 4-azasteroids derivatives; antiandrogens; alpha-substituted steroids; carbonylamino-benzimidazole; 17-hydroxy 4-aza androstan-3-ones; antiandrogenic biphenyls; goserelin; nilutamid; decursin; flutamide; p,p'-DDE; vinclozolin; cyproterone acetate; and linuron. In other embodiments, the NRL is an antagonist that acts by binding to the active site or an allosteric site and preventing activation of, or de-activating, the NR.

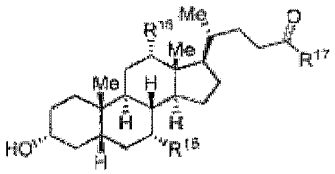
[29] As used herein, "steroids and derivatives thereof refers to compounds, either naturally occurring or synthesized, having a structure of Formula A:



wherein R^1 and R^2, when present, are independently moieties that permit or promote agonist or antagonist activity upon binding of the compound of Formula A to a nuclear hormone receptor; R^3 and R^4 are independently moieties that permit or promote agonist or antagonist activity upon binding of the compound of Formula A to a nuclear hormone receptor; and each dashed line represents an optional double bond. Formula A may further comprise one or more substituents at one or more of positions 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 14, 15, 16, and 17. Contemplated optional substituents include, but are not limited to, OH, NH_2, ketone, and C_1-C_{18} alkyl groups. Specific, nonlimiting examples of steroids and derivatives thereof include cholesterol, cholic acid estradiol, testosterone, and hydrocortisone.

[30] As used herein, "anti-androgen" refers to a group of hormone receptor antagonist compounds that are capable of preventing or inhibiting the biologic effects of androgens, male sex hormones, on normally responsive tissues in the body. An "anti-androgen" can be any pharmaceutically acceptable active agent that inhibits competitively the effect of androgens at their target site of action. Examples of antiandrogenic hormones that can be used in the present invention include, but are not limited to, coumarins, hydroxyflutamide, nilutamide, cyproterone acetate, ketoconazole, finasteride, bicalutamide, novaldex, nilandron, flutamide, progesterone, spironolactone, fluconazole, dutasteride, harman, norharman, harmine, harmaline, tetrahydroharmine, harmol, harmalol, ethyl harmol, n-butyl harmol and other beta-carboline derivatives or combinations thereof.

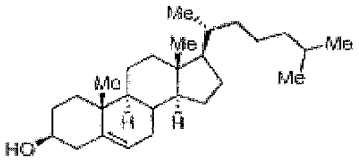
[31] As used herein, "bile acids and derivatives thereof refers to compounds, either naturally occurring or synthesized, of Formula M:



Formula M

wherein each of R^{15}, R^{16}, and R^{17} are independently moieties that permit or promote agonist or antagonist activity upon binding of the compound of Formula M to a nuclear hormone receptor. In some embodiments, each of R^{15} and R^{16} are independently hydrogen, (C_0-C_8 alkyl)halo, C_1-C_{18} alkyl, C_2-C_{18} alkenyl, C_2-C_{18} alkynyl, heteroalkyl, or (C_0-C_8 alkyl)OH; and R^{17} is OH, (C_0-C_8 alkyl)NH(C_1-C_4 alkyl)SO_3H, or (C_0-C_8 alkyl)NH(C_1-C_4 alkyl)COOH. Formula M may further comprise one or more substituents at one or more of positions 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 14, 15, 16, and 17. Nonlimiting examples of bile acids include cholic acid, deoxycholic acid, lithocholic acid, chenodeoxycholic acid, taurocolic acid, and glycocholic acid.

[32] As used herein, "cholesterol and derivatives thereof refers to compounds, either naturally occurring or synthesized, comprising a structure similar to that of cholesterol, as shown below:

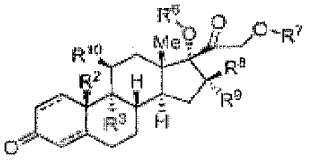


Derivatives of cholesterol can include oxysterols, such as hydroxycholesterol, 24(S)-hydroxycholesterol, 27-hydroxycholesterol, and cholestenoic acid.

[33] As used herein, "fatty acids and derivatives thereof" refers to carboxylic acids comprising a long unbranched C_1 to C_{28} alkyl or C_2 to C_{28} alkenyl moiety and can optionally comprise one or more halo substituents and/or optionally comprise one or more substituents other than halo. In some embodiments, the long unbranched alkyl or alkenyl moiety can be wholly halo substituted (e.g., all hydrogens replaced with halo atoms). A short chain fatty acid comprises 1-5 carbon atoms. A medium chain fatty acid comprises 6-12 carbon. A long chain fatty acid comprises 13-22 carbon atoms. A very long chain fatty acid comprises 23-28 carbon atoms. Specific, nonlimiting examples of fatty acids include formic acid, acetic acid, n-caproic acid, heptanoic acid, caprylic acid, nonanoic acid, capric acid, undecanoic acid, lauric acid, tridecanoic acid, myristic acid, pentadeconoic acid, palmitic acid, heptadecanoic acid, stearic acid, nonadecanoic acid, arachidic acid, heneicosanoic acid, behenic acid,

tricosanoic acid, mead acid, myristoleic acid, palmitoleic acid, sapienic acid, oleic acid, linoleic acid, a-linolenic acid, elaidic acid, petroselinic acid, arachidonic acid, dihydroxyeicosatetraenoic acid (DiHETE), octadecynoic acid, eicosatriynoic acid, eicosadienoic acid, eicosatrienoic acid, eicosapentaenoic acid, erucic acid, dihomolinolenic acid, docosatrienoic acid, docosapentaenoic acid, docosahexaenoic acid, and adrenic acid.

[34] As used herein, "Cortisol and derivatives thereof refers to compounds, either naturally occurring or synthesized, of Formula C:



wherein R^2, R^3, R^6, R^7, R^8, R^9, and R^{10} are each independently moieties that permit or promote agonist or antagonist activity upon the binding of the compound of Formula C to a nuclear hormone receptor; and each dash respresents an optional double bond. In some embodiments, the structure of Formula C is substituted with one or more substituents at one or more positions of the tetracyclic ring, such as, for example, positions 1, 2, 4, 5, 6, 7, 8, 11, 12, 14, and 15. Specific, nonlimiting examples of derivatives of Cortisol and derivatives thereof include Cortisol, cortisone acetate, beclometasone, prednisone, prednisolone, methylprednisolone, betamethasone, trimcinolone, and dexamethasone.

[35] As used herein, "linking group" is a molecule or group of molecules that binds two separate entities to one another. Linking groups may provide for optimal spacing of the two entities or may further supply a labile linkage that allows the two entities to be separated from each other. Labile linkages include hydrolyzable groups, photocleavable groups, acid-labile moieties, base-labile moieties and enzyme cleavable groups.

[36] As used herein, a "dipeptide" is the result of the linkage of an a-amino acid or a-hydroxyl acid to another amino acid, through a peptide bond.

[37] As used herein the term "chemical cleavage" absent any further designation encompasses a non-enzymatic reaction that results in the breakage of a covalent chemical bond.

[38] The term "about" as used herein means greater or lesser than the value or range of values stated by 10 percent, but is not intended to designate any value or range of values to only this broader definition. Each value or range of values preceded by the term "about" is also intended to encompass the embodiment of the stated absolute value or range of values.

[39] The terms "aldol-based linkage" or "mixed aldol-based linkage" refers to the acid- or base-catalyzed condensation of one carbonyl compound with the enolate/enol of another carbonyl compound, which may or may not be the same, to generate a β-hydroxy carbonyl compound—an aldol.

[40] The term "affinity label," as used herein, refers to a label which reversibly or irreversibly binds another molecule, either to modify it, destroy it, or form a compound with it. By way of example, affinity labels include enzymes and their substrates, or antibodies and their antigens.

[41] The terms "alkoxy," "alkylamino" and "alkylthio" (or thioalkoxy) are used in their conventional sense, and refer to those alkyl groups linked to molecules via an oxygen atom, an amino group, or a sulfur atom, respectively.

[42] The term "alkyl," by itself or as part of another molecule means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent radicals, having the number of carbon atoms designated (*i.e.* C_1-C_{10} means one to ten carbons). Examples of saturated hydrocarbon radicals include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, homologs and isomers of, for example, n-pentyl, n-hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butynyl, and the higher homologs and isomers. The term "alkyl," unless otherwise noted, is also meant to include those derivatives of alkyl defined in more detail herein, such as "heteroalkyl", "haloalkyl" and "homoalkyl".

[43] The term "alkylene" by itself or as part of another molecule means a divalent radical derived from an alkane, as exemplified, by $(–CH_2–)_n$, wherein n may be 1 to about 24. By way of example only, such groups include, but are not limited to, groups having 10 or fewer carbon atoms such as the structures $–CH_2CH_2–$ and $–CH_2CH_2CH_2CH_2–$. A "lower alkyl" or "lower alkylene" is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms. The term "alkylene," unless otherwise noted, is also meant to include those groups described herein as "heteroalkylene."

[44] The term "amino acid" refers to naturally occurring and non-natural amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the

naturally occurring amino acids. Naturally encoded amino acids are the 20 common amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) and pyrolysine and selenocysteine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, by way of example only, an α-carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group. Such analogs may have modified R groups (by way of example, norleucine) or may have modified peptide backbones while still retaining the same basic chemical structure as a naturally occurring amino acid. Non-limiting examples of amino acid analogs include homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium.

[45] Amino acids may be referred to herein by either their name, their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Additionally, nucleotides, may be referred to by their commonly accepted single-letter codes.

[46] An "amino terminus modification group" refers to any molecule that can be attached to a terminal amine group. By way of example, such terminal amine groups may be at the end of polymeric molecules, wherein such polymeric molecules include, but are not limited to, polypeptides, polynucleotides, and polysaccharides. Terminus modification groups include but are not limited to, various water soluble polymers, peptides or proteins. By way of example only, terminus modification groups include polyethylene glycol or serum albumin. Terminus modification groups may be used to modify therapeutic characteristics of the polymeric molecule, including but not limited to increasing the serum half-life of peptides.

[47] The term "antigen-binding fragment", as used herein, refers to one or more fragments of an antibody that retain the ability to bind to an antigen. It has been shown that the antigen-binding function of an antibody can be performed by fragments of an intact antibody. Examples of binding fragments encompassed within the term "antigen-binding fragment" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V.sub.L, V.sub.H, C.sub.L and C.sub.H1 domains; (ii) a F(ab').sub.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 V.sub.H and C.sub.H1 domains; (iv) a Fv fragment consisting of the V.sub.L and V.sub.H domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a V.sub.H domain; (vi) an

isolated complementarity determining region (CDR), e.g., V.sub.H CDR3 comprising or not additional sequence (linker, framework region(s) etc.) and (v) a combination of two to six isolated CDRs comprising or not additional sequence (linker, framework region(s) etc.). Furthermore, although the two domains of the Fv fragment, V.sub.L and V.sub.H, 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 polypeptide chain in which the V.sub.L and V.sub.H 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 fragment" of an antibody. Furthermore, the antigen-binding fragments include binding-domain immunoglobulin fusion proteins comprising (i) a binding domain polypeptide (such as a heavy chain variable region, a light chain variable region, or a heavy chain variable region fused to a light chain variable region via a linker peptide) that is fused to an immunoglobulin hinge region polypeptide, (ii) an immunoglobulin heavy chain CH2 constant region fused to the hinge region, and (iii) an immunoglobulin heavy chain CH3 constant region fused to the CH2 constant region. The hinge region may be modified by replacing one or more cysteine residues with serine residues so as to prevent dimerization. Such binding-domain immunoglobulin fusion proteins are further disclosed in US 2003/0118592 and US 2003/0133939. 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.

[48] A typical antigen binding site is comprised of the variable regions formed by the pairing of a light chain immunoglobulin and a heavy chain immunoglobulin. The structure of the antibody variable regions is very consistent and exhibits very similar structures. These variable regions are typically comprised of relatively homologous framework regions (FR) interspaced with three hypervariable regions termed Complementarity Determining Regions (CDRs). The overall binding activity of the antigen binding fragment is often dictated by the sequence of the CDRs. The FRs often play a role in the proper positioning and alignment in three dimensions of the CDRs for optimal antigen binding.

[49] In fact, because CDR sequences are responsible for most antibody-antigen interactions, it is possible to express recombinant antibodies that shows the properties of specific naturally occurring antibodies by constructing expression vectors that include CDR sequences from the specific naturally occurring antibody grafted onto framework sequences

from a different antibody with different properties (see, e.g., Riechmann, L. et al., 1998, Nature 332:323-327; Jones, P. et al., 1986, Nature 321:522-525; and Queen, C. et al., 1989, Proc. Natl. Acad. See. U.S.A. 86:10029-10033). Such framework sequences can be obtained from public DNA databases that include germline antibody gene sequences. These germline sequences will differ from mature antibody gene sequences because they will not include completely assembled variable genes, which are formed by V(D)J joining during B cell maturation. Germline gene sequences will also differ from the sequences of a high affinity secondary repertoire antibody which contains mutations throughout the variable gene but typically clustered in the CDRs. For example, somatic mutations are relatively infrequent in the amino terminal portion of framework region 1 and in the carboxy-terminal portion of framework region 4. Furthermore, many somatic mutations do not significantly alter the binding properties of the antibody. For this reason, it is not necessary to obtain the entire DNA sequence of a particular antibody in order to recreate an intact recombinant antibody having binding properties similar to those of the original antibody. Partial heavy and light chain sequence spanning the CDR regions is typically sufficient for this purpose. The partial sequence is used to determine which germline variable and joining gene segments contributed to the recombined antibody variable genes. The germline sequence is then used to fill in missing portions of the variable regions. Heavy and light chain leader sequences are cleaved during protein maturation and do not contribute to the properties of the final antibody. To add missing sequences, cloned cDNA sequences can be combined with synthetic oligonucleotides by ligation or PCR amplification. Alternatively, the entire variable region can be synthesized to create an entirely synthetic variable region clone. This process has certain advantages such as elimination or inclusion of particular restriction sites, or optimization of particular codons.

[50] By "antibody" herein is meant a protein consisting of one or more polypeptides substantially encoded by all or part of the antibody genes. The immunoglobulin genes include, but are not limited to, the kappa, lambda, alpha, gamma (IgG1, IgG2, IgG3, and IgG4), delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Antibody herein is meant to include full-length antibodies and antibody fragments, and include antibodies that exist naturally in any organism or are engineered (e.g. are variants).

[51] The term "antibody" refers to a substantially intact antibody molecule. As used herein, the phrase "antibody fragment" refers to a functional fragment of an antibody that is capable of binding to a surface marker of the present invention. Suitable antibody fragments

for practicing the present invention include a complementarity-determining region (CDR) of an immunoglobulin light chain (referred to herein as "light chain"), a complementarity-determining region of an immunoglobulin heavy chain (referred to herein as "heavy chain"), a variable region of a light chain, a variable region of a heavy chain, a light chain, a heavy chain, an Fd fragment, and antibody fragments comprising essentially whole variable regions of both light and heavy chains such as an Fv, a single chain Fv, an Fab, an Fab', and an $F(ab')_2$. Functional antibody fragments comprising whole or essentially whole variable regions of both light and heavy chains are defined as follows:

(i) Fv, defined as a genetically engineered fragment consisting of the variable region of the light chain and the variable region of the heavy chain expressed as two chains;

(ii) single chain Fv ("scFv"), a genetically engineered single chain molecule including the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker.

(iii) Fab, a fragment of an antibody molecule containing a monovalent antigen-binding portion of an antibody molecule which can be obtained by treating whole antibody with the enzyme papain to yield the intact light chain and the Fd fragment of the heavy chain which consists of the variable and C.sub.H1 domains thereof;

(iv) Fab', a fragment of an antibody molecule containing a monovalent antigen-binding portion of an antibody molecule which can be obtained by treating whole antibody with the enzyme pepsin, followed by reduction (two Fab' fragments are obtained
per antibody molecule); and

(v) $F(ab')_2$, a fragment of an antibody molecule containing a monovalent antigen-binding portion of an antibody molecule which can be obtained by treating whole antibody with the enzyme pepsin (i.e., a dimer of Fab' fragments held together by two disulfide bonds).

[52] Methods of generating antibodies (i.e., monoclonal and polyclonal) are well known in the art. Antibodies may be generated via any one of several methods known in the art, which methods can employ induction of in-vivo production of antibody molecules, screening of immunoglobulin libraries (Orlandi D. R. et al., 1989. Proc. Natl. Acad. Sci. U.S.A. 86:3833-3837; Winter G. et al., 1991. Nature 349:293-299) or generation of monoclonal antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the Epstein-Barr virus (EBV)-hybridoma technique (Kohler G. et al., 1975. Nature 256:495-497; Kozbor D. et al., 1985. J. Immunol. Methods 81:31-42; Cote R J. et al., 1983. Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030; Cole S P. et al., 1984. Mol. Cell. Biol. 62:109-120).

[53] In cases where target antigens are too small to elicit an adequate immunogenic response when generating antibodies in-vivo, such antigens (haptens) can be coupled to antigenically neutral carriers such as keyhole limpet hemocyanin (KLH) or serum albumin [e.g., bovine serum albumin (BSA)] carriers (see, for example, U.S. Pat. Nos. 5,189,178 and 5,239,078]. Coupling a hapten to a carrier can be effected using methods well known in the art. For example, direct coupling to amino groups can be effected and optionally followed by reduction of the imino linkage formed. Alternatively, the carrier can be coupled using condensing agents such as dicyclohexyl carbodiimide or other carbodiimide dehydrating agents. Linker compounds can also be used to effect the coupling; both homobifunctional and heterobifunctional linkers are available from Pierce Chemical Company, Rockford, Ill. The resulting immunogenic complex can then be injected into suitable mammalian subjects such as mice, rabbits, and the like. Suitable protocols involve repeated injection of the immunogen in the presence of adjuvants according to a schedule which boosts production of antibodies in the serum. The titers of the immune serum can readily be measured using immunoassay procedures which are well known in the art. The antisera obtained can be used directly or monoclonal antibodies may be obtained as described hereinabove. Antibody fragments can be obtained using methods well known in the art [(see, for example, Harlow and Lane, "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory, New York, (1988)]. For example, antibody fragments according to the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in E. coli or mammalian cells (e.g., Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment.

[54] Alternatively, antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. As described hereinabove, an $(Fab')_2$ antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages to produce 3.5S Fab' monovalent fragments. Alternatively, enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. Ample guidance for practicing such methods is provided in the literature of the art (for example, refer to: Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647; Porter, R R., 1959. Biochem. J. 73:119-126). Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

[55] As described hereinabove, an Fv is composed of paired heavy chain variable and light chain variable domains. This association may be noncovalent (see, for example, Inbar et al., 1972. Proc. Natl. Acad. Sci. USA. 69:2659-62). Alternatively, as described hereinabove the variable domains can be linked to generate a single chain Fv by an intermolecular disulfide bond, or alternately, such chains may be cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv is a single chain Fv. Single chain Fv's are prepared by constructing a structural gene comprising DNA sequences encoding the heavy chain variable and light chain variable domains connected by an oligonucleotide encoding a peptide linker. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as E. coli. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two variable domains. Ample guidance forproducing single chain Fv's is provided in the literature of the art (for example, refer to: Whitlow and Filpula, 1991. Methods 2:97-105; Bird et al., 1988. Science 242:423-426; Pack et al., 1993. Bio/Technology 11:1271-77; and Ladner et al., U.S. Pat. No. 4,946,778). Isolated complementarity determining region peptides can be obtained by constructing genes encoding the complementarity determining region of an antibody of interest. Such genes may be prepared, for example, by RT-PCR of mRNA of an antibody-producing cell. Ample guidance for practicing such methods is provided in the literature of the art (for example, refer to Larrick and Fry, 1991. Methods 2:106-10).

[56] It will be appreciated that for human therapy or diagnostics, humanized antibodies are preferably used. Humanized forms of non human (e.g., murine) antibodies are genetically engineered chimeric antibodies or antibody fragments having-preferably minimal-portions derived from non human antibodies. Humanized antibodies include antibodies in which complementary determining regions of a human antibody (recipient antibody) are replaced by residues from a complementarity determining region of a non human species (donor antibody) such as mouse, rat or rabbit having the desired functionality. In some instances, Fv framework residues of the human antibody are replaced by corresponding non human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported complementarity determining region or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two,variable domains, in which all or substantially all of the complementarity determining regions correspond to those of a non human antibody and all, or substantially all, of the framework regions correspond to those of a relevant human consensus sequence. Humanized antibodies optimally also include at least a portion of an antibody constant region, such as an Fc region, typically derived from a human antibody (see, for example, Jones et al., 1986. Nature 321:522-525; Riechmann et al., 1988. Nature 332:323-329; and Presta, 1992. Curr. Op. Struct. Biol. 2:593-596).

[57] Methods for humanizing non human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non human. These non human amino acid residues are often referred to as imported residues which are typically taken from an imported variable domain. Humanization can be essentially performed as described (see, for example: Jones et al., 1986. Nature 321:522-525; Riechmann et al., 1988. Nature 332:323-327; Verhoeyen et al., 1988. Science 239:1534-1536; U.S. Pat. No. 4,816,567) by substituting human complementarity determining regions with corresponding rodent complementarity determining regions. Accordingly, such humanized antibodies are chimeric antibodies, wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non human species. In practice, humanized antibodies may be typically human antibodies in which some complementarity determining region residues and possibly some framework residues are substituted by residues from analogous sites in rodent antibodies.

[58] Human antibodies can also be produced using various techniques known in the art, including phage display libraries [see, for example, Hoogenboom and Winter, 1991. J. Mol.

Biol. 227:381; Marks et al., 1991. J. Mol. Biol. 222:581; Cole et al., "Monoclonal Antibodies and Cancer Therapy", Alan R. Liss, pp. 77 (1985); Boerner et al., 1991. J. Immunol. 147:86-95). Humanized antibodies can also be made by introducing sequences encoding human immunoglobulin loci into transgenic animals, e.g., Into mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon antigenic challenge, human antibody production is observed in such animals which closely resembles that seen in humans in all respects, including gene rearrangement, chain assembly, and antibody repertoire. Ample guidance for practicing such an approach is provided in the literature of the art (for example, refer to; U.S. Pat. Nos. 5,545,807, 5,545,806, 5,569,825, 5,625,126, 5,633,425, and 5,661,016; Marks et al., 1992. Bio/Technology 10:779-783; Lonberg et al., 1994. Nature 368:856-859; Morrison, 1994. Nature 368:812-13; Fishwild et al., 1996. Nature Biotechnology 14:845-51; Neuberger, 1996. Nature Biotechnology 14:826; Lonberg and Huszar, 1995. Intern. Rev. Immunol. 13:65-93). Once antibodies are obtained, they may be tested for activity, for example via ELISA. As described hereinabove, since a targeting moiety capable of targeting to essentially any desired surface marker can be obtained by the ordinarily skilled artisan, the method of the present invention may be employed to kill a target cell/tissue specifically displaying essentially any such surface marker, and, as such, can be used for treating essentially any disease associated with a cell/tissue displaying such a surface marker.

[59] Ample guidance regarding surface markers specifically overexpressed in diseases such as cancer, and antibodies specific for such surface markers is provided in the literature of the art (for example, refer to: A M Scott, C Renner. "Tumour Antigens Recognised by Antibodies." In: Encyclopedia of Life Sciences, Nature Publishing Group, Macmillan, London, UK, wwwdotelsdotnet, 2001). Preferably, the method is used to treat a disease associated with a target cell/tissue specifically displaying a surface marker which is a growth factor receptor and/or a tumor associated antigen (TAA).

[60] Diseases associated with a target cell/tissue specifically displaying a growth factor receptor/TAA surface marker which are amenable to treatment by the method of the present invention include, for example, some of the numerous diseases which specifically display growth factor receptors/TAAs, such as EGF receptor, platelet derived growth factor (PDGF) receptor, insulin like growth factor receptor, vascular endothelial growth factor (VEGF) receptor, fibroblast growth factor (FGF) receptor, transferrin receptor,

and folic acid receptor. Specific examples of such diseases and the growth factor receptors/TAAs which these specifically display are listed in Table 1, below.

[61] By "antibody fragment" is meant any form of an antibody other than the full-length form. Antibody fragments herein include antibodies that are smaller components that exist within full-length antibodies, and antibodies that have been engineered. Antibody fragments include but are not limited to Fv, Fc, Fab, and (Fab')2, single chain Fv (scFv), diabodies, triabodies, tetrabodies, bifunctional hybrid antibodies, CDR1, CDR2, CDR3, combinations of CDR's, variable regions, framework regions, constant regions, heavy chains, light chains, and variable regions, and alternative scaffold non-antibody molecules, bispecific antibodies, and the like (Maynard & Georgiou, 2000, Annu. Rev. Biomed. Eng. 2:339-76; Hudson, 1998, Curr. Opin. Biotechnol. 9:395-402). Another functional substructure is a single chain Fv (scFv), comprised of the variable regions of the immunoglobulin heavy and light chain, covalently connected by a peptide linker (S-z Hu et al., 1996, Cancer Research, 56, 3055-3061). These small (Mr 25,000) proteins generally retain specificity and affinity for antigen in a single polypeptide and can provide a convenient building block for larger, antigen-specific molecules. Unless specifically noted otherwise, statements and claims that use the term "antibody" or "antibodies" specifically includes "antibody fragment" and "antibody fragments."

[62] In certain embodiments, the antibody or antigen-binding fragment thereof is selected for its ability to bind live cells, such as a tumor cell or a prostate cell, for example LNCaP cells. In other embodiments, the antibody or antigen-binding fragment thereof mediates cytolysis of cells expressing PSMA. In some embodiments cytolysis of cells expressing PSMA is mediated by effector cells or is complement mediated in the presence of effector cells.

[63] In other embodiments, the antibody or antigen-binding fragment thereof inhibits the growth of cells expressing PSMA. In some embodiments, the antibody or antigen-binding fragment thereof does not require cell lysis to bind to the extracellular domain of PSMA.

[64] In further embodiments, the antibody or antigen-binding fragment thereof is selected from the group consisting of IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgAsec, IgD, IgE or has immunoglobulin constant and/or variable domain of IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgAsec, IgD or IgE. In other embodiments, the antibody is a bispecific or multispecific antibody.

[65] In still other embodiments, the antibody is a recombinant antibody, a polyclonal antibody, a monoclonal antibody, a humanized antibody or a chimeric antibody, or a mixture of these. In particularly preferred embodiments, the antibody is a human antibody, e.g., a monoclonal antibody, polyclonal antibody or a mixture of monoclonal and polyclonal antibodies. In still other embodiments, the antibody is a bispecific or multispecific antibody. In one embodiment of the present invention, antigen-binding fragments include a Fab fragment, a F(ab').sub.2 fragment, and a Fv fragment CDR3.

[66] In certain other embodiments, the antibody or antigen-binding fragment thereof binds to a conformational epitope and/or is internalized into a cell along with the prostate specific membrane antigen. In other embodiments, the isolated antibody or antigen-binding fragment thereof is bound to a label, in some embodiments the label is selected from the group consisting of a fluorescent label, an enzyme label, a radioactive label, a nuclear magnetic resonance active label, a luminescent label, and a chromophore label.

[67] In still other embodiments, the isolated antibody or antigen-binding fragment thereof is bound to at least one therapeutic moiety, such as a drug, preferably a cytotoxic drug, a replication-selective virus, a toxin or a fragment thereof, or an enzyme or a fragment thereof. Preferred cytotoxic drug include: calicheamicin, esperamicin, methotrexate, doxorubicin, melphalan, chlorambucil, ARA-C, vindesine, mitomycin C, cis-platinum, etoposide, bleomycin, 5-fluorouracil, estramustine, vincristine, etoposide, doxorubicin, paclitaxel, docetaxel, dolastatin 10, auristatin E and auristatin PHE. In other embodiments, the therapeutic moiety is an immunostimulatory or immunomodulating agent, preferably one selected from the group consisting of: a cytokine, chemokine and adjuvant.

[68] In some embodiments, the antibodies or antigen-binding fragments of the invention specifically bind cell-surface PSMA and/or rsPSMA with a binding affinity of about 1 x 10^{-9}M or less. In some embodiments, the binding affinity is about 1 x 10^{-10}M or less, In some embodiments the binding affinity is about 1 x 10^{-11}M or less. In other embodiments the binding affinity is less than about 5 x 10^{-10}M. In additional embodiments, the antibodies or antigen-binding fragments of the invention mediate specific cell killing of PSMA-expressing cells with an IC_{50}s of less than about 1 x 10^{-10}M. In some embodiments the IC_{50} is less than about 1 x 10^{-11}M. In some embodiments the IC_{50} is less than about 1 x 10^{-12}M. In other embodiments the IC_{50} is less than about 1.5 x 10^{-11}M.

[69] In one embodiment, the modified antibody or functional antibody fragment is an anti-PSMA minibody. In one embodiment, the anti-PSMA antibody is a J591 minibody. The anti-

PSMA minibody has an anti-PSMA antibody fragment with optimized pharmacodynamic properties for in vivo imaging and biodistribution as described below. A "minibody" is a homodimer, wherein each monomer is a single-chain variable fragment (scFv) linked to a human IgG1 CH3 domain by a linker, such as ana hinge sequence. In another embodiment, the anti-PSMA antibody fragment comprises one non-naturally encoded amino acid. In other embodiments, the anti-PSMA minibody comprises more than one non-naturally encoded amino acid.

[70] In another embodiment, the modified antibody or functional antibody fragment is an anti-PSMA cys-diabody (CysDB) is provided. A "diabody" comprises a first polypeptide chain which comprises a heavy (VH) chain variable domain connected to a light chain variable domain (VL) on the first polypeptide chain (VH-VL) connected by a peptide linker that is too short to allow pairing between the two domains on the first polypeptide chain and a second polypeptide chain comprising a light chain variable domain (VL) linked to a heavy chain variable domain VH on the second polypeptide chain (VL-VH) connected by a peptide linker that is too short to allow pairing between the two domains on the second polypeptide chain. In another embodiment, the diabody comprises a non-naturally encoded amino acid. In another embodiment, the diabody contains more than one non-naturally encoded amino acid. The short linkages force chain pairing between the complementary domains of the first and the second polypeptide chains and promotes the assembly of a dimeric molecule with two functional antigen binding sites. Therefore, a peptide linker may be any suitable length that promotes such assembly, for example, between 5 and 10 amino acids in length. As described further below, some cys-diabodies may include a peptide linker that is 5 or 8 amino acids in length. In another embodiment, the linker contains a non-naturally encoded amino acid. In other embodiments,t he linker contains more than one non-naturally occurring amino acid. The anti-PSMA CysDB is a homodimer antibody format formed with two identical monomers that include single chain Fv (scFv) fragments with an approximate molecular weight of 55 kDa. In one embodiment, the anti-PSMA is a J591 CysDB. Like the anti-PSMA minibodies described above, the anti-PSMA CysDBs described herein have an anti-PSMA antibody fragment with optimized pharmacodynamic properties that may be used for in vivo imaging and biodistribution.

[71] By "antibody-drug conjugate, or "ADC", as used herein, refers to an antibody molecule, or fragment thereof, that is covalently bonded to one or more biologically active

molecule(s). The biologically active molecule may be conjugated to the antibody through a linker, polymer, or other covalent bond.

[72] As used herein an "acylated" amino acid is an amino acid comprising an acyl group which is non-native to a naturally-occurring amino acid, regardless by the means by which it is produced. Exemplary methods of producing acylated amino acids and acylated peptides are known in the art and include acylating an amino acid before inclusion in the peptide or peptide synthesis followed by chemical acylation of the peptide. In some embodiments, the acyl group causes the peptide to have one or more of (i) a prolonged half-life in circulation, (ii) a delayed onset of action, (iii) an extended duration of action, (iv) an improved resistance to proteases, such as DPP-IV, and (v) increased potency at the glucagon superfamily peptide receptor.

[73] As used herein, an "alkylated" amino acid is an amino acid comprising an alkyl group which is non-native to a naturally-occurring amino acid, regardless of the means by which it is produced. Exemplary methods of producing alkylated amino acids and alkylated peptides are known in the art and including alkylating an amino acid before inclusion in the peptide or peptide synthesis followed by chemical alkylation of the peptide. Without being held to any particular theory, it is believed that alkylation of peptides will achieve similar, if not the same, effects as acylation of the peptides, e.g., a prolonged half-life in circulation, a delayed onset of action, an extended duration of action, an improved resistance to proteases, such as DPP-IV, and increased potency at the glucagon superfamily peptide receptor.

[74] The term "C_1-C_n alkyl" wherein n can be from 1 through 18, as used herein, represents a branched or linear alkyl group having from one to the specified number of carbon atoms. For example, C_1-C_6 alkyl represents a branched or linear alkyl group having from 1 to 6 carbon atoms. Typical C_1-C_{18} alkyl groups include, but are not limited to, methyl, ethyl, n-propyl, iso-propyl, butyl, iso-butyl, sec -butyl, tert-butyl, pentyl, hexyl and the like. Alkyl groups optionally can be substituted, for example, with hydroxy (OH), halo, aryl, carboxyl, thio, C_3-C_8 cycloalkyl, and amino.

[75] The term "C_0-C_n alkyl" wherein n can be from 1-18, as used herein, represents a branched or linear alkyl group having up to 18 carbon atoms. For example, the term "(C_0-C_6 alkyl)OH" represents a hydroxyl parent moiety attached to an alkyl substituent having up to 6 carbon atoms (e.g. -OH, -CH_2OH, -C_2H_4OH, -C_3H_6OH, -C_4H_8OH, -$C_5H_{10}OH$, -$C_6H_{12}OH$).

[76] The term "C_2-C_n alkenyl" wherein n can be from 2 through 18, as used herein, represents an unsaturated branched or linear group having from 2 to the specified number of

carbon atoms and at least one double bond. Examples of such groups include, but are not limited to, 1-propenyl, 2-propenyl (-CH_2-CH=CH_2), 1,3-butadienyl, (-CH=CHCH=CH_2), 1-butenyl (-CH=$CHCH_2CH_3$), hexenyl, pentenyl, and the like. Alkenyl groups optionally can be substituted, for example, with hydroxy (OH), halo, aryl, carboxyl, thio, C_3-C_8 cycloalkyl, and amino.

[77] The term "C_2-C_n alkynyl" wherein n can be from 2 to 18, refers to an unsaturated branched or linear group having from 2 to n carbon atoms and at least one triple bond. Examples of such groups include, but are not limited to, 1-propynyl, 2-propynyl, 1-butynyl, 2-butynyl, 1-pentynyl, and the like. Alkynyl groups optionally can be substituted, for example, with hydroxy (OH), halo, aryl, carboxyl, thio, C_3-C_8 cycloalkyl, and amino.

[78] The term "aromatic" or "aryl", as used herein, refers to a closed ring structure which has at least one ring having a conjugated pi electron system and includes both carbocyclic aryl and heterocyclic aryl (or "heteroaryl" or "heteroaromatic") groups. The carbocyclic or heterocyclic aromatic group may contain from 5 to 20 ring atoms. The term includes monocyclic rings linked covalently or fused-ring polycyclic (i.e., rings which share adjacent pairs of carbon atoms) groups. An aromatic group can be unsubstituted or substituted. Non-limiting examples of "aromatic" or "aryl", groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, anthracenyl, and phenanthracenyl. Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described herein.

[79] For brevity, the term "aromatic" or "aryl" when used in combination with other terms (including but not limited to, aryloxy, arylthioxy, aralkyl) includes both aryl and heteroaryl rings as defined above. Thus, the term "aralkyl" or "alkaryl" is meant to include those radicals in which an aryl group is attached to an alkyl group (including but not limited to, benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (including but not limited to, a methylene group) has been replaced by a heteroatom, by way of example only, by an oxygen atom. Examples of such aryl groups include, but are not limited to, phenoxymethyl, 2-pyridyloxymethyl, 3-(1-naphthyloxy)propyl, and the like.

[80] The term "arylene", as used herein, refers to a divalent aryl radical. Non-limiting examples of "arylene" include phenylene, pyridinylene, pyrimidinylene and thiophenylene. Substituents for arylene groups are selected from the group of acceptable substituents described herein.

[81] A "bifunctional polymer", also referred to as a "bifunctional linker", refers to a polymer comprising two functional groups that are capable of reacting specifically with other moieties to form covalent or non-covalent linkages. Such moieties may include, but are not limited to, the side groups on natural or non-natural amino acids or peptides which contain such natural or non-natural amino acids. The other moieties that may be linked to the bifunctional linker or bifunctional polymer may be the same or different moieties. By way of example only, a bifunctional linker may have a functional group reactive with a group on a first peptide, and another functional group which is reactive with a group on a second peptide, whereby forming a conjugate that includes the first peptide, the bifunctional linker and the second peptide. Many procedures and linker molecules for attachment of various compounds to peptides are known. See, e.g., European Patent Application No. 188,256; U.S. Patent Nos. 4,671,958, 4,659,839, 4,414,148, 4,699,784; 4,680,338; and 4,569,789 which are incorporated by reference herein in their entirety. A "multi-functional polymer" also referred to as a "multi-functional linker", refers to a polymer comprising two or more functional groups that are capable of reacting with other moieties. Such moieties may include, but are not limited to, the side groups on natural or non-natural amino acids or peptides which contain such natural or non-natural amino acids. (including but not limited to, amino acid side groups) to form covalent or non-covalent linkages. A bi-functional polymer or multi-functional polymer may be any desired length or molecular weight, and may be selected to provide a particular desired spacing or conformation between one or more molecules linked to a compound and molecules it binds to or the compound.

[82] The term "bioavailability," as used herein, refers to the rate and extent to which a substance or its active moiety is delivered from a pharmaceutical dosage form and becomes available at the site of action or in the general circulation. Increases in bioavailability refers to increasing the rate and extent a substance or its active moiety is delivered from a pharmaceutical dosage form and becomes available at the site of action or in the general circulation. By way of example, an increase in bioavailability may be indicated as an increase in concentration of the substance or its active moiety in the blood when compared to other substances or active moieties. A non-limiting example of a method to evaluate increases in bioavailability is given in examples 21-25. This method may be used for evaluating the bioavailability of any polypeptide.

[83] The term "biologically active molecule", "biologically active moiety" or "biologically active agent" when used herein means any substance which can affect any physical or

biochemical properties of a biological system, pathway, molecule, or interaction relating to an organism, including but not limited to, viruses, bacteria, bacteriophage, transposon, prion, insects, fungi, plants, animals, and humans. In particular, as used herein, biologically active molecules include but are not limited to any substance intended for diagnosis, cure, mitigation, treatment, or prevention of disease in humans or other animals, or to otherwise enhance physical or mental well-being of humans or animals. Examples of biologically active molecules include, but are not limited to, peptides, proteins, enzymes, small molecule drugs, hard drugs, soft drugs, prodrugs, carbohydrates, inorganic atoms or molecules, dyes, lipids, nucleosides, radionuclides, oligonucleotides, cells, viruses, liposomes, microparticles and micelles. Classes of biologically active agents that are suitable for use with the methods and compositions described herein include, but are not limited to, drugs, prodrugs, radionuclides, imaging agents, polymers, antibiotics, fungicides, anti-viral agents, anti-inflammatory agents, anti-tumor agents, cardiovascular agents, anti-anxiety agents, hormones, growth factors, steroidal agents, and the like.

[84] By "modulating biological activity" is meant increasing or decreasing the reactivity of a polypeptide, altering the selectivity of the polypeptide, enhancing or decreasing the substrate selectivity of the polypeptide. Analysis of modified biological activity can be performed by comparing the biological activity of the non-natural polypeptide to that of the natural polypeptide.

[85] The term "biomaterial," as used herein, refers to a biologically-derived material, including but not limited to material obtained from bioreactors and/or from recombinant methods and techniques.

[86] The term "biophysical probe," as used herein, refers to probes which can detect or monitor structural changes in molecules. Such molecules include, but are not limited to, proteins and the "biophysical probe" may be used to detect or monitor interaction of proteins with other macromolecules. Examples of biophysical probes include, but are not limited to, spin-labels, a fluorophores, and photoactivatible groups.

[87] The term "biosynthetically," as used herein, refers to any method utilizing a translation system (cellular or non-cellular), including use of at least one of the following components: a polynucleotide, a codon, a tRNA, and a ribosome. By way of example, non-natural amino acids may be "biosynthetically incorporated" into non-natural amino acid polypeptides using the methods and techniques described herein, "In vivo generation of polypeptides comprising non-natural amino acids", and in the non-limiting example 20.

Additionally, the methods for the selection of useful non-natural amino acids which may be "biosynthetically incorporated" into non-natural amino acid polypeptides are described in the non-limiting examples 20.

[88] The term "biotin analogue," or also referred to as "biotin mimic", as used herein, is any molecule, other than biotin, which bind with high affinity to avidin and/or streptavidin.

[89] The term "carbonyl" as used herein refers to a group containing at a moiety selecting from the group consisting of -C(O)-, -S(O)-, -S(O)2-, and –C(S)-, including, but not limited to, groups containing a least one ketone group, and/or at least one aldehyde groups, and/or at least one ester group, and/or at least one carboxylic acid group, and/or at least one thioester group. Such carbonyl groups include ketones, aldehydes, carboxylic acids, esters, and thioesters. In addition, such groups may be part of linear, branched, or cyclic molecules.

[90] The term "carboxy terminus modification group" refers to any molecule that can be attached to a terminal carboxy group. By way of example, such terminal carboxy groups may be at the end of polymeric molecules, wherein such polymeric molecules include, but are not limited to, polypeptides, polynucleotides, and polysaccharides. Terminus modification groups include but are not limited to, various water soluble polymers, peptides or proteins. By way of example only, terminus modification groups include polyethylene glycol or serum albumin. Terminus modification groups may be used to modify therapeutic characteristics of the polymeric molecule, including but not limited to increasing the serum half-life of peptides.

[91] The term "chemically cleavable group," also referred to as "chemically labile", as used herein, refers to a group which breaks or cleaves upon exposure to acid, base, oxidizing agents, reducing agents, chemical inititiators, or radical initiators.

[92] The term "chemiluminescent group," as used herein, refers to a group which emits light as a result of a chemical reaction without the addition of heat. By way of example only, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) reacts with oxidants like hydrogen peroxide (H_2O_2) in the presence of a base and a metal catalyst to produce an excited state product (3-aminophthalate, 3-APA).

[93] The term "chromophore," as used herein, refers to a molecule which absorbs light of visible wavelengths, UV wavelengths or IR wavelengths.

[94] The term "cofactor," as used herein, refers to an atom or molecule essential for the action of a large molecule. Cofactors include, but are not limited to, inorganic ions,

coenzymes, proteins, or some other factor necessary for the activity of enzymes. Examples include, heme in hemoglobin, magnesium in chlorophyll, and metal ions for proteins.

[95] "Cofolding," as used herein, refers to refolding processes, reactions, or methods which employ at least two molecules which interact with each other and result in the transformation of unfolded or improperly folded molecules to properly folded molecules. By way of example only, "cofolding," employ at least two polypeptides which interact with each other and result in the transformation of unfolded or improperly folded polypeptides to native, properly folded polypeptides. Such polypeptides may contain natural amino acids and/or at least one non-natural amino acid.

[96] A "comparison window," as used herein, refers a segment of any one of contiguous positions used to compare a sequence to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Such contiguous positions include, but are not limited to a group consisting of from about 20 to about 600 sequential units, including about 50 to about 200 sequential units, and about 100 to about 150 sequential units. By way of example only, such sequences include polypeptides and polypeptides containing non-natural amino acids, with the sequential units include, but are not limited to natural and non-natural amino acids. In addition, by way of example only, such sequences include polynucleotides with nucleotides being the corresponding sequential units. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, including but not limited to, by the local homology algorithm of Smith and Waterman (1970) Adv. Appl. Math. 2:482c, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48:443, by the search for similarity method of Pearson and Lipman (1988) Proc. Nat'l. Acad. Sci. USA 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, e.g., Ausubel et al., Current Protocols in Molecular Biology (1995 supplement)).

[97] By way of example, an algorithm which may be used to determine percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1997) Nuc. Acids Res. 25:3389-3402, and Altschul et al. (1990) J. Mol. Biol. 215:403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN

program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) Proc. Natl. Acad. Sci. USA 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands. The BLAST algorithm is typically performed with the "low complexity" filter turned off.

[98] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5787). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, or less than about 0.01, or less than about 0.001.

[99] The term "conservatively modified variants" applies to both natural and non-natural amino acid and natural and non-natural nucleic acid sequences, and combinations thereof. With respect to particular nucleic acid sequences, "conservatively modified variants" refers to those natural and non-natural nucleic acids which encode identical or essentially identical natural and non-natural amino acid sequences, or where the natural and non-natural nucleic acid does not encode a natural and non-natural amino acid sequence, to essentially identical sequences. By way of example, because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Thus by way of example every natural or non-natural nucleic acid sequence herein which encodes a natural or non-natural polypeptide also describes every possible silent variation of the natural or non-natural nucleic acid. One of ordinary skill in the art will recognize that each codon in a natural or non-natural nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each

silent variation of a natural and non-natural nucleic acid which encodes a natural and non-natural polypeptide is implicit in each described sequence.

[100] As to amino acid sequences, individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single natural and non-natural amino acid or a small percentage of natural and non-natural amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the deletion of an amino acid, addition of an amino acid, or substitution of a natural and non-natural amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar natural amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the methods and compositions described herein.

[101] Conservative substitution tables providing functionally similar amino acids are known to those of ordinary skill in the art. The following eight groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Glycine (G);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
7) Serine (S), Threonine (T); and
8) Cysteine (C), Methionine (M)

(see, e.g., Creighton, Proteins:Structures and Molecular Properties (W H Freeman & Co.; 2nd edition (December 1993)

[102] The terms "cycloalkyl" and "heterocycloalkyl", by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of "alkyl" and "heteroalkyl", respectively. Thus, a cycloalkyl or heterocycloalkyl include saturated, partially unsaturated and fully unsaturated ring linkages. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. The heteroatom may include, but is not limited to, oxygen, nitrogen or sulfur. Examples of cycloalkyl include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of heterocycloalkyl include, but are not limited to, 1-(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-

morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1–piperazinyl, 2-piperazinyl, and the like. Additionally, the term encompasses multicyclic structures, including but not limited to, bicyclic and tricyclic ring structures. Similarly, the term "heterocycloalkylene" by itself or as part of another molecule means a divalent radical derived from heterocycloalkyl, and the term "cycloalkylene" by itself or as part of another molecule means a divalent radical derived from cycloalkyl.

[103] The term "cyclodextrin," as used herein, refers to cyclic carbohydrates consisting of at least six to eight glucose molecules in a ring formation. The outer part of the ring contains water soluble groups; at the center of the ring is a relatively nonpolar cavity able to accommodate small molecules.

[104] The term "cytotoxic," as used herein, refers to a compound which harms cells.

[105] "Denaturing agent" or "denaturant," as used herein, refers to any compound or material which will cause a reversible unfolding of a polymer. By way of example only, "denaturing agent" or "denaturants," may cause a reversible unfolding of a protein. The strength of a denaturing agent or denaturant will be determined both by the properties and the concentration of the particular denaturing agent or denaturant. By way of example, denaturing agents or denaturants include, but are not limited to, chaotropes, detergents, organic, water miscible solvents, phospholipids, or a combination thereof. Non-limiting examples of chaotropes include, but are not limited to, urea, guanidine, and sodium thiocyanate. Non-limiting examples of detergents may include, but are not limited to, strong detergents such as sodium dodecyl sulfate, or polyoxyethylene ethers (e.g. Tween or Triton detergents), Sarkosyl, mild non-ionic detergents (e.g., digitonin), mild cationic detergents such as N-›2,3-(Dioleyoxy)-propyl-N,N,N-trimethylammonium, mild ionic detergents (e.g. sodium cholate or sodium deoxycholate) or zwitterionic detergents including, but not limited to, sulfobetaines (Zwittergent), 3-(3-chlolamidopropyl)dimethylammonio-1-propane sulfate (CHAPS), and 3-(3-chlolamidopropyl)dimethylammonio-2-hydroxy-1-propane sulfonate (CHAPSO). Non-limiting examples of organic, water miscible solvents include, but are not limited to, acetonitrile, lower alkanols (especially C2 - C4 alkanols such as ethanol or isopropanol), or lower alkandiols (C2 - C4 alkandiols such as ethylene-glycol) may be used as denaturants. Non-limiting examples of phospholipids include, but are not limited to, naturally occurring phospholipids such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, and phosphatidylinositol or synthetic phospholipid derivatives or variants such as dihexanoylphosphatidylcholine or diheptanoylphosphatidylcholine.

[106] The term "desired functionality" as used herein refers to any group selected from a label; a dye; a polymer; a water-soluble polymer; a derivative of polyethylene glycol; a photocrosslinker; an affinity label; a photoaffinity label; a reactive compound; a resin; a second protein or polypeptide or polypeptide analog; an antibody or antibody fragment; a metal chelator; a cofactor; a fatty acid; a carbohydrate; a polynucleotide; a DNA; a RNA; an antisense polynucleotide; a saccharide, a water-soluble dendrimer, a cyclodextrin, a biomaterial; a nanoparticle; a spin label; a fluorophore; a metal-containing moiety; a radioactive moiety; a novel functional group; a group that covalently or noncovalently interacts with other molecules; a photocaged moiety; an actinic radiation excitable moiety; a ligand; a photoisomerizable moiety; biotin; a biotin analogue; a moiety incorporating a heavy atom; a chemically cleavable group; a photocleavable group; an elongated side chain; a carbon-linked sugar; a redox-active agent; an amino thioacid; an isotopically labeled moiety; a biophysical probe; a phosphorescent group; a chemiluminescent group; an electron dense group; a magnetic group; an intercalating group; a chromophore; an energy transfer agent; a biologically active agent (in which case, the biologically active agent can include an agent with therapeutic activity and the non-natural amino acid polypeptide or modified non-natural amino acid can serve either as a co-therapeutic agent with the attached therapeutic agent or as a means for delivery the therapeutic agent to a desired site within an organism); a detectable label; a small molecule; an inhibitory ribonucleic acid; a radionucleotide; a neutron-capture agent; a derivative of biotin; quantum dot(s); a nanotransmitter; a radiotransmitter; an abzyme, an activated complex activator, a virus, an adjuvant, an aglycan, an allergan, an angiostatin, an antihormone, an antioxidant, an aptamer, a guide RNA, a saponin, a shuttle vector, a macromolecule, a mimotope, a receptor, a reverse micelle, and any combination thereof.

[107] The term "diamine,"as used herein, refers to groups/molecules comprising at least two amine functional groups, including, but not limited to, a hydrazine group, an amidine group, an imine group, a 1,1-diamine group, a 1,2-diamine group, a 1,3-diamine group, and a 1,4-diamine group. In addition, such groups may be part of linear, branched, or cyclic molecules.

[108] The term "detectable label," as used herein, refers to a label which may be observable using analytical techniques including, but not limited to, fluorescence, chemiluminescence, electron-spin resonance, ultraviolet/visible absorbance spectroscopy, mass spectrometry, nuclear magnetic resonance, magnetic resonance, and electrochemical methods.

[109] The term "dicarbonyl" as used herein refers to a group containing at least two moieties selected from the group consisting of -C(O)-, -S(O)-, -$S(O)_2$-, and –C(S)-, including, but not limited to, 1,2-dicarbonyl groups, a 1,3-dicarbonyl groups, and 1,4-dicarbonyl groups, and groups containing a least one ketone group, and/or at least one aldehyde groups, and/or at least one ester group, and/or at least one carboxylic acid group, and/or at least one thioester group. Such dicarbonyl groups include diketones, ketoaldehydes, ketoacids, ketoesters, and ketothioesters. In addition, such groups may be part of linear, branched, or cyclic molecules. The two moieties in the dicarbonyl group may be the same or different, and may include substituents that would produce, by way of example only, an ester, a ketone, an aldehyde, a thioester, or an amide, at either of the two moieties.

[110] The term "drug," as used herein, refers to any substance used in the prevention, diagnosis, alleviation, treatment, or cure of a disease or condition.

[111] The term "dye," as used herein, refers to a soluble, coloring substance which contains a chromophore.

[112] The term "effective amount," as used herein, refers to a sufficient amount of an agent or a compound being administered which will relieve to some extent one or more of the symptoms of the disease or condition being treated. The result can be reduction and/or alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. By way of example, an agent or a compound being administered includes, but is not limited to, a natural amino acid polypeptide, non-natural amino acid polypeptide, modified natural amino acid polypeptide, or modified non-amino acid polypeptide. Compositions containing such natural amino acid polypeptides, non-natural amino acid polypeptides, modified natural amino acid polypeptides, or modified non-natural amino acid polypeptides can be administered for prophylactic, enhancing, and/or therapeutic treatments. An appropriate "effective" amount in any individual case may be determined using techniques, such as a dose escalation study.

[113] The term "electron dense group," as used herein, refers to a group which scatters electrons when irradiated with an electron beam. Such groups include, but are not limited to, ammonium molybdate, bismuth subnitrate cadmium iodide, 99%, carbohydrazide, ferric chloride hexahydrate, hexamethylene tetramine, 98.5%, indium trichloride anhydrous, lanthanum nitrate, lead acetate trihydrate, lead citrate trihydrate, lead nitrate, periodic acid, phosphomolybdic acid, phosphotungstic acid, potassium ferricyanide, potassium ferrocyanide, ruthenium red, silver nitrate, silver proteinate (Ag Assay: 8.0-8.5%) "Strong",

silver tetraphenylporphin (S-TPPS), sodium chloroaurate, sodium tungstate, thallium nitrate, thiosemicarbazide (TSC), uranyl acetate, uranyl nitrate, and vanadyl sulfate.

[114] The term "energy transfer agent," as used herein, refers to a molecule which can either donate or accept energy from another molecule. By way of example only, fluorescence resonance energy transfer (FRET) is a dipole-dipole coupling process by which the excited-state energy of a fluorescence donor molecule is non-radiatively transferred to an unexcited acceptor molecule which then fluorescently emits the donated energy at a longer wavelength.

[115] The terms "enhance" or "enhancing" means to increase or prolong either in potency or duration a desired effect. By way of example, "enhancing" the effect of therapeutic agents refers to the ability to increase or prolong, either in potency or duration, the effect of therapeutic agents on during treatment of a disease, disorder or condition. An "enhancing-effective amount," as used herein, refers to an amount adequate to enhance the effect of a therapeutic agent in the treatment of a disease, disorder or condition. When used in a patient, amounts effective for this use will depend on the severity and course of the disease, disorder or condition, previous therapy, the patient's health status and response to the drugs, and the judgment of the treating physician.

[116] As used herein, the term "eukaryote" refers to organisms belonging to the phylogenetic domain Eucarya, including but not limited to animals (including but not limited to, mammals, insects, reptiles, birds, etc.), ciliates, plants (including but not limited to, monocots, dicots, and algae), fungi, yeasts, flagellates, microsporidia, and protists.

[117] The term "fatty acid," as used herein, refers to carboxylic acids with about C6 or longer hydrocarbon side chain.

[118] The term "fluorophore," as used herein, refers to a molecule which upon excitation emits photons and is thereby fluorescent.

[119] The terms "functional group", "active moiety", "activating group", "leaving group", "reactive site", "chemically reactive group" and "chemically reactive moiety," as used herein, refer to portions or units of a molecule at which chemical reactions occur. The terms are somewhat synonymous in the chemical arts and are used herein to indicate the portions of molecules that perform some function or activity and are reactive with other molecules.

[120] The term "halogen" includes fluorine, chlorine, iodine, and bromine.

[121] The term "haloacyl," as used herein, refers to acyl groups which contain halogen moieties, including, but not limited to, $-C(O)CH_3$, $-C(O)CF_3$, $-C(O)CH_2OCH_3$, and the like.

[122] The term "haloalkyl," as used herein, refers to alkyl groups which contain halogen moieties, including, but not limited to, $-CF_3$ and $-CH_2CF_3$ and the like.

[123] The term "heteroalkyl," as used herein, refers to straight or branched chain, or cyclic hydrocarbon radicals, or combinations thereof, consisting of an alkyl group and at least one heteroatom selected from the group consisting of O, N, Si and S, and wherein the nitrogen and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N and S and Si may be placed at any interior position of the heteroalkyl group or at the position at which the alkyl group is attached to the remainder of the molecule. Examples include, but are not limited to, $-CH_2-CH_2-O-CH_3$, $-CH_2-CH_2-NH-CH_3$, $-CH_2-CH_2-N(CH_3)-CH_3$, $-CH_2-S-CH_2-CH_3$, $-CH_2-CH_2,-S(O)-CH_3$, $-CH_2-CH_2-S(O)_2-CH_3$, $-CH=CH-O-CH_3$, $-Si(CH_3)_3$, $-CH_2-CH=N-OCH_3$, and $-CH=CH-N(CH_3)-CH_3$. In addition, up to two heteroatoms may be consecutive, such as, by way of example, $-CH_2-NH-OCH_3$ and $-CH_2-O-Si(CH_3)_3$.

[124] The terms "heterocyclic-based linkage" or "heterocycle linkage" refers to a moiety formed from the reaction of a dicarbonyl group with a diamine group. The resulting reaction product is a heterocycle, including a heteroaryl group or a heterocycloalkyl group. The resulting heterocycle group serves as a chemical link between a non-natural amino acid or non-natural amino acid polypeptide and another functional group. In one embodiment, the heterocycle linkage includes a nitrogen-containing heterocycle linkage, including by way of example only a pyrazole linkage, a pyrrole linkage, an indole linkage, a benzodiazepine linkage, and a pyrazalone linkage.

[125] Similarly, the term "heteroalkylene" refers to a divalent radical derived from heteroalkyl, as exemplified, but not limited by, $-CH_2-CH_2-S-CH_2-CH_2-$ and $-CH_2-S-CH_2-CH_2-NH-CH_2-$. For heteroalkylene groups, the same or different heteroatoms can also occupy either or both of the chain termini (including but not limited to, alkyleneoxy, alkylenedioxy, alkyleneamino, alkylenediamino, aminooxyalkylene, and the like). Still further, for alkylene and heteroalkylene linking groups, no orientation of the linking group is implied by the direction in which the formula of the linking group is written. By way of example, the formula $-C(O)_2R'-$ represents both $-C(O)_2R'-$ and $-R'C(O)_2-$.

[126] The term "heteroaryl" or "heteroaromatic," as used herein, refers to aryl groups which contain at least one heteroatom selected from N, O, and S; wherein the nitrogen and sulfur atoms may be optionally oxidized, and the nitrogen atom(s) may be optionally quaternized. Heteroaryl groups may be substituted or unsubstituted. A heteroaryl group may be attached to

the remainder of the molecule through a heteroatom. Non-limiting examples of heteroaryl groups include 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalinyl, 5-quinoxalinyl, 3-quinolyl, and 6-quinolyl.

[127] The term "homoalkyl," as used herein refers to alkyl groups which are hydrocarbon groups.

[128] The term "identical," as used herein, refers to two or more sequences or subsequences which are the same. In addition, the term "substantially identical," as used herein, refers to two or more sequences which have a percentage of sequential units which are the same when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using comparison algorithms or by manual alignment and visual inspection. By way of example only, two or more sequences may be "substantially identical" if the sequential units are about 60% identical, about 65% identical, about 70% identical, about 75% identical, about 80% identical, about 85% identical, about 90% identical, or about 95% identical over a specified region. Such percentages to describe the "percent identity" of two or more sequences. The identity of a sequence can exist over a region that is at least about 75-100 sequential units in length, over a region that is about 50 sequential units in length, or, where not specified, across the entire sequence. This definition also refers to the complement of a test sequence. By way of example only, two or more polypeptide sequences are identical when the amino acid residues are the same, while two or more polypeptide sequences are "substantially identical" if the amino acid residues are about 60% identical, about 65% identical, about 70% identical, about 75% identical, about 80% identical, about 85% identical, about 90% identical, or about 95% identical over a specified region. The identity can exist over a region that is at least about 75 to about 100 amino acids in length, over a region that is about 50 amino acids in length, or, where not specified, across the entire sequence of a polypeptide sequence. In addition, by way of example only, two or more polynucleotide sequences are identical when the nucleic acid residues are the same, while two or more polynucleotide sequences are "substantially identical" if the nucleic acid residues are about 60% identical, about 65% identical, about 70% identical, about 75% identical, about 80% identical, about 85% identical, about 90% identical, or about 95%

identical over a specified region. The identity can exist over a region that is at least about 75 to about 100 nucleic acids in length, over a region that is about 50 nucleic acids in length, or, where not specified, across the entire sequence of a polynucleotide sequence.

[129] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[130] The term "immunogenicity," as used herein, refers to an antibody response to administration of a therapeutic drug. The immunogenicity toward therapeutic non-natural amino acid polypeptides can be obtained using quantitative and qualitative assays for detection of anti-non-natural amino acid polypeptides antibodies in biological fluids. Such assays include, but are not limited to, Radioimmunoassay (RIA), Enzyme-linked immunosorbent assay (ELISA), luminescent immunoassay (LIA), and fluorescent immunoassay (FIA). Analysis of immunogenicity toward therapeutic non-natural amino acid polypeptides involves comparing the antibody response upon administration of therapeutic non-natural amino acid polypeptides to the antibody response upon administration of therapeutic natural amino acid polypeptides.

[131] The term "intercalating agent," also referred to as "intercalating group," as used herein, refers to a chemical that can insert into the intramolecular space of a molecule or the intermolecular space between molecules. By way of example only an intercalating agent or group may be a molecule which inserts into the stacked bases of the DNA double helix.

[132] The term "isolated," as used herein, refers to separating and removing a component of interest from components not of interest. Isolated substances can be in either a dry or semi-dry state, or in solution, including but not limited to an aqueous solution. The isolated component can be in a homogeneous state or the isolated component can be a part of a pharmaceutical composition that comprises additional pharmaceutically acceptable carriers and/or excipients. Purity and homogeneity may be determined using analytical chemistry techniques including, but not limited to, polyacrylamide gel electrophoresis or high performance liquid chromatography. In addition, when a component of interest is isolated and is the predominant species present in a preparation, the component is described herein as

substantially purified. The term "purified," as used herein, may refer to a component of interest which is at least 85% pure, at least 90% pure, at least 95% pure, at least 99% or greater pure. By way of example only, nucleic acids or proteins are "isolated" when such nucleic acids or proteins are free of at least some of the cellular components with which it is associated in the natural state, or that the nucleic acid or protein has been concentrated to a level greater than the concentration of its in vivo or in vitro production. Also, by way of example, a gene is isolated when separated from open reading frames which flank the gene and encode a protein other than the gene of interest.

[133] The term "label," as used herein, refers to a substance which is incorporated into a compound and is readily detected, whereby its physical distribution may be detected and/or monitored.

[134] The term "linkage," as used herein to refer to bonds or chemical moiety formed from a chemical reaction between the functional group of a linker and another molecule. Such bonds may include, but are not limited to, covalent linkages and non-covalent bonds, while such chemical moieties may include, but are not limited to, esters, carbonates, imines phosphate esters, hydrazones, acetals, orthoesters, peptide linkages, and oligonucleotide linkages. Hydrolytically stable linkages means that the linkages are substantially stable in water and do not react with water at useful pH values, including but not limited to, under physiological conditions for an extended period of time, perhaps even indefinitely. Hydrolytically unstable or degradable linkages means that the linkages are degradable in water or in aqueous solutions, including for example, blood. Enzymatically unstable or degradable linkages means that the linkage can be degraded by one or more enzymes. By way of example only, PEG and related polymers may include degradable linkages in the polymer backbone or in the linker group between the polymer backbone and one or more of the terminal functional groups of the polymer molecule. Such degradable linkages include, but are not limited to, ester linkages formed by the reaction of PEG carboxylic acids or activated PEG carboxylic acids with alcohol groups on a biologically active agent, wherein such ester groups generally hydrolyze under physiological conditions to release the biologically active agent. Other hydrolytically degradable linkages include but are not limited to carbonate linkages; imine linkages resulted from reaction of an amine and an aldehyde; phosphate ester linkages formed by reacting an alcohol with a phosphate group; hydrazone linkages which are reaction product of a hydrazide and an aldehyde; acetal linkages that are the reaction product of an aldehyde and an alcohol; orthoester linkages that are the reaction product of a

formate and an alcohol; peptide linkages formed by an amine group, including but not limited to, at an end of a polymer such as PEG, and a carboxyl group of a peptide; and oligonucleotide linkages formed by a phosphoramidite group, including but not limited to, at the end of a polymer, and a 5' hydroxyl group of an oligonucleotide.

[135] The terms "medium" or "media," as used herein, refer to any culture medium used to grow and harvest cells and/or products expressed and/or secreted by such cells. Such "medium" or "media" include, but are not limited to, solution, solid, semi-solid, or rigid supports that may support or contain any host cell, including, by way of example, bacterial host cells, yeast host cells, insect host cells, plant host cells, eukaryotic host cells, mammalian host cells, CHO cells, prokaryotic host cells, E. coli, or Pseudomonas host cells, and cell contents. Such "medium" or "media" includes, but is not limited to, medium or media in which the host cell has been grown into which a polypeptide has been secreted, including medium either before or after a proliferation step. Such "medium" or "media" also includes, but is not limited to, buffers or reagents that contain host cell lysates, by way of example a polypeptide produced intracellularly and the host cells are lysed or disrupted to release the polypeptide.

[136] The term "metabolite," as used herein, refers to a derivative of a compound, by way of example natural amino acid polypeptide, a non-natural amino acid polypeptide, a modified natural amino acid polypeptide, or a modified non-natural amino acid polypeptide, that is formed when the compound, by way of example natural amino acid polypeptide, non-natural amino acid polypeptide, modified natural amino acid polypeptide, or modified non-natural amino acid polypeptide, is metabolized. The term "pharmaceutically active metabolite" or "active metabolite" refers to a biologically active derivative of a compound, by way of example natural amino acid polypeptide, a non-natural amino acid polypeptide, a modified natural amino acid polypeptide, or a modified non-natural amino acid polypeptide, that is formed when such a compound, by way of example a natural amino acid polypeptide, non-natural amino acid polypeptide, modified natural amino acid polypeptide, or modified non-natural amino acid polypeptide, is metabolized.

[137] The term "metabolized," as used herein, refers to the sum of the processes by which a particular substance is changed by an organism. Such processes include, but are not limited to, hydrolysis reactions and reactions catalyzed by enzymes. Further information on metabolism may be obtained from The Pharmacological Basis of Therapeutics, 9th Edition, McGraw-Hill (1996). By way of example only, metabolites of natural amino acid

polypeptides, non-natural amino acid polypeptides, modified natural amino acid polypeptides, or modified non-natural amino acid polypeptides may be identified either by administration of the natural amino acid polypeptides, non-natural amino acid polypeptides, modified natural amino acid polypeptides, or modified non-natural amino acid polypeptides to a host and analysis of tissue samples from the host, or by incubation of natural amino acid polypeptides, non-natural amino acid polypeptides, modified natural amino acid polypeptides, or modified non-natural amino acid polypeptides with hepatic cells in vitro and analysis of the resulting compounds.

[138] The term "metal chelator," as used herein, refers to a molecule which forms a metal complex with metal ions. By way of example, such molecules may form two or more coordination bonds with a central metal ion and may form ring structures.

[139] The term "metal-containing moiety," as used herein, refers to a group which contains a metal ion, atom or particle. Such moieties include, but are not limited to, cisplatin, chelated metals ions (such as nickel, iron, and platinum), and metal nanoparticles (such as nickel, iron, and platinum).

[140] The term "moiety incorporating a heavy atom," as used herein, refers to a group which incorporates an ion of atom which is usually heavier than carbon. Such ions or atoms include, but are not limited to, silicon, tungsten, gold, lead, and uranium.

[141] The term "modified," as used herein refers to the presence of a change to a natural amino acid, a non-natural amino acid, a natural amino acid polypeptide or a non-natural amino acid polypeptide. Such changes, or modifications, may be obtained by post synthesis modifications of natural amino acids, non-natural amino acids, natural amino acid polypeptides or non-natural amino acid polypeptides, or by co-translational, or by post-translational modification of natural amino acids, non-natural amino acids, natural amino acid polypeptides or non-natural amino acid polypeptides. The form "modified or unmodified" means that the natural amino acid, non-natural amino acid, natural amino acid polypeptide or non-natural amino acid polypeptide being discussed are optionally modified, that is, he natural amino acid, non-natural amino acid, natural amino acid polypeptide or non-natural amino acid polypeptide under discussion can be modified or unmodified.

[142] As used herein, the term "modulated serum half-life" refers to positive or negative changes in the circulating half-life of a modified biologically active molecule relative to its non-modified form. By way of example, the modified biologically active molecules include, but are not limited to, natural amino acid, non-natural amino acid, natural amino acid

polypeptide or non-natural amino acid polypeptide. By way of example, serum half-life is measured by taking blood samples at various time points after administration of the biologically active molecule or modified biologically active molecule, and determining the concentration of that molecule in each sample. Correlation of the serum concentration with time allows calculation of the serum half-life. By way of example, modulated serum half-life may be an increased in serum half-life, which may enable an improved dosing regimens or avoid toxic effects. Such increases in serum may be at least about two fold, at least about three-fold, at least about five-fold, or at least about ten-fold. A non-limiting example of a method to evaluate increases in serum half-life is given in example 33. This method may be used for evaluating the serum half-life of any polypeptide.

[143] The term "modulated therapeutic half-life," as used herein, refers to positive or negative change in the half-life of the therapeutically effective amount of a modified biologically active molecule, relative to its non-modified form. By way of example, the modified biologically active molecules include, but are not limited to, natural amino acid, non-natural amino acid, natural amino acid polypeptide or non-natural amino acid polypeptide. By way of example, therapeutic half-life is measured by measuring pharmacokinetic and/or pharmacodynamic properties of the molecule at various time points after administration. Increased therapeutic half-life may enable a particular beneficial dosing regimen, a particular beneficial total dose, or avoids an undesired effect. By way of example, the increased therapeutic half-life may result from increased potency, increased or decreased binding of the modified molecule to its target, an increase or decrease in another parameter or mechanism of action of the non-modified molecule, or an increased or decreased breakdown of the molecules by enzymes such as, by way of example only, proteases. A non-limiting example of a method to evaluate increases in therapeutic half-life is given in example 33. This method may be used for evaluating the therapeutic half-life of any polypeptide.

[144] The term "nanoparticle," as used herein, refers to a particle which has a particle size between about 500 nm to about 1 nm.

[145] The term "near-stoichiometric," as used herein, refers to the ratio of the moles of compounds participating in a chemical reaction being about 0.75 to about 1.5.

[146] As used herein, the term "non-eukaryote" refers to non-eukaryotic organisms. By way of example, a non-eukaryotic organism may belong to the Eubacteria, (which includes but is not limited to, Escherichia coli, Thermus thermophilus, or Bacillus stearothermophilus, Pseudomonas fluorescens, Pseudomonas aeruginosa, Pseudomonas putida), phylogenetic

domain, or the Archaea, which includes, but is not limited to, Methanococcus jannaschii, Methanobacterium thermoautotrophicum, Archaeoglobus fulgidus, Pyrococcus furiosus, Pyrococcus horikoshii, Aeuropyrum pernix, or Halobacterium such as Haloferax volcanii and Halobacterium species NRC-1, or phylogenetic domain.

[147] A "non-natural amino acid" refers to an amino acid that is not one of the 20 common amino acids or pyrolysine or selenocysteine. Other terms that may be used synonymously with the term "non-natural amino acid" is "non-naturally encoded amino acid," "unnatural amino acid," "non-naturally-occurring amino acid," and variously hyphenated and non-hyphenated versions thereof. The term "non-natural amino acid" includes, but is not limited to, amino acids which occur naturally by modification of a naturally encoded amino acid (including but not limited to, the 20 common amino acids or pyrrolysine and selenocysteine) but are not themselves incorporated into a growing polypeptide chain by the translation complex. Examples of naturally-occurring amino acids that are not naturally-encoded include, but are not limited to, N-acetylglucosaminyl-L-serine, N-acetylglucosaminyl-L-threonine, and O-phosphotyrosine. Additionally, the term "non-natural amino acid" includes, but is not limited to, amino acids which do not occur naturally and may be obtained synthetically or may be obtained by modification of non-natural amino acids.

[148] The term "nucleic acid," as used herein, refers to deoxyribonucleotides, deoxyribonucleosides, ribonucleosides or ribonucleotides and polymers thereof in either single- or double-stranded form. By way of example only, such nucleic acids and nucleic acid polymers include, but are not limited to, (i) analogues of natural nucleotides which have similar binding properties as a reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides; (ii) oligonucleotide analogs including, but are not limited to, PNA (peptidonucleic acid), analogs of DNA used in antisense technology (phosphorothioates, phosphoroamidates, and the like); (iii) conservatively modified variants thereof (including but not limited to, degenerate codon substitutions) and complementary sequences and sequence explicitly indicated. By way of example, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka et al., J. Biol. Chem. 260:2605-2608 (1985); and Rossolini et al., Mol. Cell. Probes 8:91-98 (1994)).

[149] The term "oxidizing agent," as used herein, refers to a compound or material which is capable of removing an electron from a compound being oxidized. By way of example

oxidizing agents include, but are not limited to, oxidized glutathione, cystine, cystamine, oxidized dithiothreitol, oxidized erythreitol, and oxygen. A wide variety of oxidizing agents are suitable for use in the methods and compositions described herein.

[150] The term "pharmaceutically acceptable", as used herein, refers to a material, including but not limited, to a salt, carrier or diluent, which does not abrogate the biological activity or properties of the compound, and is relatively nontoxic, i.e., the material may be administered to an individual without causing undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

[151] The term "photoaffinity label," as used herein, refers to a label with a group, which, upon exposure to light, forms a linkage with a molecule for which the label has an affinity. By way of example only, such a linkage may be covalent or non-covalent.

[152] The term "photocaged moiety," as used herein, refers to a group which, upon illumination at certain wavelengths, covalently or non-covalently binds other ions or molecules.

[153] The term "photocleavable group," as used herein, refers to a group which breaks upon exposure to light.

[154] The term "photocrosslinker," as used herein, refers to a compound comprising two or more functional groups which, upon exposure to light, are reactive and form a covalent or non-covalent linkage with two or more monomeric or polymeric molecules.

[155] The term "photoisomerizable moiety," as used herein, refers to a group wherein upon illumination with light changes from one isomeric form to another.

[156] The term "polyalkylene glycol," as used herein, refers to linear or branched polymeric polyether polyols. Such polyalkylene glycols, including, but are not limited to, polyethylene glycol, polypropylene glycol, polybutylene glycol, and derivatives thereof. Other exemplary embodiments are listed, for example, in commercial supplier catalogs, such as Shearwater Corporation's catalog "Polyethylene Glycol and Derivatives for Biomedical Applications" (2001). By way of example only, such polymeric polyether polyols have average molecular weights between about 0.05 kDa to about 100 kDa. By way of example, such polymeric polyether polyols include, but are not limited to, between about 50 Da and about 100,000 Da or more. The molecular weight of the polymer may be between about 50 Da and about 100,000 Da, including but not limited to, about 100,000 Da, about 95,000 Da, about 90,000 Da, about 85,000 Da, about 80,000 Da, about 75,000 Da, about 70,000 Da, about 65,000 Da, about 60,000 Da, about 55,000 Da, about 50,000 Da, about 45,000 Da, about 40,000 Da,

about 35,000 Da, about 30,000 Da, about 25,000 Da, about 20,000 Da, about 15,000 Da, about 10,000 Da, about 9,000 Da, about 8,000 Da, about 7,000 Da, about 6,000 Da, about 5,000 Da, about 4,000 Da, about 3,000 Da, about 2,000 Da, about 1,000 Da, about 900 Da, about 800 Da, about 700 Da, about 600 Da, about 500 Da, 400 Da, about 300 Da, about 200 Da, about 100 Da, and about 50 Da. In some embodiments molecular weight of the polymer is between about 50 Da and about 50,000 Da. In some embodiments, the molecular weight of the polymer is between about 50 Da and about 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 50 Da and about 1,000 Da. In some embodiments, the molecular weight of the polymer is between about 100 Da and about 500 Da. In some embodiments, the molecular weight of the polymer is between about 1,000 Da and about 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 2,000 to about 50,000 Da. In some embodiments, the molecular weight of the polymer is between about 5,000 Da and about 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 10,000 Da and about 40,000 Da. In some embodiments, the poly(ethylene glycol) molecule is a branched polymer. The molecular weight of the branched chain PEG may be between about 50 Da and about 100,000 Da, including but not limited to, about 100,000 Da, about 95,000 Da, about 90,000 Da, about 85,000 Da, about 80,000 Da, about 75,000 Da, about 70,000 Da, about 65,000 Da, about 60,000 Da, about 55,000 Da, about 50,000 Da, about 45,000 Da, about 40,000 Da, about 35,000 Da, about 30,000 Da, about 25,000 Da, about 20,000 Da, about 15,000 Da, about 10,000 Da, about 9,000 Da, about 8,000 Da, about 7,000 Da, about 6,000 Da, about 5,000 Da, about 4,000 Da, about 3,000 Da, about 2,000 Da, about 1,000 Da, about 900 Da, about 800 Da, about 700 Da, about 600 Da, about 500 Da, about 400 Da, about 300 Da, about 250 Da, about 200 Da, about 150 Da, about 100 Da, about 75 Da, and about 50 Da. In some embodiments, the molecular weight of the branched chain PEG is between about 50 Da and about 50,000 Da. In some embodiments, the molecular weight of the branched chain PEG is between about 100 Da and about 1,000 Da. In some embodiments, the molecular weight of the branched chain PEG is between about 5,000 Da and about 40,000 Da. In some embodiments, the molecular weight of the branched chain PEG is between about 5,000 Da and about 20,000 Da. In other embodiments, the molecular weight of the branched chain PEG is between about 2,000 to about 50,000 Da.

[157] The term "polymer," as used herein, refers to a molecule composed of repeated subunits. Such molecules include, but are not limited to, polypeptides, polynucleotides, or polysaccharides or polyalkylene glycols.

[158] The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. That is, a description directed to a polypeptide applies equally to a description of a peptide and a description of a protein, and vice versa. The terms apply to naturally occurring amino acid polymers as well as amino acid polymers in which one or more amino acid residues is a non-natural amino acid. Additionally, such "polypeptides," "peptides" and "proteins" include amino acid chains of any length, including full length proteins, wherein the amino acid residues are linked by covalent peptide bonds.

[159] As used herein, "partly non-peptidic" refers to a molecule wherein a portion of the molecule is a chemical compound or substituent that has biological activity and that does not comprises a sequence of amino acids.

[160] As used herein, "non-peptidic" refers to a molecule has biological activity and that does not comprise a sequence of amino acids.

[161] The term "post-translationally modified" refers to any modification of a natural or non-natural amino acid which occurs after such an amino acid has been translationally incorporated into a polypeptide chain. Such modifications include, but are not limited to, co-translational in vivo modifications, co-translational in vitro modifications (such as in a cell-free translation system), post-translational in vivo modifications, and post-translational in vitro modifications.

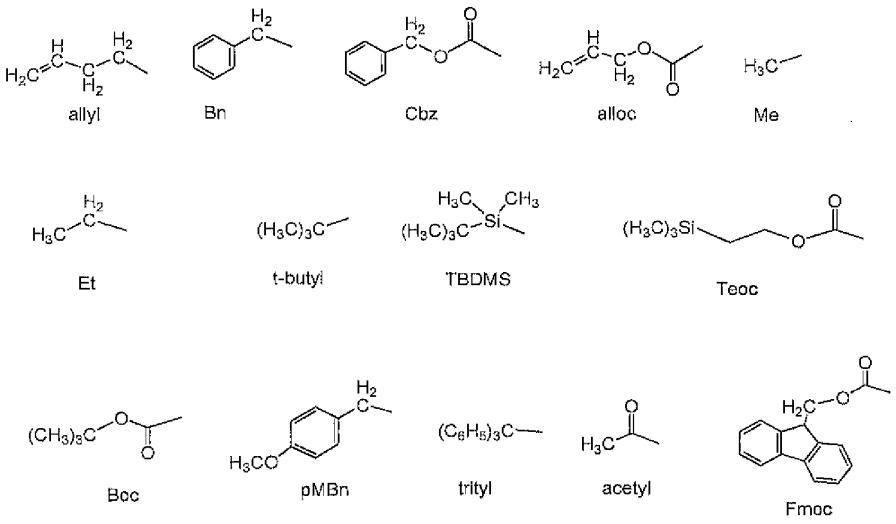
[162] The terms "prodrug" or "pharmaceutically acceptable prodrug," as used herein, refers to an agent that is converted into the parent drug in vivo or in vitro, wherein which does not abrogate the biological activity or properties of the drug, and is relatively nontoxic, i.e., the material may be administered to an individual without causing undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained. Prodrugs are generally drug precursors that, following administration to a subject and subsequent absorption, are converted to an active, or a more active species via some process, such as conversion by a metabolic pathway. Some prodrugs have a chemical group present on the prodrug that renders it less active and/or confers solubility or some other property to the drug. Once the chemical group has been cleaved and/or modified from the prodrug the active drug is generated. Prodrugs are converted into active drug within the body through enzymatic or non-enzymatic reactions. Prodrugs may provide improved physiochemical properties such as better solubility, enhanced delivery characteristics, such as specifically targeting a particular cell, tissue, organ or ligand, and improved therapeutic value of the drug. The benefits of such prodrugs include, but are not limited to, (i) ease of

administration compared with the parent drug; (ii) the prodrug may be bioavailable by oral administration whereas the parent is not; and (iii) the prodrug may also have improved solubility in pharmaceutical compositions compared with the parent drug. A pro-drug includes a pharmacologically inactive, or reduced-activity, derivative of an active drug. Prodrugs may be designed to modulate the amount of a drug or biologically active molecule that reaches a desired site of action through the manipulation of the properties of a drug, such as physiochemical, biopharmaceutical, or pharmacokinetic properties. An example, without limitation, of a prodrug would be a non-natural amino acid polypeptide which is administered as an ester (the "prodrug") to facilitate transmittal across a cell membrane where water solubility is detrimental to mobility but which then is metabolically hydrolyzed to the carboxylic acid, the active entity, once inside the cell where water solubility is beneficial. Prodrugs may be designed as reversible drug derivatives, for use as modifiers to enhance drug transport to site-specific tissues.

[163] The term "prophylactically effective amount," as used herein, refers that amount of a composition containing at least one non-natural amino acid polypeptide or at least one modified non-natural amino acid polypeptide prophylactically applied to a patient which will relieve to some extent one or more of the symptoms of a disease, condition or disorder being treated. In such prophylactic applications, such amounts may depend on the patient's state of health, weight, and the like. It is considered well within the skill of the art for one to determine such prophylactically effective amounts by routine experimentation, including, but not limited to, a dose escalation clinical trial.

[164] The term "protected," as used herein, refers to the presence of a "protecting group" or moiety that prevents reaction of the chemically reactive functional group under certain reaction conditions. The protecting group will vary depending on the type of chemically reactive group being protected. By way of example only, (i) if the chemically reactive group is an amine or a hydrazide, the protecting group may be selected from tert-butyloxycarbonyl (t-Boc) and 9-fluorenylmethoxycarbonyl (Fmoc); (ii) if the chemically reactive group is a thiol, the protecting group may be orthopyridyldisulfide; and (iii) if the chemically reactive group is a carboxylic acid, such as butanoic or propionic acid, or a hydroxyl group, the protecting group may be benzyl or an alkyl group such as methyl, ethyl, or tert-butyl.

[165] By way of example only, blocking/protecting groups may be selected from:



[166] Additionally, protecting groups include, but are not limited to, including photolabile groups such as Nvoc and MeNvoc and other protecting groups known in the art. Other protecting groups are described in Greene and Wuts, Protective Groups in Organic Synthesis, 3rd Ed., John Wiley & Sons, New York, NY, 1999, which is incorporated herein by reference in its entirety.

[167] The term "radioactive moiety," as used herein, refers to a group whose nuclei spontaneously give off nuclear radiation, such as alpha, beta, or gamma particles; wherein, alpha particles are helium nuclei, beta particles are electrons, and gamma particles are high energy photons.

[168] The term "reactive compound," as used herein, refers to a compound which under appropriate conditions is reactive toward another atom, molecule or compound.

[169] The term "recombinant host cell," also referred to as "host cell," refers to a cell which includes an exogenous polynucleotide, wherein the methods used to insert the exogenous polynucleotide into a cell include, but are not limited to, direct uptake, transduction, f-mating, or other methods known in the art to create recombinant host cells. By way of example only, such exogenous polynucleotide may be a nonintegrated vector, including but not limited to a plasmid, or may be integrated into the host genome.

[170] The term "redox-active agent," as used herein, refers to a molecule which oxidizes or reduces another molecule, whereby the redox active agent becomes reduced or oxidized. Examples of redox active agent include, but are not limited to, ferrocene, quinones, $Ru^{2+/3+}$ complexes, $Co^{2+/3+}$ complexes, and $Os^{2+/3+}$ complexes.

[171] The term "reducing agent," as used herein, refers to a compound or material which is capable of adding an electron to a compound being reduced. By way of example reducing agents include, but are not limited to, dithiothreitol (DTT), 2-mercaptoethanol, dithioerythritol, cysteine, cysteamine (2-aminoethanethiol), and reduced glutathione. Such reducing agents may be used, by way of example only, to maintain sulfhydryl groups in the reduced state and to reduce intra- or intermolecular disulfide bonds.

[172] "Refolding," as used herein describes any process, reaction or method which transforms an improperly folded or unfolded state to a native or properly folded conformation. By way of example only, refolding transforms disulfide bond containing polypeptides from an improperly folded or unfolded state to a native or properly folded conformation with respect to disulfide bonds. Such disulfide bond containing polypeptides may be natural amino acid polypeptides or non-natural amino acid polypeptides.

[173] The term "resin," as used herein, refers to high molecular weight, insoluble polymer beads. By way of example only, such beads may be used as supports for solid phase peptide synthesis, or sites for attachment of molecules prior to purification.

[174] The term "saccharide," as used herein, refers to a series of carbohydrates including but not limited to sugars, monosaccharides, oligosaccharides, and polysaccharides.

[175] The term "safety" or "safety profile," as used herein, refers to side effects that might be related to administration of a drug relative to the number of times the drug has been administered. By way of example, a drug which has been administered many times and produced only mild or no side effects is said to have an excellent safety profile. A non-limiting example of a method to evaluate the safety profile is given in example 26. This method may be used for evaluating the safety profile of any polypeptide.

[176] The phrase "selectively hybridizes to" or "specifically hybridizes to," as used herein, refers to the binding, duplexing, or hybridizing of a molecule to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture including but not limited to, total cellular or library DNA or RNA.

[177] The term "spin label," as used herein, refers to molecules which contain an atom or a group of atoms exhibiting an unpaired electron spin (i.e. a stable paramagnetic group) that can be detected by electron spin resonance spectroscopy and can be attached to another molecule. Such spin-label molecules include, but are not limited to, nitryl radicals and nitroxides, and may be single spin-labels or double spin-labels.

[178] The term "stoichiometric," as used herein, refers to the ratio of the moles of compounds participating in a chemical reaction being about 0.9 to about 1.1.

[179] The term "stoichiometric-like," as used herein, refers to a chemical reaction which becomes stoichiometric or near-stoichiometric upon changes in reaction conditions or in the presence of additives. Such changes in reaction conditions include, but are not limited to, an increase in temperature or change in pH. Such additives include, but are not limited to, accelerants.

[180] The phrase "stringent hybridization conditions" refers to hybridization of sequences of DNA, RNA, PNA or other nucleic acid mimics, or combinations thereof, under conditions of low ionic strength and high temperature. By way of example, under stringent conditions a probe will hybridize to its target subsequence in a complex mixture of nucleic acid (including but not limited to, total cellular or library DNA or RNA) but does not hybridize to other sequences in the complex mixture. Stringent conditions are sequence-dependent and will be different in different circumstances. By way of example, longer sequences hybridize specifically at higher temperatures. Stringent hybridization conditions include, but are not limited to, (i) about 5-10 ^{o}C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH; (ii) the salt concentration is about 0.01 M to about 1.0 M at about pH 7.0 to about pH 8.3 and the temperature is at least about 30 ^{o}C for short probes (including but not limited to, about 10 to about 50 nucleotides) and at least about 60 ^{o}C for long probes (including but not limited to, greater than 50 nucleotides); (iii) the addition of destabilizing agents including, but not limited to, formamide, (iv) 50% formamide, 5X SSC, and 1% SDS, incubating at 42 ^{o}C, or 5X SSC, about 1% SDS, incubating at 65 ^{o}C, with wash in 0.2X SSC, and about 0.1% SDS at 65 ^{o}C for between about 5 minutes to about 120 minutes. By way of example only, detection of selective or specific hybridization, includes, but is not limited to, a positive signal at least two times background. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993).

[181] The term "subject" as used herein, refers to an animal which is the object of treatment, observation or experiment. By way of example only, a subject may be, but is not limited to, a mammal including, but not limited to, a human.

[182] The term "substantially purified," as used herein, refers to a component of interest that may be substantially or essentially free of other components which normally accompany

or interact with the component of interest prior to purification. By way of example only, a component of interest may be "substantially purified" when the preparation of the component of interest contains less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1% (by dry weight) of contaminating components. Thus, a "substantially purified" component of interest may have a purity level of about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or greater. By way of example only, a natural amino acid polypeptide or a non-natural amino acid polypeptide may be purified from a native cell, or host cell in the case of recombinantly produced natural amino acid polypeptides or non-natural amino acid polypeptides. By way of example a preparation of a natural amino acid polypeptide or a non-natural amino acid polypeptide may be "substantially purified" when the preparation contains less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1% (by dry weight) of contaminating material. By way of example when a natural amino acid polypeptide or a non-natural amino acid polypeptide is recombinantly produced by host cells, the natural amino acid polypeptide or non-natural amino acid polypeptide may be present at about 30%, about 25%, about 20%, about 15%, about 10%, about 5%, about 4%, about 3%, about 2%, or about 1% or less of the dry weight of the cells. By way of example when a natural amino acid polypeptide or a non-natural amino acid polypeptide is recombinantly produced by host cells, the natural amino acid polypeptide or non-natural amino acid polypeptide may be present in the culture medium at about 5g/L, about 4g/L, about 3g/L, about 2g/L, about 1g/L, about 750mg/L, about 500mg/L, about 250mg/L, about 100mg/L, about 50mg/L, about 10mg/L, or about 1mg/L or less of the dry weight of the cells. By way of example, "substantially purified" natural amino acid polypeptides or non-natural amino acid polypeptides may have a purity level of 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 95%, about 99% or greater as determined by appropriate methods, including, but not limited to, SDS/PAGE analysis, RP-HPLC, SEC, and capillary electrophoresis.

[183] The term "substituents" also referred to as "non-interfering substituents" "refers to groups which may be used to replace another group on a molecule. Such groups include, but are not limited to, halo, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, C_1-C_{10} alkoxy, C_5-C_{12}

aralkyl, C_3-C_{12} cycloalkyl, C_4-C_{12} cycloalkenyl, phenyl, substituted phenyl, toluolyl, xylenyl, biphenyl, C_2-C_{12} alkoxyalkyl, C_5-C_{12} alkoxyaryl, C_5-C_{12} aryloxyalkyl, C_7-C_{12} oxyaryl, C_1-C_6 alkylsulfinyl, C_1-C_{10} alkylsulfonyl, -$(CH_2)_m$-O-(C_1-C_{10} alkyl) wherein m is from 1 to 8, aryl, substituted aryl, substituted alkoxy, fluoroalkyl, heterocyclic radical, substituted heterocyclic radical, nitroalkyl, -NO_2, -CN, -NRC(O)-(C_1-C_{10} alkyl), -C(O)-(C_1-C_{10} alkyl), C_2-C_{10} alkthioalkyl, -C(O)O-(C_1-C_{10} alkyl), -OH, -SO_2, =S, -COOH, -NR_2, carbonyl, -C(O)-(C_1-C_{10} alkyl)-CF_3, -C(O)-CF_3, -C(O)NR_2, -(C_1-C_{10} aryl)-S-(C_6-C_{10} aryl), -C(O)-(C_6-C_{10} aryl), -$(CH_2)_m$-O-$(CH_2)_m$-O-(C_1-C_{10} alkyl) wherein each m is from 1 to 8, -C(O)NR_2, -C(S)NR_2, -SO_2NR_2, -NRC(O)NR_2, -NRC(S)NR_2, salts thereof, and the like. Each R group in the preceding list includes, but is not limited to, H, alkyl or substituted alkyl, aryl or substituted aryl, or alkaryl. Where substituent groups are specified by their conventional chemical formulas, written from left to right, they equally encompass the chemically identical substituents that would result from writing the structure from right to left; for example, -CH_2O- is equivalent to –OCH_2-.

[184] By way of example only, substituents for alkyl and heteroalkyl radicals (including those groups referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) includes, but is not limited to: -OR, =O, =NR, =N-OR, -NR_2, -SR, -halogen, -SiR_3, -OC(O)R, -C(O)R, -CO_2R, -$CONR_2$, -OC(O)NR_2, -NRC(O)R, -NRC(O)NR_2, -$NR(O)_2R$, -NR-C(NR_2)=NR, -S(O)R, -$S(O)_2R$, -$S(O)_2NR_2$, -$NRSO_2R$, -CN and –NO_2. Each R group in the preceding list includes, but is not limited to, hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, including but not limited to, aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or aralkyl groups. When two R groups are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, -NR_2 is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl.

[185] By way of example, substituents for aryl and heteroaryl groups include, but are not limited to, -OR, =O, =NR, =N-OR, -NR_2, -SR, -halogen, -SiR_3, -OC(O)R, -C(O)R, -CO_2R, -$CONR_2$, -OC(O)NR_2, -NRC(O)R, -NRC(O)NR_2, -$NR(O)_2R$, -NR-C(NR_2)=NR, -S(O)R, -$S(O)_2R$, -$S(O)_2NR_2$, -$NRSO_2R$, -CN, –NO_2, -R, -N_3, -$CH(Ph)_2$, fluoro(C_1-C_4)alkoxy, and fluoro(C_1-C_4)alkyl, in a number ranging from zero to the total number of open valences on the aromatic ring system; and where each R group in the preceding list includes, but is not limited to, hydrogen, alkyl, heteroalkyl, aryl and heteroaryl.

[186] The term "therapeutically effective amount," as used herein, refers to the amount of a composition containing at least one non-natural amino acid polypeptide and/or at least one modified non-natural amino acid polypeptide administered to a patient already suffering from a disease, condition or disorder, sufficient to cure or at least partially arrest, or relieve to some extent one or more of the symptoms of the disease, disorder or condition being treated. The effectiveness of such compositions depend conditions including, but not limited to, the severity and course of the disease, disorder or condition, previous therapy, the patient's health status and response to the drugs, and the judgment of the treating physician. By way of example only, therapeutically effective amounts may be determined by routine experimentation, including but not limited to a dose escalation clinical trial.

[187] The term "thioalkoxy," as used herein, refers to sulfur containing alkyl groups linked to molecules via an oxygen atom.

[188] The term "thermal melting point" or Tm is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of probes complementary to a target hybridize to the target sequence at equilibrium.

[189] The terms "treat," "treating" or "treatment", as used herein, include alleviating, abating or ameliorating a disease or condition symptoms, preventing additional symptoms, ameliorating or preventing the underlying metabolic causes of symptoms, inhibiting the disease or condition, e.g., arresting the development of the disease or condition, relieving the disease or condition, causing regression of the disease or condition, relieving a condition caused by the disease or condition, or stopping the symptoms of the disease or condition. The terms "treat," "treating" or "treatment", include, but are not limited to, prophylactic and/or therapeutic treatments.

[190] As used herein, the term "water soluble polymer" refers to any polymer that is soluble in aqueous solvents. Such water soluble polymers include, but are not limited to, polyethylene glycol, polyethylene glycol propionaldehyde, mono C_1-C_{10} alkoxy or aryloxy derivatives thereof (described in U.S. Patent No. 5,252,714 which is incorporated by reference herein), monomethoxy-polyethylene glycol, polyvinyl pyrrolidone, polyvinyl alcohol, polyamino acids, divinylether maleic anhydride, N-(2-Hydroxypropyl)-methacrylamide, dextran, dextran derivatives including dextran sulfate, polypropylene glycol, polypropylene oxide/ethylene oxide copolymer, polyoxyethylated polyol, heparin, heparin fragments, polysaccharides, oligosaccharides, glycans, cellulose and cellulose derivatives, including but not limited to methylcellulose and carboxymethyl cellulose, serum albumin,

starch and starch derivatives, polypeptides, polyalkylene glycol and derivatives thereof, copolymers of polyalkylene glycols and derivatives thereof, polyvinyl ethyl ethers, and alpha-beta-poly[(2-hydroxyethyl)-DL-aspartamide, and the like, or mixtures thereof. By way of example only, coupling of such water soluble polymers to natural amino acid polypeptides or non-natural polypeptides may result in changes including, but not limited to, increased water solubility, increased or modulated serum half-life, increased or modulated therapeutic half-life relative to the unmodified form, increased bioavailability, modulated biological activity, extended circulation time, modulated immunogenicity, modulated physical association characteristics including, but not limited to, aggregation and multimer formation, altered receptor binding, altered binding to one or more binding partners, and altered receptor dimerization or multimerization. In addition, such water soluble polymers may or may not have their own biological activity.

[191] Unless otherwise indicated, conventional methods of mass spectroscopy, NMR, HPLC, protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art are employed.

[192] Compounds, (including, but not limited to non-natural amino acids, non-natural amino acid polypeptides, modified non-natural amino acid polypeptides, and reagents for producing the aforementioned compounds) presented herein include isotopically-labeled compounds, which are identical to those recited in the various formulas and structures presented herein, but for the fact that one or more atoms are replaced by an atom having an atomic mass or mass number different from the atomic mass or mass number usually found in nature. Examples of isotopes that can be incorporated into the present compounds include isotopes of hydrogen, carbon, nitrogen, oxygen, fluorine and chlorine, such as ^{2}H, ^{3}H, ^{13}C, ^{14}C, ^{15}N, ^{18}O, ^{17}O, ^{35}S, ^{18}F, ^{36}Cl, respectively. Certain isotopically-labeled compounds described herein, for example those into which radioactive isotopes such as ^{3}H and ^{14}C are incorporated, are useful in drug and/or substrate tissue distribution assays. Further, substitution with isotopes such as deuterium, i.e., ^{2}H, can afford certain therapeutic advantages resulting from greater metabolic stability, for example increased in vivo half-life or reduced dosage requirements.

[193] Some of the compounds herein (including, but not limited to non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides, and reagents for producing the aforementioned compounds) have asymmetric carbon atoms and can therefore exist as enantiomers or diastereomers. Diasteromeric mixtures can be separated

into their individual diastereomers on the basis of their physical chemical differences by methods known, for example, by chromatography and/or fractional crystallization. Enantiomers can be separated by converting the enantiomeric mixture into a diastereomeric mixture by reaction with an appropriate optically active compound (e.g., alcohol), separating the diastereomers and converting (e.g., hydrolyzing) the individual diastereomers to the corresponding pure enantiomers. All such isomers, including diastereomers, enantiomers, and mixtures thereof are considered as part of the compositions described herein.

[194] In additional or further embodiments, the compounds described herein (including, but not limited to non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides, and reagents for producing the aforementioned compounds) are used in the form of pro-drugs. In additional or further embodiments, the compounds described herein ((including, but not limited to non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides, and reagents for producing the aforementioned compounds) are metabolized upon administration to an organism in need to produce a metabolite that is then used to produce a desired effect, including a desired therapeutic effect. In further or additional embodiments are active metabolites of non-natural amino acids and "modified or unmodified" non-natural amino acid polypeptides.

[195] The methods and formulations described herein include the use of N-oxides, crystalline forms (also known as polymorphs), or pharmaceutically acceptable salts of non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides. In certain embodiments, non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides may exist as tautomers. All tautomers are included within the scope of the non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides presented herein. In addition, the non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides described herein can exist in unsolvated as well as solvated forms with pharmaceutically acceptable solvents such as water, ethanol, and the like. The solvated forms of the non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides presented herein are also considered to be disclosed herein.

[196] Some of the compounds herein (including, but not limited to non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides and

reagents for producing the aforementioned compounds) may exist in several tautomeric forms. All such tautomeric forms are considered as part of the compositions described herein. Also, for example all enol-keto forms of any compounds (including, but not limited to non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides and reagents for producing the aforementioned compounds) herein are considered as part of the compositions described herein.

[197] Some of the compounds herein (including, but not limited to non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides and reagents for producing either of the aforementioned compounds) are acidic and may form a salt with a pharmaceutically acceptable cation. Some of the compounds herein (including, but not limited to non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides and reagents for producing the aforementioned compounds) can be basic and accordingly, may form a salt with a pharmaceutically acceptable anion. All such salts, including di-salts are within the scope of the compositions described herein and they can be prepared by conventional methods. For example, salts can be prepared by contacting the acidic and basic entities, in either an aqueous, non-aqueous or partially aqueous medium. The salts are recovered by using at least one of the following techniques: filtration, precipitation with a non-solvent followed by filtration, evaporation of the solvent, or, in the case of aqueous solutions, lyophilization.

[198] Pharmaceutically acceptable salts of the non-natural amino acid polypeptides disclosed herein may be formed when an acidic proton present in the parent non-natural amino acid polypeptides either is replaced by a metal ion, by way of example an alkali metal ion, an alkaline earth ion, or an aluminum ion; or coordinates with an organic base. In addition, the salt forms of the disclosed non-natural amino acid polypeptides can be prepared using salts of the starting materials or intermediates. The non-natural amino acid polypeptides described herein may be prepared as a pharmaceutically acceptable acid addition salt (which is a type of a pharmaceutically acceptable salt) by reacting the free base form of non-natural amino acid polypeptides described herein with a pharmaceutically acceptable inorganic or organic acid. Alternatively, the non-natural amino acid polypeptides described herein may be prepared as pharmaceutically acceptable base addition salts (which are a type of a pharmaceutically acceptable salt) by reacting the free acid form of non-natural amino acid polypeptides described herein with a pharmaceutically acceptable inorganic or organic base.

[199] The type of pharmaceutical acceptable salts, include, but are not limited to: (1) acid addition salts, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like; or formed with organic acids such as acetic acid, propionic acid, hexanoic acid, cyclopentanepropionic acid, glycolic acid, pyruvic acid, lactic acid, malonic acid, succinic acid, malic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, 3-(4-hydroxybenzoyl)benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, 1,2-ethanedisulfonic acid, 2-hydroxyethanesulfonic acid, benzenesulfonic acid, 2-naphthalenesulfonic acid, 4-methylbicyclo-[2.2.2]oct-2-ene-1-carboxylic acid, glucoheptonic acid, 4,4'-methylenebis-(3-hydroxy-2-ene-1 -carboxylic acid), 3-phenylpropionic acid, trimethylacetic acid, tertiary butylacetic acid, lauryl sulfuric acid, gluconic acid, glutamic acid, hydroxynaphthoic acid, salicylic acid, stearic acid, muconic acid, and the like; (2) salts formed when an acidic proton present in the parent compound either is replaced by a metal ion, e.g., an alkali metal ion, an alkaline earth ion, or an aluminum ion; or coordinates with an organic base. Acceptable organic bases include ethanolamine, diethanolamine, triethanolamine, tromethamine, N-methylglucamine, and the like. Acceptable inorganic bases include aluminum hydroxide, calcium hydroxide, potassium hydroxide, sodium carbonate, sodium hydroxide, and the like.

[200] The corresponding counterions of the non-natural amino acid polypeptide pharmaceutical acceptable salts may be analyzed and identified using various methods including, but not limited to, ion exchange chromatography, ion chromatography, capillary electrophoresis, inductively coupled plasma, atomic absorption spectroscopy, mass spectrometry, or any combination thereof. In addition, the therapeutic activity of such non-natural amino acid polypeptide pharmaceutical acceptable salts may be tested using the techniques and methods described in examples 87-91.

[201] It should be understood that a reference to a salt includes the solvent addition forms or crystal forms thereof, particularly solvates or polymorphs. Solvates contain either stoichiometric or non-stoichiometric amounts of a solvent, and are often formed during the process of crystallization with pharmaceutically acceptable solvents such as water, ethanol, and the like. Hydrates are formed when the solvent is water, or alcoholates are formed when the solvent is alcohol. Polymorphs include the different crystal packing arrangements of the same elemental composition of a compound. Polymorphs usually have different X-ray diffraction patterns, infrared spectra, melting points, density, hardness, crystal shape, optical and electrical properties, stability, and solubility. Various factors such as the recrystallization

solvent, rate of crystallization, and storage temperature may cause a single crystal form to dominate.

[202] The screening and characterization of non-natural amino acid polypeptide pharmaceutical acceptable salts polymorphs and/or solvates may be accomplished using a variety of techniques including, but not limited to, thermal analysis, x-ray diffraction, spectroscopy, vapor sorption, and microscopy. Thermal analysis methods address thermo chemical degradation or thermo physical processes including, but not limited to, polymorphic transitions, and such methods are used to analyze the relationships between polymorphic forms, determine weight loss, to find the glass transition temperature, or for excipient compatibility studies. Such methods include, but are not limited to, Differential scanning calorimetry (DSC), Modulated Differential Scanning Calorimetry (MDCS), Thermogravimetric analysis (TGA), and Thermogravi-metric and Infrared analysis (TG/IR). X-ray diffraction methods include, but are not limited to, single crystal and powder diffractometers and synchrotron sources. The various spectroscopic techniques used include, but are not limited to, Raman, FTIR, UVIS, and NMR (liquid and solid state). The various microscopy techniques include, but are not limited to, polarized light microscopy, Scanning Electron Microscopy (SEM) with Energy Dispersive X-Ray Analysis (EDX), Environmental Scanning Electron Microscopy with EDX (in gas or water vapor atmosphere), IR microscopy, and Raman microscopy.

INCORPORATION BY REFERENCE

[203] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.ate of crystallization, and storage temperature may cause a single crystal form to dominate.

BRIEF DESCRIPTION OF THE DRAWINGS

[204] 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 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:

[205] Figure 1 presents dexamethasone-hydroxylamine linker conjugation with para-acetyl phenylalanine (pAF).

[206] Figure 2 (A) is an SDS-PAGE analysis of the Figure 1 conjugation. The left-most arrow shows pAF; the middle arrow shows dexamethasone-hydroxylamine; the peak indicated by the right-most arrow shows the dexamethasone-hydroxylamine linker conjugation with pAF.

[207] Figure 2 (B) is an SDS-PAGE analysis of the Figure 1 conjugation. The left-most arrow shows pAF; the middle arrow shows dexamethasone-hydroxylamine; the peak indicated by the right-most arrow shows the dexamethasone-hydroxylamine linker conjugation with pAF.

[208] Figure 2 (C) is an SDS-PAGE analysis of the Figure 1 conjugation. The peak indicated by the right-most arrow shows the dexamethasone-hydroxylamine linker conjugation with pAF.

[209] Figure 3 (A) is a mass spectra analysis of the intact mass of the heavy chain of the monoclonal antibody plus dexamethasone conjugation reaction (reduced) and the peaks represent different conjugations including, in the far right peak, dexamethasone-linker oligomers.

[210] Figure 3 (B) is a mass spectra analysis of the intact mass of the light chain of the monoclonal antibody plus dexamethasone conjugation reaction (reduced).

[211] Figure 4 is a schematic of dexamethasone and cleavable linkers with [2+3] chemistry.

[212] Figure 5 is a schematic showing new analogs and linkers based on Mometasone furoate.

[213] Figure 6 is a schematic of a non-limiting example of a linker designed for dexamethasone.

[214] Figure 7 is a schematic of the chemical structures of SAR and Dexamethasone analogs, including: Dexamethasone (receptor affinity of 100); Budesonide (receptor affinity 855); Mometasone furoate (receptor affinity 2245); and Fluticasone furoate (receptor affinity 2989).

[215] Figure 8 is a schematic of the synthesis detailed in Example 1 (below).

[216] Figure 9 is a schematic of the synthesis detailed in Example 2 (below).

[217] Figure 10 is a schematic of the synthesis detailed in Example 3 (below).

[218] Figure 11 is a schematic of the synthesis detailed in Example 4 (below).

[219] Figure 12 is a schematic of the synthesis detailed in Example 5 (below).

[220] Figure 13 is a schematic of the synthesis detailed in Example 6 (below).

DETAILED DESCRIPTION OF THE INVENTION

[221] 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.

I. Introduction

[222] Recently, an entirely new technology in the protein sciences has been reported, which promises to overcome many of the limitations associated with site-specific modifications of proteins. Specifically, new components have been added to the protein biosynthetic machinery of the prokaryote *Escherichia coli* (*E. coli*) (e.g., L. Wang, et al., (2001), Science 292:498-500) and the eukaryote *Saccharomyces cerevisiae* (*S. cerevisiae*) (e.g., J. Chin et al., Science 301:964-7 (2003)), which has enabled the incorporation of non-natural amino acids to proteins *in vivo*. A number of new amino acids with novel chemical, physical or biological properties, including photoaffinity labels and photoisomerizable amino acids, keto amino acids, and glycosylated amino acids have been incorporated efficiently and with high fidelity into proteins in *E. coli* and in yeast in response to the amber codon, TAG, using this methodology. *See, e.g.,* J. W. Chin et al., (2002), Journal of the American Chemical Society 124:9026-9027 (incorporated by reference in its entirety); J. W. Chin, & P. G. Schultz, (2002), ChemBioChem 3(11):1135-1137 (incorporated by reference in its entirety); J. W. Chin, et al., (2002), PNAS United States of America 99(17):11020-11024 (incorporated by reference in its entirety); and, L. Wang, & P. G. Schultz, (2002), Chem. Comm., 1-11 (incorporated by reference in its entirety). These studies have demonstrated that it is possible to selectively and routinely introduce chemical functional groups that are not found in proteins, that are chemically inert to all of the functional groups found in the 20 common, genetically-encoded amino acids and that may be used to react efficiently and selectively to form stable covalent linkages.

II. Overview

[223] At one level, described herein are the tools (methods, compositions, techniques) for creating and using NRL conjugates including nuclear receptor ligand (NRL) linker

derivatives or analogs, comprising at least one carbonyl, dicarbonyl, oxime, hydroxylamine, aldehyde, protected aldehyde, ketone, protected ketone, thioester, ester, dicarbonyl, hydrazine, azide, amidine, imine, diamine, keto-amine, keto-alkyne, alkyne, cycloalkyne, or ene-dione. At another level, described herein are the tools (methods, compositions, techniques) for creating and using NRL conjugates including NRL linker derivatives or analogs, comprising at least one non-natural amino acid or modified non-natural amino acid with an oxime, aromatic amine, heterocycle (e.g., indole, quinoxaline, phenazine, pyrazole, triazole, etc.).

[224] Such NRL conjugates comprising non-natural amino acids may contain further functionality, including but not limited to, a polymer; a water-soluble polymer; a derivative of polyethylene glycol; a second protein or polypeptide or polypeptide analog; an antibody or antibody fragment; and any combination thereof. Note that the various aforementioned functionalities are not meant to imply that the members of one functionality cannot be classified as members of another functionality. Indeed, there will be overlap depending upon the particular circumstances. By way of example only, a water-soluble polymer overlaps in scope with a derivative of polyethylene glycol, however the overlap is not complete and thus both functionalities are cited above.

III. Nuclear Receptor Ligand Conjugates and Derivatives

[225] At one level, described herein are the tools (methods, compositions, techniques) for creating and using NRL conjugates, including NRL linker derivatives or analogs, comprising at least one non-natural amino acid or modified non-natural amino acid with a carbonyl, dicarbonyl, oxime or hydroxylamine group. Such NRL conjugates comprising non-natural amino acids may contain further functionality, including but not limited to, a polymer; a water-soluble polymer; a derivative of polyethylene glycol; a second protein or polypeptide or polypeptide analog; an antibody or antibody fragment; and any combination thereof. Note that the various aforementioned functionalities are not meant to imply that the members of one functionality cannot be classified as members of another functionality. Indeed, there will be overlap depending upon the particular circumstances. By way of example only, a water-soluble polymer overlaps in scope with a derivative of polyethylene glycol, however the overlap is not complete and thus both functionalities are cited above.

[226] In one aspect are methods for selecting and designing NRL conjugates including NRL linker derivatives to be modified using the methods, compositions and techniques

described herein. The new NRL conjugate or NRL linker derivative may be designed de novo, including by way of example only, as part of high-throughput screening process (in which case numerous polypeptides may be designed, synthesized, characterized and/or tested) or based on the interests of the researcher. The new NRL conjugate may also be designed based on the structure of a known or partially characterized polypeptide. The principles for selecting which amino acid(s) to substitute and/or modify are described separately herein. The choice of which modification to employ is also described herein, and can be used to meet the need of the experimenter or end user. Such needs may include, but are not limited to, manipulating the therapeutic effectiveness of the polypeptide, improving the safety profile of the polypeptide, adjusting the pharmacokinetics, pharmacologics and/or pharmacodynamics of the polypeptide, such as, by way of example only, increasing water solubility, bioavailability, increasing serum half-life, increasing therapeutic half-life, modulating immunogenicity, modulating biological activity, or extending the circulation time. In addition, such modifications include, by way of example only, providing additional functionality to the polypeptide, incorporating an antibody, and any combination of the aforementioned modifications.

[227] Also described herein are NRL conjugates that have or can be modified to contain an oxime, carbonyl, dicarbonyl, or hydroxylamine group. Included with this aspect are methods for producing, purifying, characterizing and using such NRL conjugates.

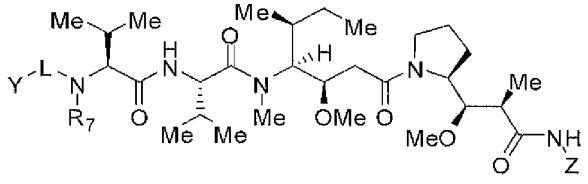
[228] The NRL conjugate may contain at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or ten or more of a carbonyl or dicarbonyl group, oxime group, hydroxylamine group, or protected forms thereof. The NRL conjugate can be the same or different, for example, there can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more different sites in the derivative that comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more different reactive groups.

A. Structure and Synthesis of Nuclear Receptor Ligand Conjugates: Electrophilic and Nucleophilic Groups

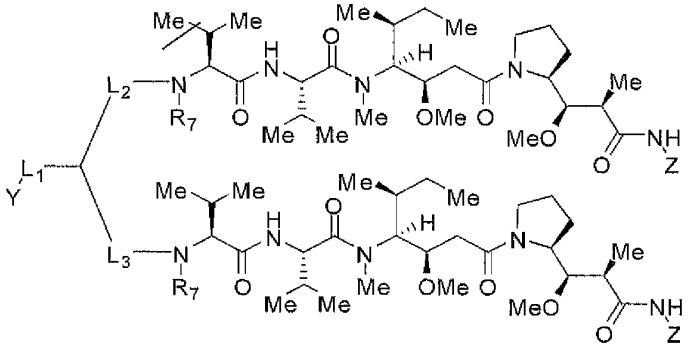
[229] Nuclear receptor ligand conjugates with linkers containing a hydroxylamine (also called an aminooxy) group allow for reaction with a variety of electrophilic groups to form conjugates (including but not limited to, with PEG or other water soluble polymers). Like hydrazines, hydrazides and semicarbazides, the enhanced nucleophilicity of the aminooxy group permits it to react efficiently and selectively with a variety of molecules that contain

carbonyl- or dicarbonyl-groups, including but not limited to, ketones, aldehydes or other functional groups with similar chemical reactivity. See, e.g., Shao, J. and Tam, J., J. Am. Chem. Soc. 117:3893-3899 (1995); H. Hang and C. Bertozzi, Acc. Chem. Res. 34(9): 727-736 (2001). Whereas the result of reaction with a hydrazine group is the corresponding hydrazone, however, an oxime results generally from the reaction of an aminooxy group with a carbonyl- or dicarbonyl-containing group such as, by way of example, a ketones, aldehydes or other functional groups with similar chemical reactivity. In some embodiments of NRL conjugates with linkers, the conjugate comprises an azide, alkyne or cycloalkyne allow for linking of molecules via cycloaddition reactions (e.g., 1,3-dipolar cycloadditions, azide-alkyne Huisgen cycloaddition, etc.). (Described in U.S. Patent No. 7,807,619 which is incorporated by reference herein to the extent relative to the reaction).

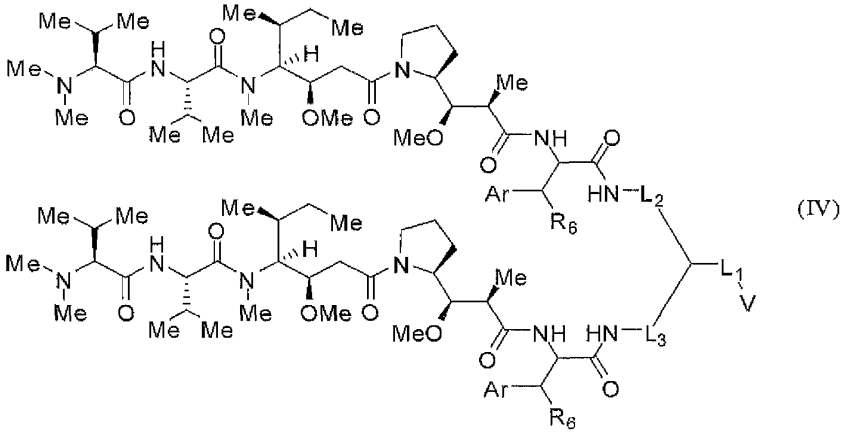
[230] Thus, in certain embodiments described herein are NRL conjugates with linkers comprising a hydroxylamine, aldehyde, protected aldehyde, ketone, protected ketone, thioester, ester, dicarbonyl, hydrazine, amidine, imine, diamine, keto-amine, keto-alkyne, and ene-dione hydroxylamine group, a hydroxylamine-like group (which has reactivity similar to a hydroxylamine group and is structurally similar to a hydroxylamine group), a masked hydroxylamine group (which can be readily converted into a hydroxylamine group), or a protected hydroxylamine group (which has reactivity similar to a hydroxylamine group upon deprotection). In some embodiments, the NRL conjugates comprise azides, alkynes or cycloalkynes. Such NRL conjugates include compounds having the structure of Formula (I), (III), (IV), (V), and (VI) wherein NRL is any nuclear receptor ligand:



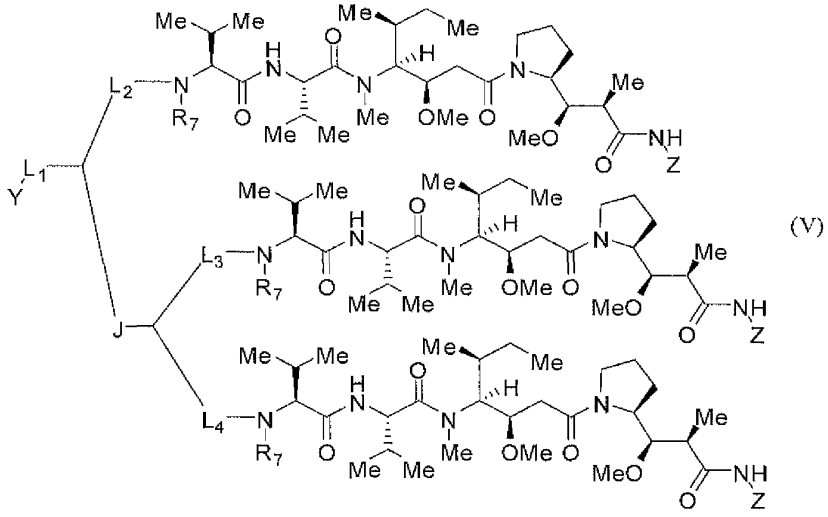
(I)



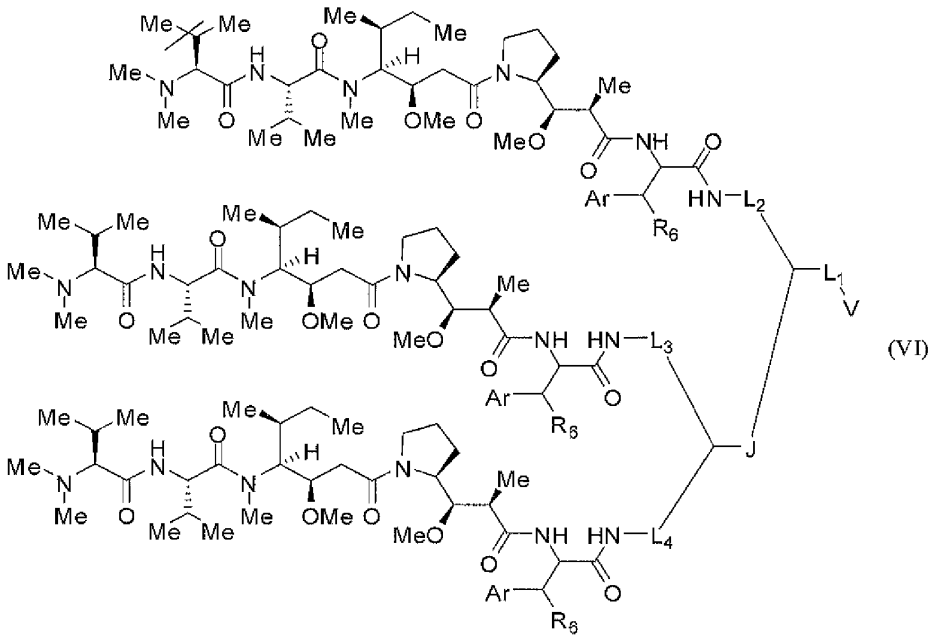
(III)



(IV)



(V)



(VI)

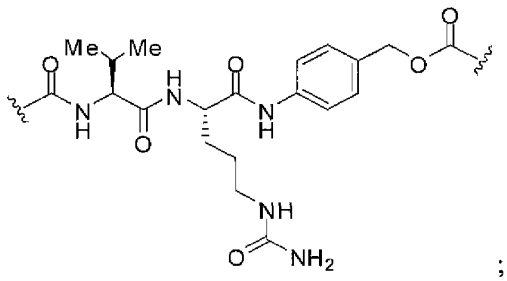
wherein:

Y and V are each selected from the group consisting of an hydroxylamine, methyl, aldehyde, protected aldehyde, ketone, protected ketone, thioester, ester, dicarbonyl, hydrazine, azide, amidine, imine, diamine, keto-amine, keto-alkyne, alkyne, cycloalkyne, and ene-dione;

L, L_1, L_2, L_3, and L_4 are each linkers selected from the group consisting of a bond, –alkylene–, –alkylene–C(O)–, –alkylene–J–, –(alkylene–O)$_n$–alkylene–, –(alkylene–O)$_n$–alkylene–C(O)–, –(alkylene–O)$_n$–J–, –(alkylene–O)$_n$–J–alkylene–, –(alkylene–O)$_n$–(CH$_2$)$_{n'}$–NHC(O)–(CH$_2$)$_{n''}$–C(Me)$_2$–S–S–(CH$_2$)$_{n'''}$–NHC(O)–(alkylene–O)$_{n''''}$–alkylene–, –(alkylene–O)$_n$–alkylene–W–, –alkylene–C(O)–W–, –(alkylene–O)$_n$–alkylene–J–, –alkylene'–J–(alkylene–O)$_n$–alkylene–, –(alkylene–O)$_n$–alkylene–J–alkylene', –J–(alkylene–O)$_n$–alkylene–, –(alkylene–O)$_n$–alkylene–J–(alkylene–O)$_{n'}$–alkylene–J'–, –W–, –alkylene–W–, alkylene'–J–(alkylene–NMe)$_n$–alkylene–W–, –J–(alkylene–NMe)$_n$–alkylene–W–, –(alkylene–O)$_n$–alkylene–U–alkylene–C(O)–, –(alkylene–O)$_n$–alkylene–U–alkylene–; –J–

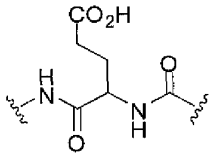
alkylene–NMe–alkylene'–NMe–alkylene''–W–, and –alkylene–J–alkylene'–NMe–alkylene''–NMe–alkylene'''–W–;

W has the structure of:

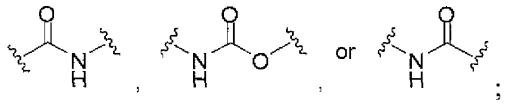


;

U has the structure of:



each J and J' independently have the structure of:

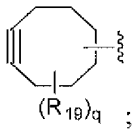


;

each n, n' n'', n''' and n'''' are independently integers greater than or equal to one.

Such NRL conjugates may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[231] In some embodiments, Y is azide. In other embodiments, Y is cycloalkyne. In specific embodiments, the cyclooctyne has a structure of:



;

each R_{19} is independently selected from the group consisting of C_1-C_6 alkyl, C_1-C_6 alkoxy, ester, ether, thioether, aminoalkyl, halogen, alkyl ester, aryl ester, amide, aryl amide, alkyl halide, alkyl amine, alkyl sulfonic acid, alkyl nitro, thioester, sulfonyl ester, halosulfonyl, nitrile, alkyl nitrile, and nitro; and

q is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11.

[232] In certain embodiments of compounds of Formula (I), (III), and (V), Y is hydroxylamine, aldehyde, protected aldehyde, ketone, protected ketone, thioester, ester, dicarbonyl, hydrazine, amidine, imine, diamine, keto-amine, keto-alkyne, or ene-dione.

[233] In certain embodiments of compounds of Formula (IV) and (VI), V is a hydroxylamine, methyl, aldehyde, protected aldehyde, ketone, protected ketone, thioester, ester, dicarbonyl, hydrazine, amidine, imine, diamine, keto-amine, keto-alkyne, and ene-dione.

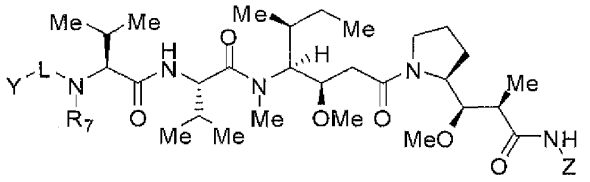
[234] In certain embodiments of compounds of Formula (I), (III), (IV), (V), and (VI), each L, L_1, L_2, L_3, and L_4 is independently a cleavable linker or non-cleavable linker. In certain embodiments of compounds of Formula (I), (III), (IV), (V), and (VI), each L, L_1, L_2, L_3, and L_4 is independently a oligo(ethylene glycol) derivatized linker.

[235] In certain embodiments of compounds of Formula (I), (III), (IV), (V), and (VI), each alkylene, alkylene', alkylene", and alkylene"' independently is $-CH_2-$, $-CH_2CH_2-$, $-CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, or $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$. In certain embodiments of compounds of Formula (XIV), (XV), (XVI), (XVII), and (XVIII), each n, n', n", n"', and n"" is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100.

B. Structure and Synthesis of Nuclear Receptor Ligand Conjugates: Hydroxylamine Groups

[236] Thus, in certain embodiments described herein are NRL conjugates comprising a hydroxylamine group, a hydroxylamine-like group (which has reactivity similar to a hydroxylamine group and is structurally similar to a hydroxylamine group), a masked hydroxylamine group (which can be readily converted into a hydroxylamine group), or a protected hydroxylamine group (which has reactivity similar to a hydroxylamine group upon

deprotection). Such NRL conjugates include compounds having the structure of Formula (I):



(I)

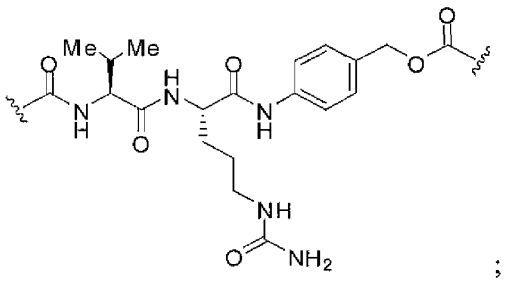
;

[237] wherein:

Y is NH_2–O– or methyl;

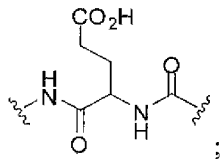
L is a linker selected from the group consisting of –alkylene–, –alkylene–C(O)–, –(alkylene–O)$_n$–alkylene–, –(alkylene–O)$_n$–alkylene–C(O)–, –(alkylene–O)$_n$–$(CH_2)_{n'}$–NHC(O)–$(CH_2)_{n'}$–C(Me)$_2$–S–S–$(CH_2)_{n'''}$–NHC(O)–(alkylene–O)$_{n''''}$–alkylene–, –(alkylene–O)$_n$–alkylene–W–, –alkylene–C(O)–W–, –(alkylene–O)$_n$–alkylene–U–alkylene–C(O)–, and –(alkylene–O)$_n$–alkylene–U–alkylene–;

W has the structure of:



;

U has the structure of:



;

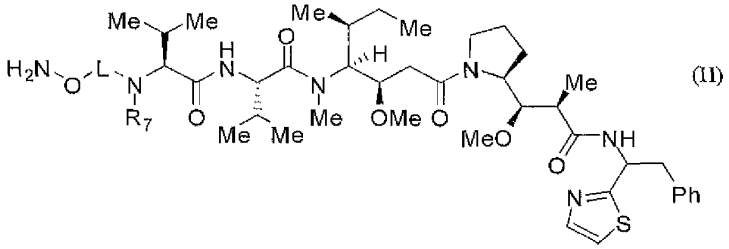
or L is absent, Y is methyl, R_5 is COR_8, and R_8 is –NH–(alkylene–O)$_n$–NH_2; and each n, n', n'', n''' and n'''' are independently integers greater than or equal to one.

[238] In certain embodiments of compounds of Formula (I), Y is hydroxylamine, aldehyde, protected aldehyde, ketone, protected ketone, thioester, ester, dicarbonyl, hydrazine, amidine, imine, diamine, keto-amine, keto-alkyne, or ene-dione. In certain embodiments of compounds of Formula (I), V is a hydroxylamine, methyl, aldehyde, protected aldehyde, ketone, protected ketone, thioester, ester, dicarbonyl, hydrazine, amidine, imine, diamine, keto-amine, keto-alkyne, and ene-dione.

[239] In certain embodiments of compounds of Formula (I), each L is independently a cleavable linker or non-cleavable linker. In certain embodiments of compounds of Formula (I), each L is independently a oligo(ethylene glycol) derivatized linker.

[240] In certain embodiments of compounds of Formula (I), alkylene is $-CH_2-$, $-CH_2CH_2-$, $-CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, or $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$. In certain embodiments of compounds of Formula (I), each n, n', n'', n''', and n'''' is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100.

[241] In certain embodiments, NRL conjugates include compounds having the structure of Formula (II):



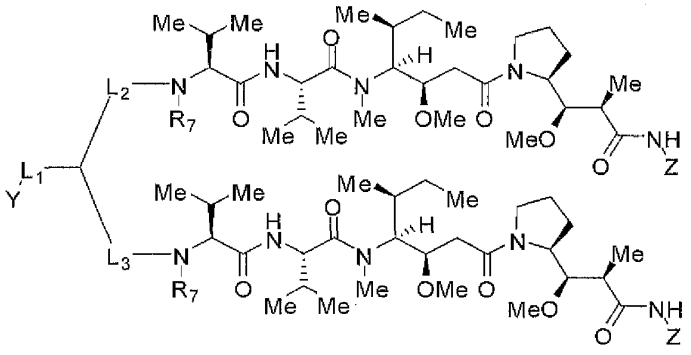
(II)

In some embodiments of compounds of Formula (II), L is $-(alkylene-O)_n-alkylene-$. In some embodiments, each alkylene is $-CH_2CH_2-$, n is equal to 3, and R_7 is methyl. In some embodiments, L is –alkylene–. In some embodiments of compounds of Formula (II), each alkylene is $-CH_2CH_2-$ and R_7 is methyl or hydrogen. In some embodiments of compounds of Formula (II), L is $-(alkylene-O)_n-alkylene-C(O)-$. In some embodiments of compounds of Formula (II), each alkylene is $-CH_2CH_2-$, n is equal to 4, and R_7 is methyl. In some embodiments of compounds of Formula (II), L is $-(alkylene-O)_n-(CH_2)_{n'}-NHC(O)-(CH_2)_{n''}-C(Me)_2-S-S-(CH_2)_{n'''}-NHC(O)-(alkylene-O)_{n''''}-alkylene-$. In some embodiments of compounds of Formula (II), each alkylene is $-CH_2CH_2-$, n is equal to 1, n' is equal to 2, n'' is equal to 1, n ''' is equal to 2, n'''' is equal to 4, and R_7 is methyl. Such NRL conjugates may be

in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

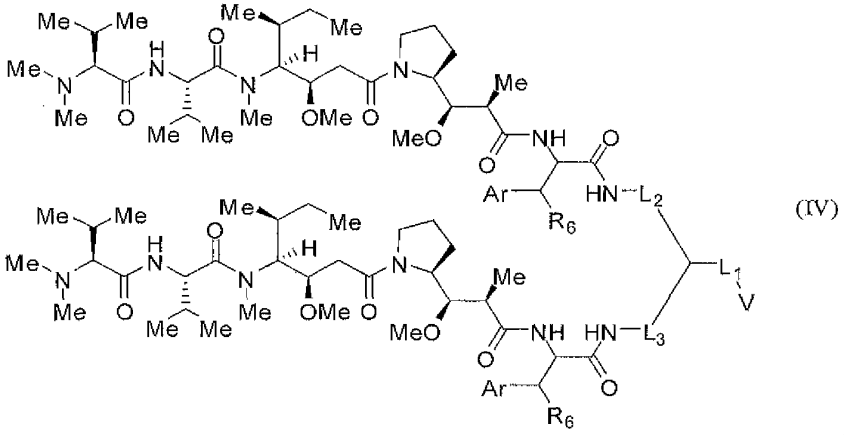
[242] In certain embodiments of compounds of Formula (II), each L is independently a cleavable linker or non-cleavable linker. In certain embodiments of compounds of Formula (II), each L is independently a oligo(ethylene glycol) derivatized linker.

[243] Such NRL conjugates include compounds having the structure of Formula (III), (IV), (V) or (VI):



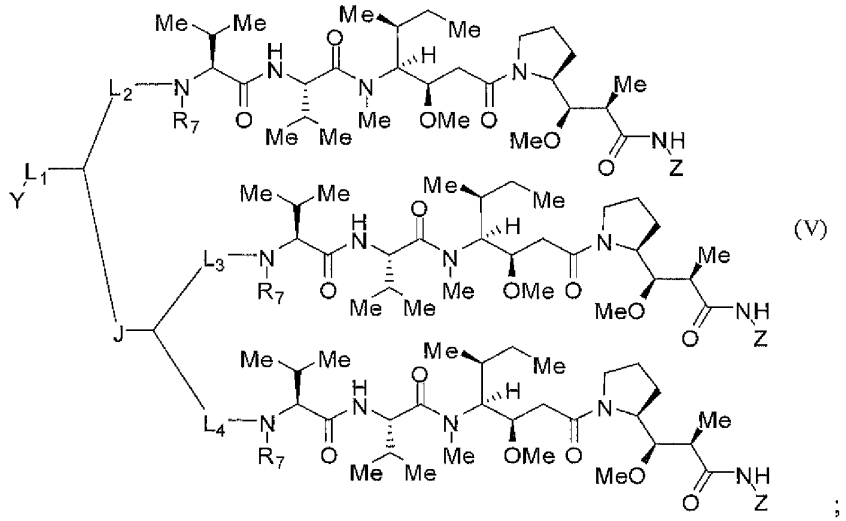
(III)

;



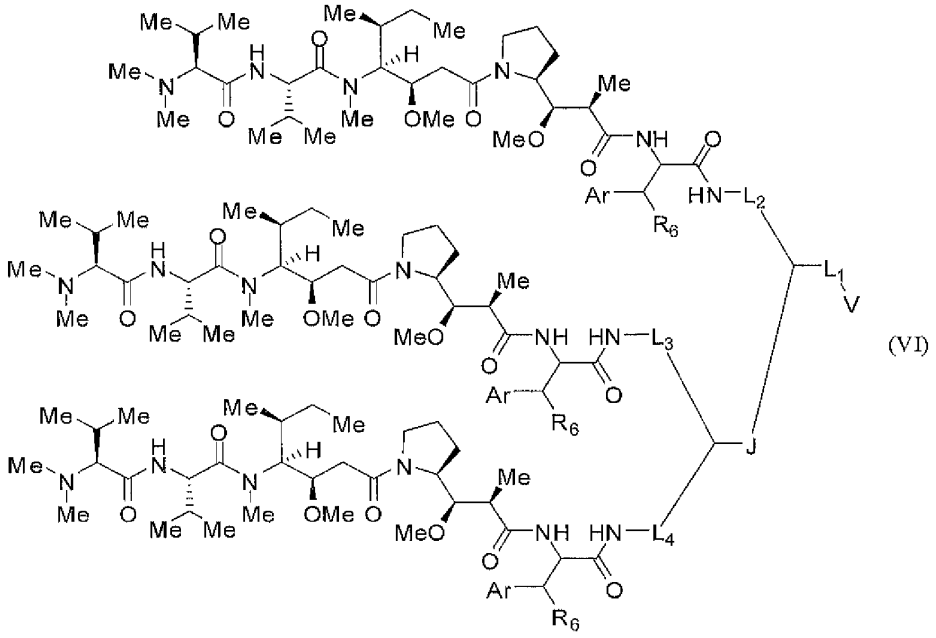
(IV)

;



(V)

;



(VI)

wherein:

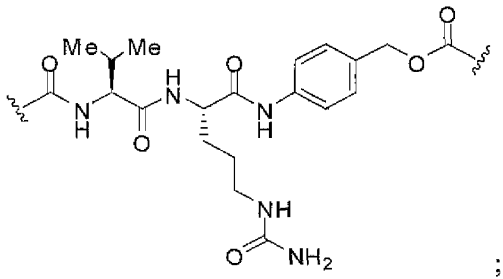
Y is NH_2–O–;

V is –O–NH_2

L_1, L_2, L_3, and L_4 are each linkers independently selected from the group consisting of a bond, –alkylene–, –(alkylene–O)$_n$–alkylene–J–, –alkylene'–J–(alkylene–O)$_n$–alkylene–, –J–(alkylene–O)$_n$–alkylene–, –(alkylene–O)$_n$–alkylene–J–(alkylene–O)$_n$'–alkylene–J'–, –(alkylene–O)$_n$–alkylene–J–alkylene'–, –W–, –alkylene–W–,

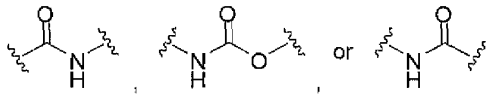
alkylene'–J–(alkylene–NMe)$_n$–alkylene–W–, –J–(alkylene–NMe)$_n$–alkylene–W–, –J–alkylene–NMe–alkylene'–NMe–alkylene"–W–, and –alkylene–J–alkylene'–NMe–alkylene"–NMe–alkylene'"–W–;

W has the structure of:



;

each J and J' independently have the structure of:



; and

each n and n' are independently integers greater than or equal to one.

Such NRL conjugates may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[244] In certain embodiments of compounds of Formula (III) and (V), Y is hydroxylamine, aldehyde, protected aldehyde, ketone, protected ketone, thioester, ester, dicarbonyl, hydrazine, amidine, imine, diamine, keto-amine, keto-alkyne, or ene-dione. In certain embodiments of compounds of Formula (IV) and (VI), V is a hydroxylamine, methyl, aldehyde, protected aldehyde, ketone, protected ketone, thioester, ester, dicarbonyl, hydrazine, amidine, imine, diamine, keto-amine, keto-alkyne, and ene-dione.

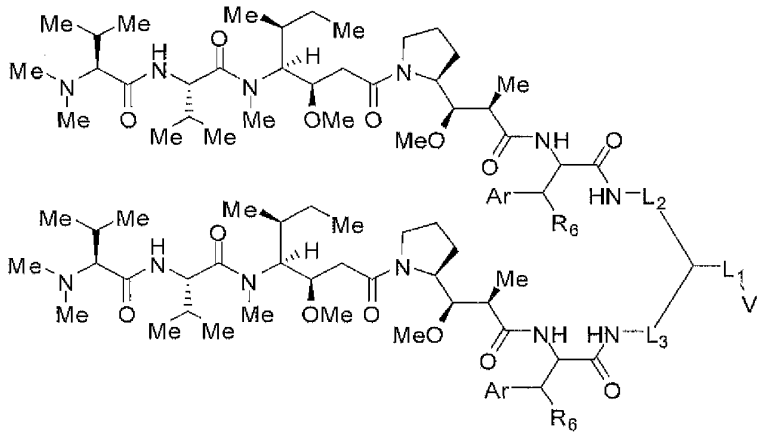
[245] In certain embodiments of compounds of Formula (XIV), (XV), (XVI), (XVII), and (XVIII), each L, L_1, L_2, L_3, and L_4 is independently a cleavable linker or non-cleavable linker. In certain embodiments of compounds of Formula (XIV), (XV), (XVI), (XVII), and (XVIII), each L, L_1, L_2, L_3, and L_4 is independently a oligo(ethylene glycol) derivatized linker.

[246] In certain embodiments of compounds of Formula (III), (IV), (V) and (VI), each alkylene, alkylene', alkylene", and alkylene'" independently is $-CH_2-$, $-CH_2CH_2-$, $-CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, –

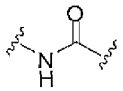
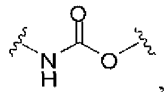
$CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2–$, $–CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2–$, $–CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2–$, or $–CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2–$. In certain embodiments of compounds of Formula (III), (IV), (V) and (VI), alkylene is methylene, ethylene, propylene, butylenes, pentylene, hexylene, or heptylene.

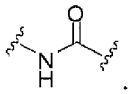
[247] In certain embodiments of compounds of Formula (III), (IV), (V) and (VI), each n and n' independently is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100.

[248] In certain embodiments, NRL conjugates include compounds having the structure of Formula (VII):



(VII)

[249] In certain embodiments of compounds of Formula (VII), L_1 is $–(alkylene–O)_n–alkylene–J–$, L_2 is $–alkylene'–J'–(alkylene–O)_{n'}–alkylene–$, L_3 is $–J''–(alkylene–O)_{n''}–alkylene–$, alkylene is $–CH_2CH_2–$, alkylene' is $–(CH_2)_4–$, n is 1, n' and n'' are 3, J has the structure of  , J' and J'' have the structure of  , and R_7 is methyl. In certain embodiments of compounds of Formula (VII), L_1 is $–J–(alkylene–O)_n–alkylene–$, L_2 is $–(alkylene–O)_{n'}–alkylene–J'–alkylene'–$, L_3 is $–(alkylene–O)_{n''}–alkylene–J''–$, alkylene is $–CH_2CH_2–$, alkylene' is $–(CH_2)_4–$, n is 1, n' and n'' are 4, and J, J' and J'' have the structure of

 . Such NRL conjugates may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[250] In certain embodiments, compounds of Formula (I)-(VII) are stable in aqueous solution for at least 1 month under mildly acidic conditions. In certain embodiments, compounds of Formula (I)-(VII) are stable for at least 2 weeks under mildly acidic conditions. In certain embodiments, compound of Formula (I)-(VII) are stable for at least 5 days under mildly acidic conditions. In certain embodiments, such acidic conditions are pH 2 to 8.

[251] The methods and compositions provided and described herein include polypeptides comprising an NRL conjugate containing at least one carbonyl or dicarbonyl group, oxime group, hydroxylamine group, or protected or masked forms thereof. Introduction of at least one reactive group into a NRL conjugate, or to any one or two components of the Ab-L-Y conjugate, can allow for the application of conjugation chemistries that involve specific chemical reactions, including, but not limited to, with one or more NRL conjguate(s) while not reacting with the commonly occurring amino acids. Once incorporated, the NRL conjugate side chains can also be modified by utilizing chemistry methodologies described herein or suitable for the particular functional groups or substituents present in the NRL conjugate.

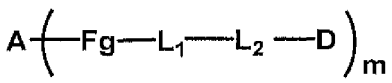
[252] The NRL conjugate methods and compositions described herein provide conjugates of substances having a wide variety of functional groups, substituents or moieties, with other substances including but not limited to a polymer; a water-soluble polymer; a derivative of polyethylene glycol; a second protein or polypeptide or polypeptide analog; an antibody or antibody fragment; and any combination thereof.

[253] In certain embodiments, the NRL conjugates, linkers and reagents described herein, including compounds of Formulas (I)-(VII) are stable in aqueous solution under mildly acidic conditions (including but not limited to pH 2 to 8). In other embodiments, such compounds are stable for at least one month under mildly acidic conditions. In other embodiments, such compounds are stable for at least 2 weeks under mildly acidic conditions. In other embodiments, such compounds are stable for at least 5 days under mildly acidic conditions.

[254] In another aspect of the compositions, methods, techniques and strategies described herein are methods for studying or using any of the aforementioned "modified or

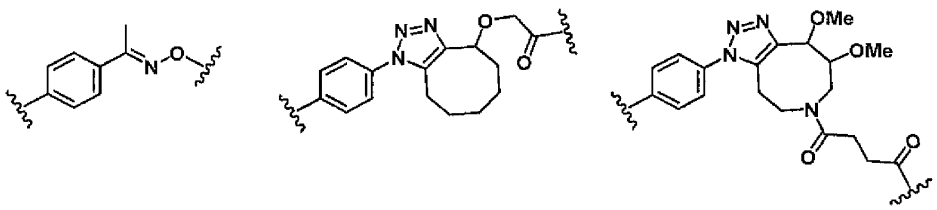
unmodified" non-natural amino acid NRL conjugates. Included within this aspect, by way of example only, are therapeutic, diagnostic, assay-based, industrial, cosmetic, plant biology, environmental, energy-production, consumer-products, and/or military uses which would benefit from a NRL conjugate comprising a "modified or unmodified" non-natural amino acid polypeptide or protein.

[255] Non-limiting examples of NRL conjugates are given below. For example, if:



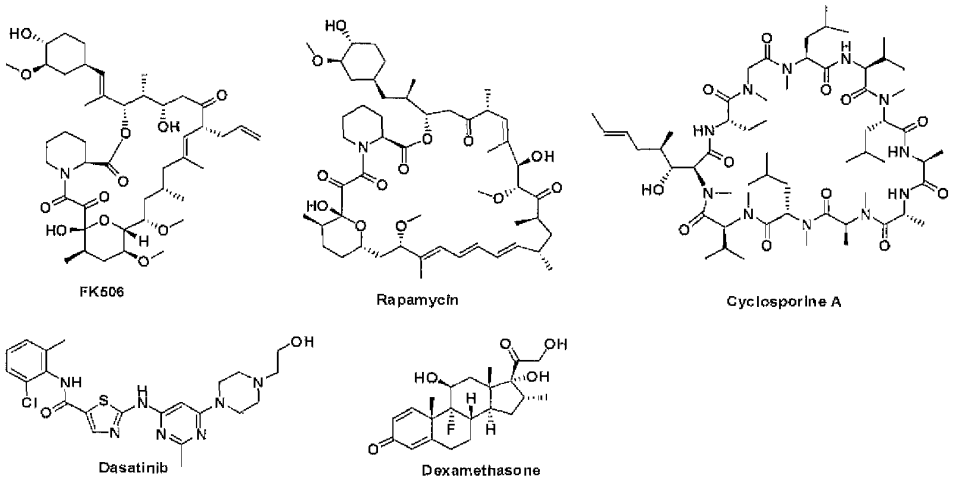
and A is a antibody;

Fg is functional group connecting antibody and linker, which is selected from:



and L1 and L2 are linkers;

[256] then non-limiting examples of D include: antiandrogens; alpha-substituted steroids; carbonylamino-benzimidazole; 17-hydroxy 4-aza androstan-3-ones; antiandrogenic biphenyls; goserelin; nilutamid; decursin; flutamide; p,p'-DDE; vinclozolin; cyproterone acetate; linuron; fluorinated 4-azasteroids; fluorinated 4-azasteroids derivatives; antiandrogens; alpha-substituted steroids; carbonylamino-benzimidazole; 17-hydroxy 4-aza androstan-3-ones; antiandrogenic biphenyls; goserelin; nilutamid; decursin; flutamide; p,p'-DDE; vinclozolin; cyproterone acetate; linuron; other kinase inhibitors, staurosporine, saracatinib, fingolimod, and other glucocorticoids

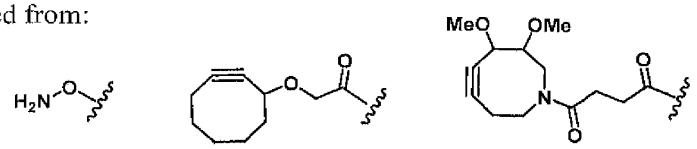


m= 1-4

[257] Other non-limiting examples of NRL conjugates are given below. For example, if:

G——L_1——L_2——D

wherein G is functional group for conjugation to connect antibody and linker, which is selected from:



L1 is selected from –J-W-, -NH-J-W-,

J is selected from: $-C_1$-C_{30} alkylene-, $-C_2$-C_{30} alkenylene- containing 0 to 20 heteroatoms selected from O, S or N; substituted $-C_1$-C_{30} alkylene, substituted $-C_2$-C_{30} alkenylene containing 0 to 20 heteroatoms selected from O, S or N;

W is selected from none, -CO-, -NHCO-,-OCO-

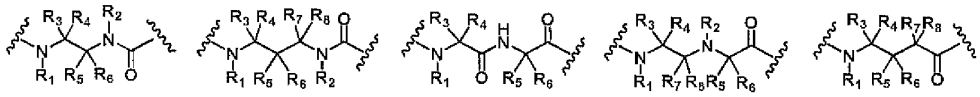
L2 is selected from $-(E\text{-}Q)_k$-,

E is an enzyme cleavage substrate: a dipeptide up to hexapeptide with or without para aminobenzyl alcohol, selected from:
-ValCit-(p-amino-benzylalcohol-CO)k-, -ValLys-(p-amino-benzylalcohol-CO)k-, -ValArg-(p-amino-benzylalcohol-CO)k-, -PheLys-(p-amino-benzylalcohol-CO)k-,

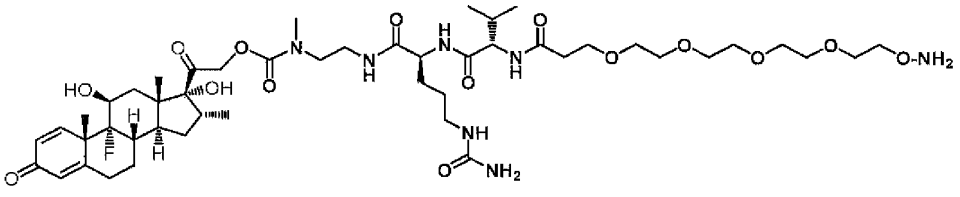
-PheArg-(p-amino-benzylalcohol-CO)k-,

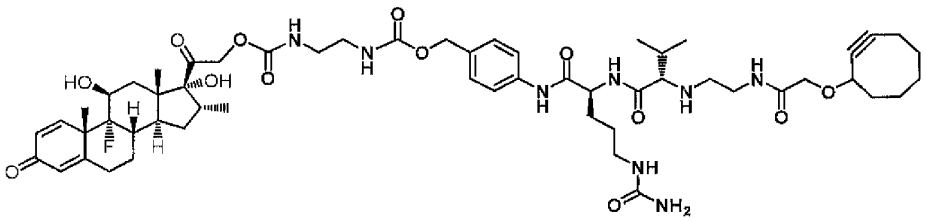
k=0,1

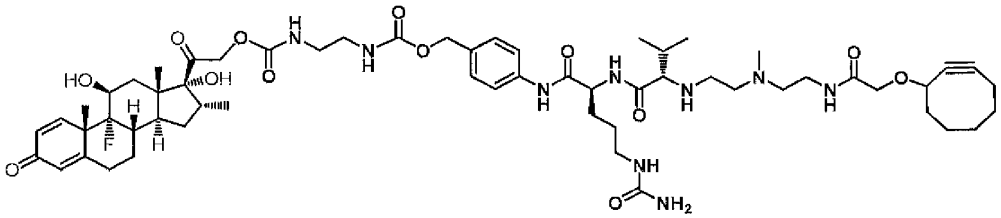
Q is a spacer, selected from:

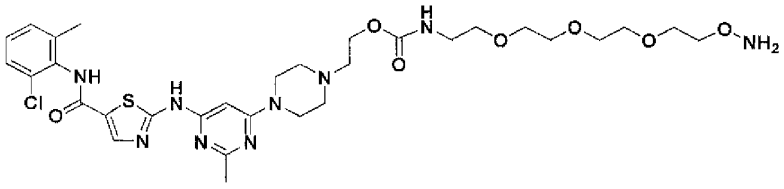


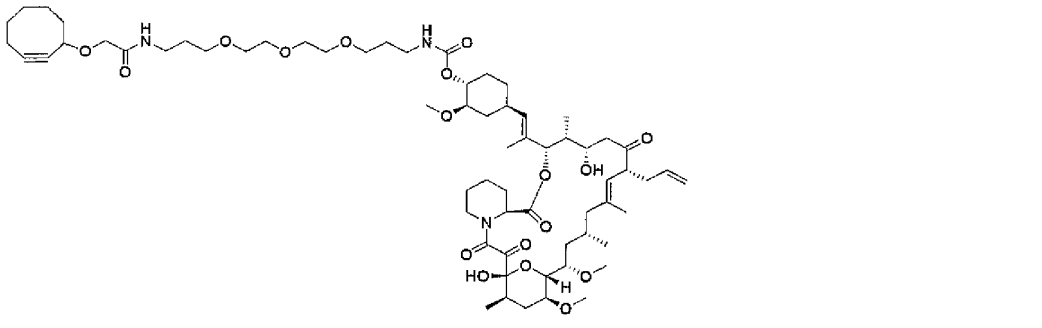
R1, R2, R3, R4, R5, R6, R7, R8 is independently selected from H, CH3, (C1-C6) alkyl

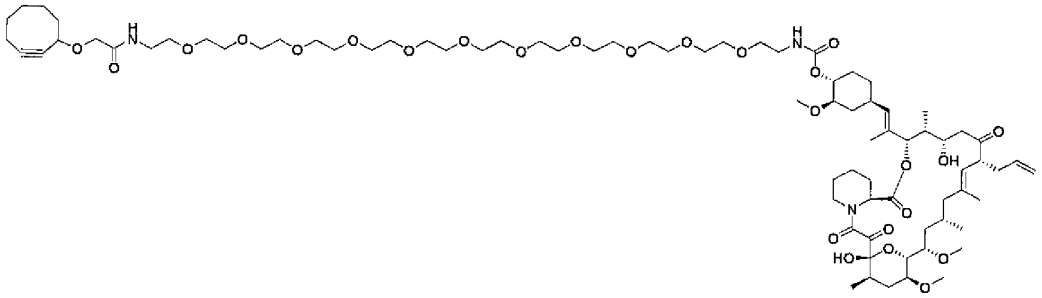


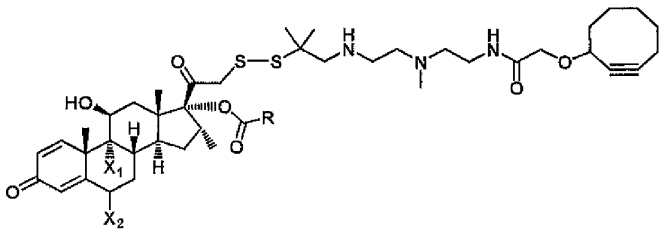


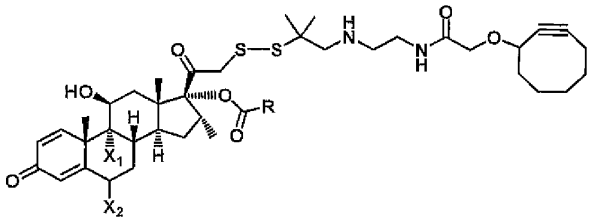


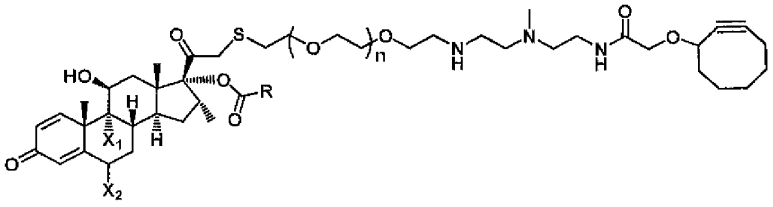


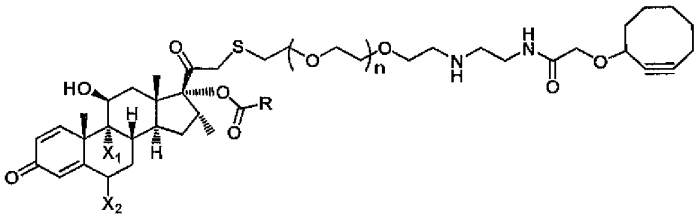








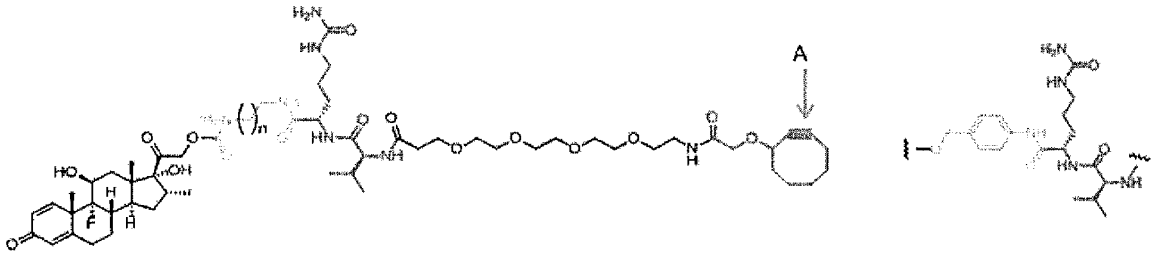




m= 1-4

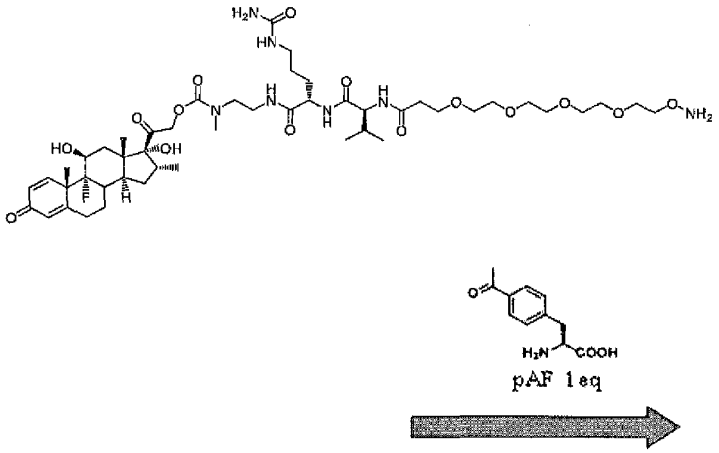
[258] Non-limiting examples of NRL conjugates include:

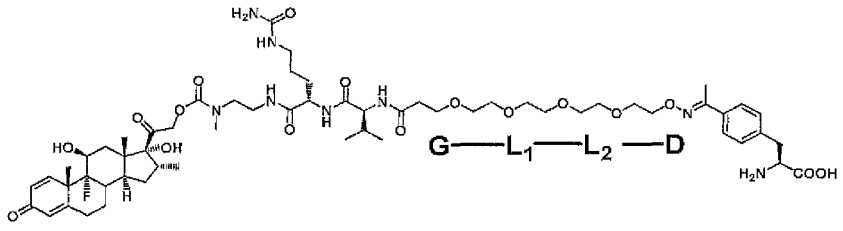
For example, NRL linker of the present invention includes below used with dexamethasone. It can also be used with SAR and Dex analogs including, but not limited to, budesonide, mometasone furoate, and fluticasone furoate and these may be used in the treatment of a variety of conditions. An example of a linker of the present invention to be used in the treatment of a chronic immune disease:



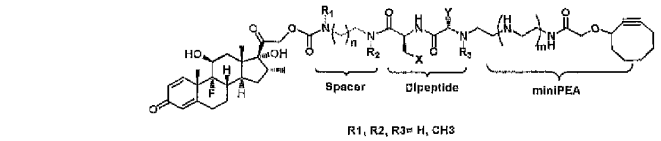
wherein A indicates where to avoid cyclooctatetraene.

For example, dexamethasone-hydroxylamine linker conjugation with pAF:





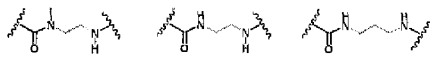
Also, by way of non-limiting example, dexamethasone and cleavable linkers with [2+3] chemistry:



R1, R2, R3= H, CH3
n= 1, 2
m= 0, 1

1. Evaluation of spacers: R1, R2 = H, CH3
n= 1, 2

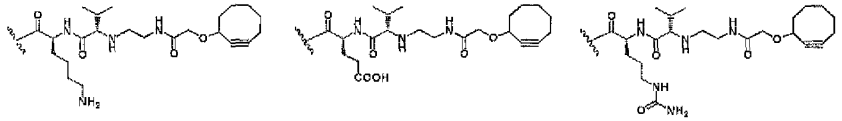
1st run examples:



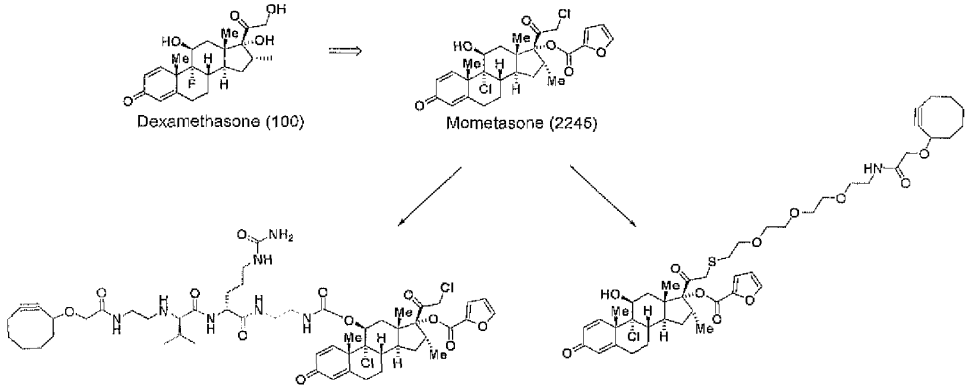
2. Evaluation of dipeptides with fixed miniPEA linker:

Val-Cit, Val/Phe-Lys, Val-Glu, Val-Asp

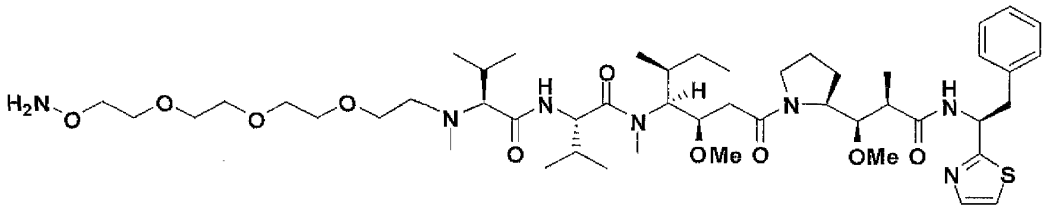
1st run examples:

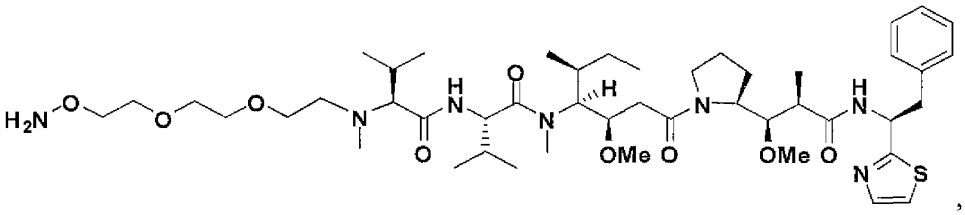


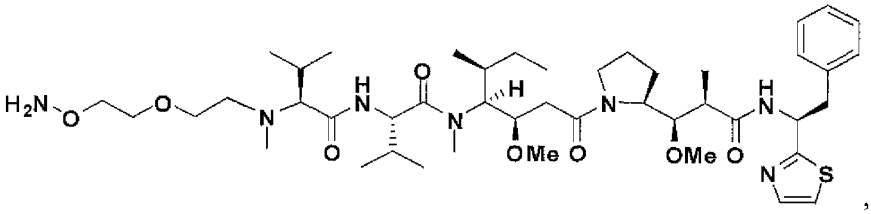
And new analogs and linkers based on dexamethasone derivative, mometasone furoate:

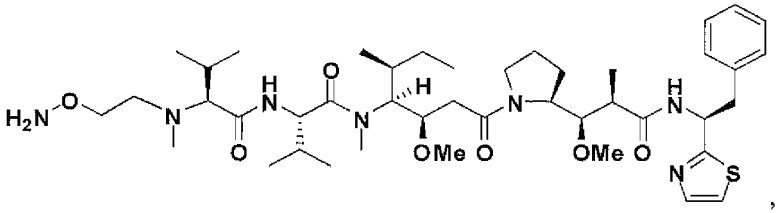


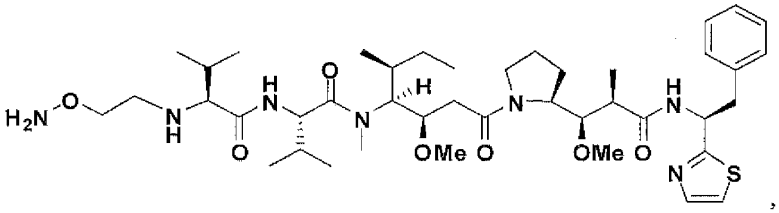
[259] Non-limiting examples of antibody conjugated glucocorticoid receptor modulator linker derivatives, and/or antibody conjugated nuclear receptor ligand linker derivatives include:

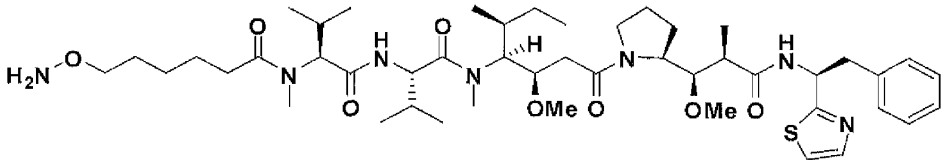


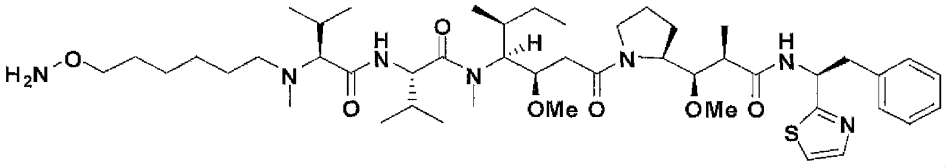


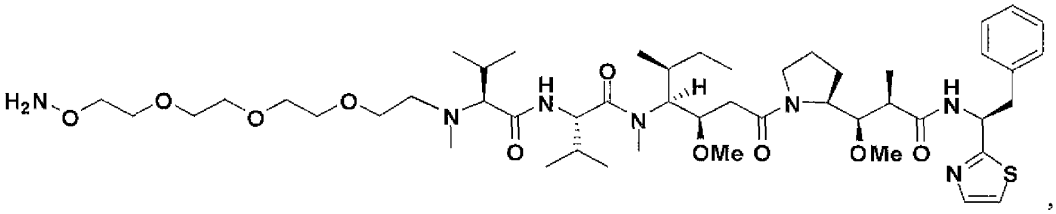


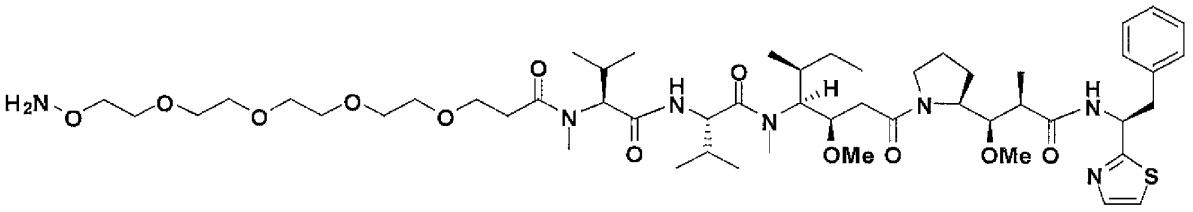




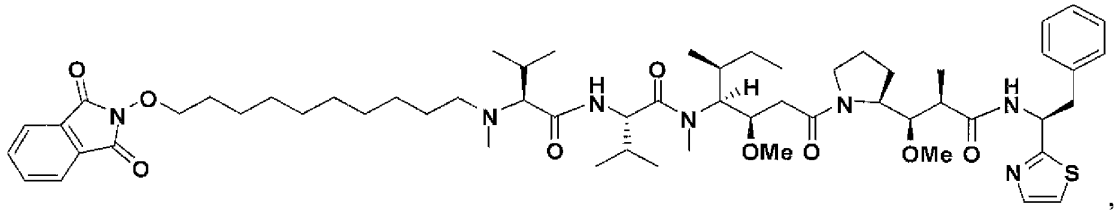




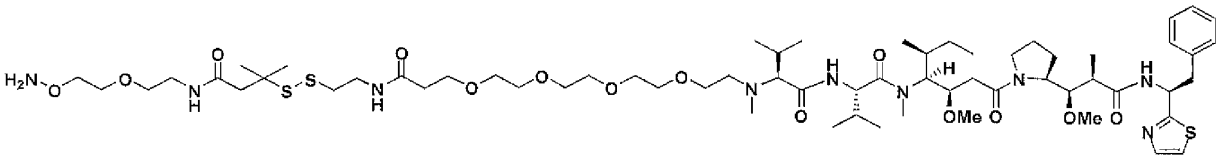




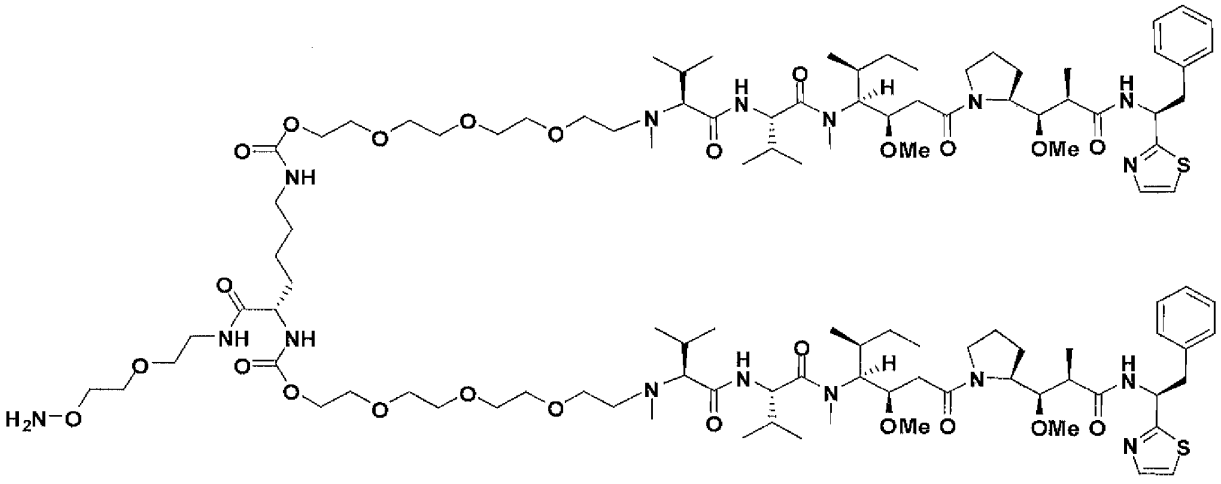
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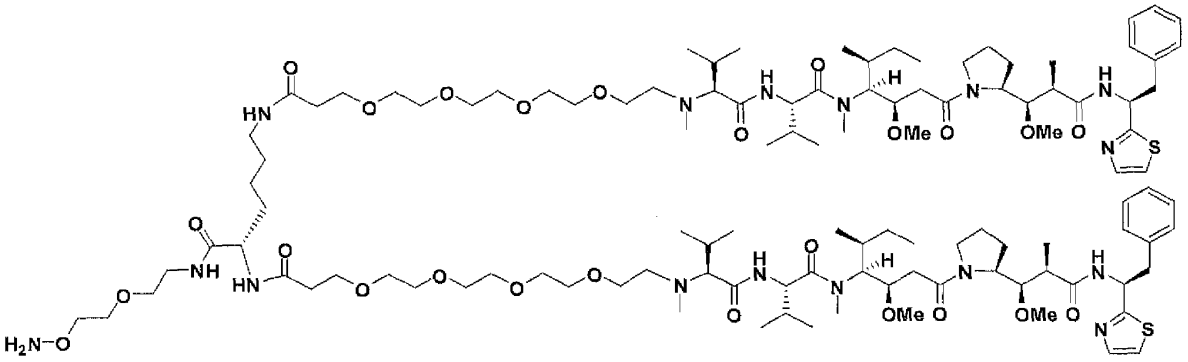
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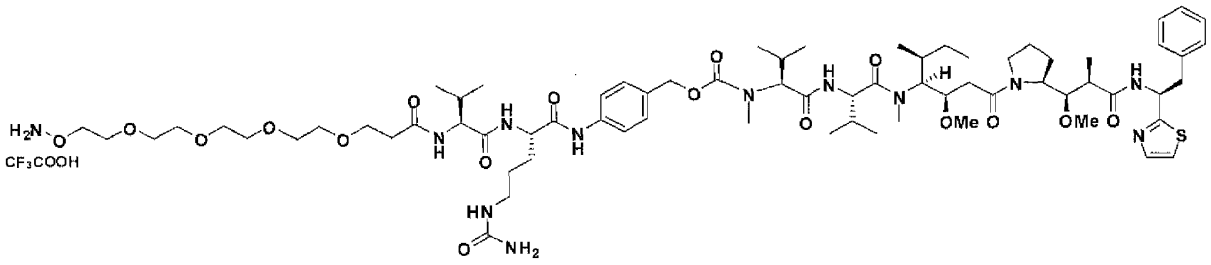
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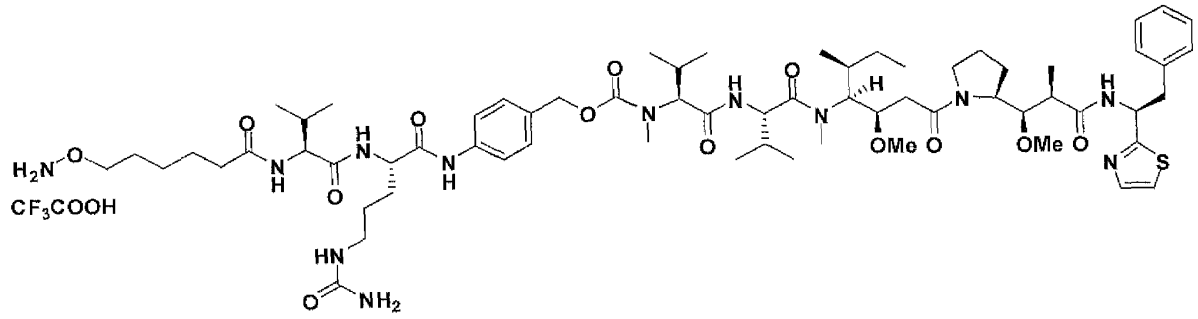
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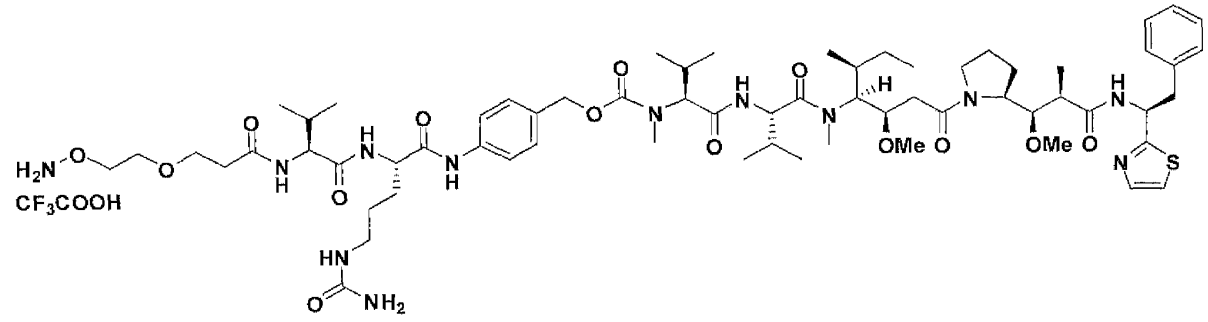
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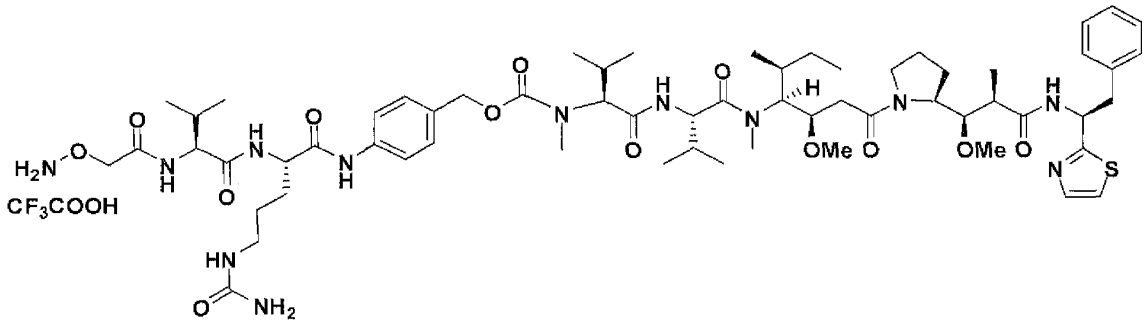
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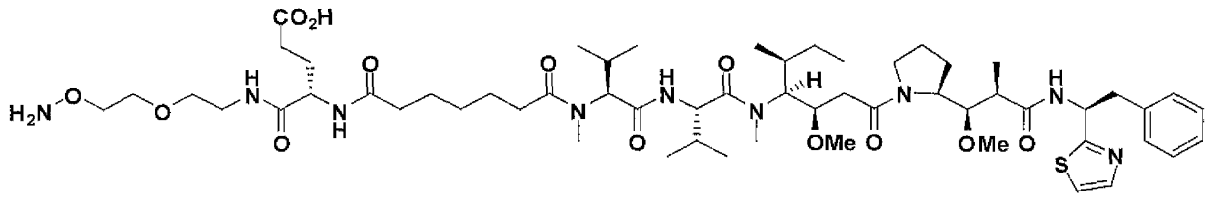
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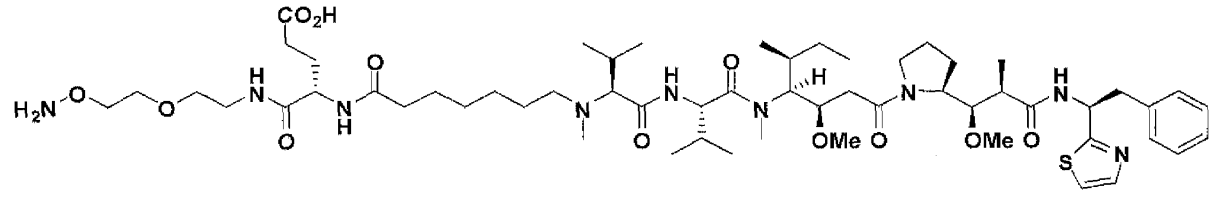
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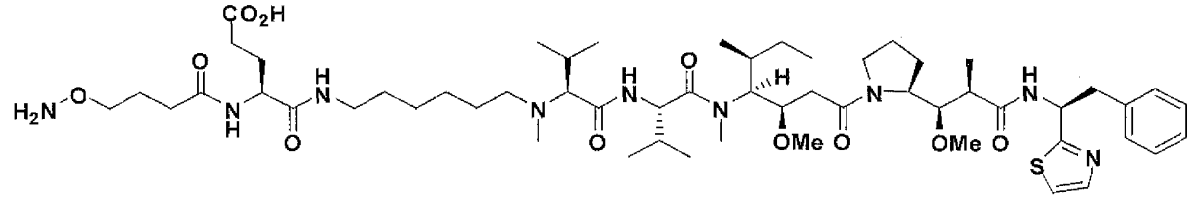
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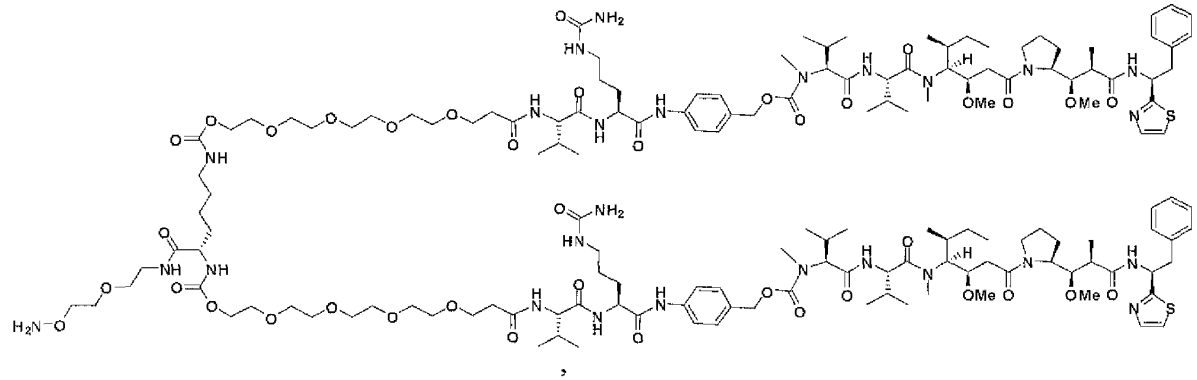
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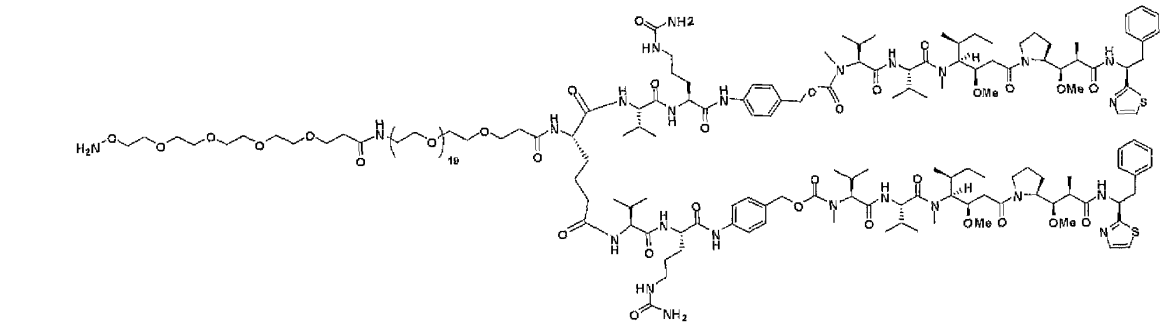
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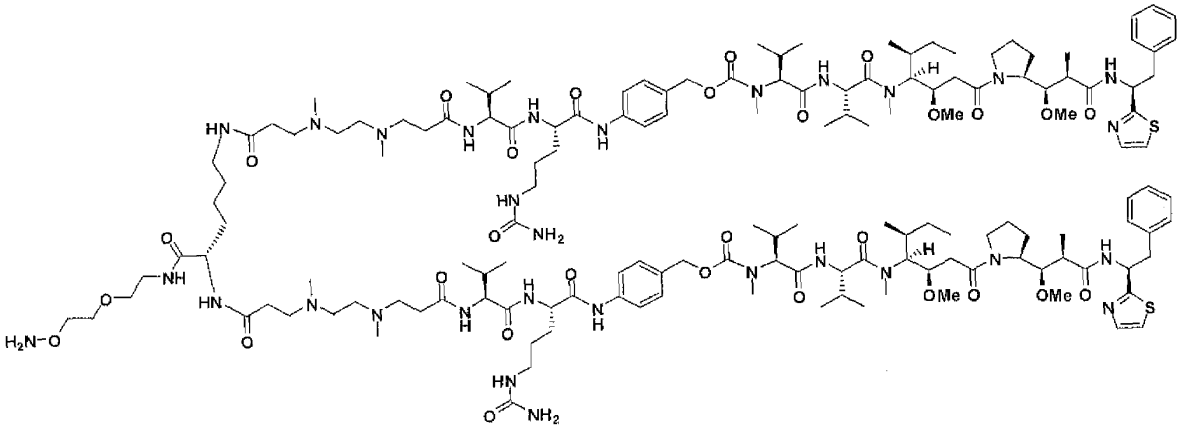
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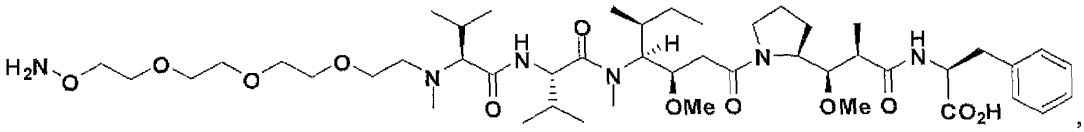
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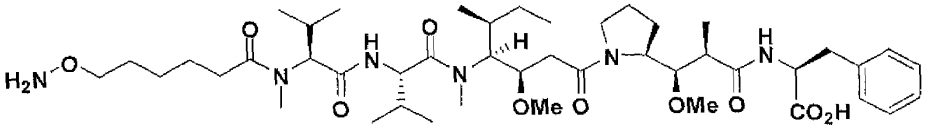
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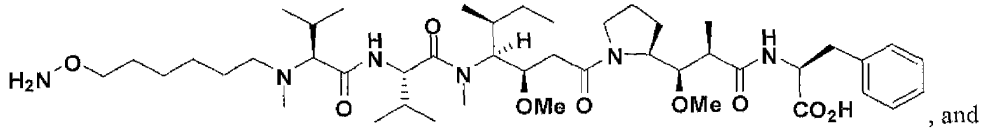
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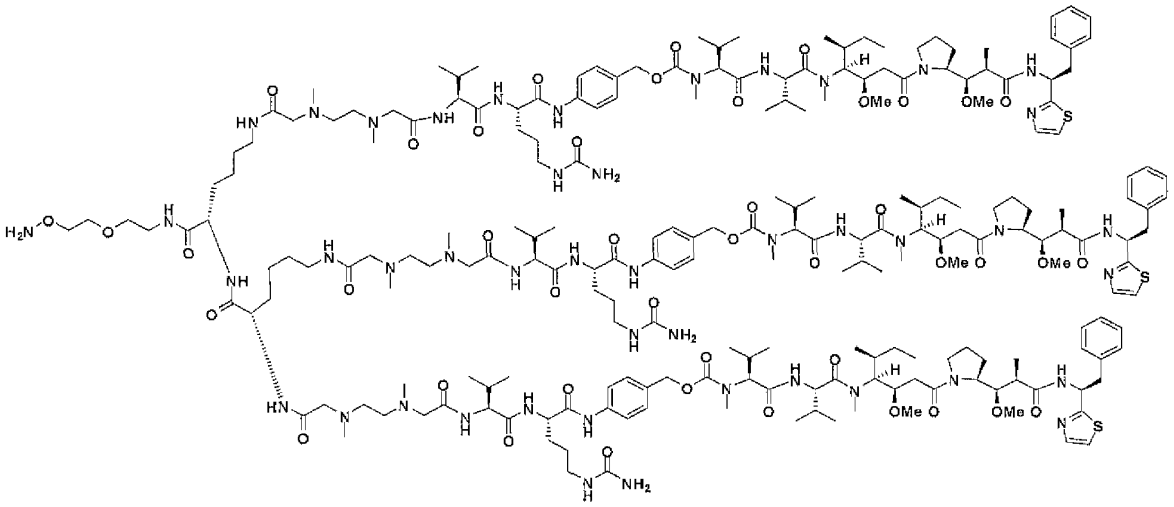
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, and



I. Non-Natural Amino Acid Derivatives

[260] The non-natural amino acids used in the methods and compositions described herein have at least one of the following four properties: (1) at least one functional group on the sidechain of the non-natural amino acid has at least one characteristics and/or activity and/or reactivity orthogonal to the chemical reactivity of the 20 common, genetically-encoded amino acids (i.e., alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine), or at least orthogonal to the chemical reactivity of the naturally occurring amino acids present in the polypeptide that includes the non-natural amino acid; (2) the introduced non-natural amino acids are substantially chemically inert toward the 20 common, genetically-encoded amino acids; (3) the non-natural amino acid can be stably incorporated into a polypeptide, preferably with the stability commensurate with the naturally-occurring amino acids or under typical physiological conditions, and further preferably such incorporation can occur via an *in vivo* system; and (4) the non-natural amino acid includes an oxime functional group or a functional group that can be transformed into an oxime group by reacting with a reagent, preferably under conditions that do not destroy the biological properties of the polypeptide that includes the non-natural amino acid (unless of course such a destruction of biological properties is the purpose of the modification/transformation), or where the transformation can occur under aqueous conditions at a pH between about 4 and about 8, or where the reactive site on the non-natural amino acid is an electrophilic site. Any number of non-natural amino acids can be introduced into the polypeptide. Non-natural amino acids may also include protected or masked oximes

or protected or masked groups that can be transformed into an oxime group after deprotection of the protected group or unmasking of the masked group. Non-natural amino acids may also include protected or masked carbonyl or dicarbonyl groups, which can be transformed into a carbonyl or dicarbonyl group after deprotection of the protected group or unmasking of the masked group and thereby are available to react with hydroxylamines or oximes to form oxime groups.

[261] Non-natural amino acids that may be used in the methods and compositions described herein include, but are not limited to, amino acids comprising a amino acids with novel functional groups, amino acids that covalently or noncovalently interact with other molecules, glycosylated amino acids such as a sugar substituted serine, other carbohydrate modified amino acids, keto-containing amino acids, aldehyde-containing amino acids, amino acids comprising polyethylene glycol or other polyethers, heavy atom substituted amino acids, chemically cleavable and/or photocleavable amino acids, amino acids with an elongated side chains as compared to natural amino acids, including but not limited to, polyethers or long chain hydrocarbons, including but not limited to, greater than about 5 or greater than about 10 carbons, carbon-linked sugar-containing amino acids, redox-active amino acids, and amino thioacid containing amino acids.

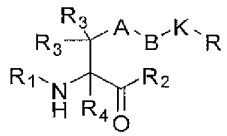
[262] In some embodiments, non-natural amino acids comprise a saccharide moiety. Examples of such amino acids include *N*-acetyl-L-glucosaminyl-L-serine, *N*-acetyl-L-galactosaminyl-L-serine, *N*-acetyl-L-glucosaminyl-L-threonine, *N*-acetyl-L-glucosaminyl-L-asparagine and *O*-mannosaminyl-L-serine. Examples of such amino acids also include examples where the naturally-occurring N- or O- linkage between the amino acid and the saccharide is replaced by a covalent linkage not commonly found in nature – including but not limited to, an alkene, an oxime, a thioether, an amide and the like. Examples of such amino acids also include saccharides that are not commonly found in naturally-occurring proteins such as 2-deoxy-glucose, 2-deoxygalactose and the like.

[263] The chemical moieties incorporated into polypeptides via incorporation of non-natural amino acids into such polypeptides offer a variety of advantages and manipulations of polypeptides. For example, the unique reactivity of a carbonyl or dicarbonyl functional group (including a keto- or aldehyde- functional group) allows selective modification of proteins with any of a number of hydrazine- or hydroxylamine-containing reagents *in vivo* and *in vitro*. A heavy atom non-natural amino acid, for example, can be useful for phasing x-ray structure data. The site-specific introduction of heavy atoms using non-natural amino acids

also provides selectivity and flexibility in choosing positions for heavy atoms. Photoreactive non-natural amino acids (including but not limited to, amino acids with benzophenone and arylazides (including but not limited to, phenylazide) side chains), for example, allow for efficient *in vivo* and *in vitro* photocrosslinking of polypeptides. Examples of photoreactive non-natural amino acids include, but are not limited to, p-azido-phenylalanine and p-benzoyl-phenylalanine. The polypeptide with the photoreactive non-natural amino acids may then be crosslinked at will by excitation of the photoreactive group-providing temporal control. In a non-limiting example, the methyl group of a non-natural amino can be substituted with an isotopically labeled, including but not limited to, with a methyl group, as a probe of local structure and dynamics, including but not limited to, with the use of nuclear magnetic resonance and vibrational spectroscopy.

A. Structure and Synthesis of Non-Natural Amino Acid Derivatives: Carbonyl, Carbonyl like, Masked Carbonyl, and Protected Carbonyl Groups

[264] Amino acids with an electrophilic reactive group allow for a variety of reactions to link molecules via various chemical reactions, including, but not limited to, nucleophilic addition reactions. Such electrophilic reactive groups include a carbonyl- or dicarbonyl-group (including a keto- or aldehyde group), a carbonyl-like- or dicarbonyl-like-group (which has reactivity similar to a carbonyl- or dicarbonyl-group and is structurally similar to a carbonyl- or dicarbonyl-group), a masked carbonyl- or masked dicarbonyl-group (which can be readily converted into a carbonyl- or dicarbonyl-group), or a protected carbonyl- or protected dicarbonyl-group (which has reactivity similar to a carbonyl- or dicarbonyl-group upon deprotection). Such amino acids include amino acids having the structure of Formula (XXXVII):

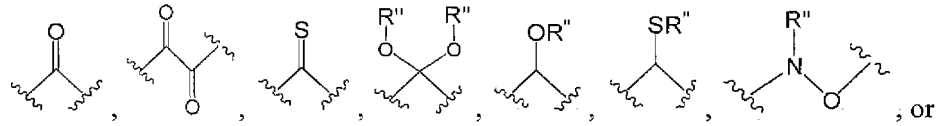
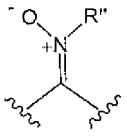


(XXXVII)

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, $-NS(O)_2-$, $-OS(O)_2-$, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, $-N(R')C(O)O-$, $-S(O)_kN(R')-$, $-N(R')C(O)N(R')-$, $-N(R')C(S)N(R')-$, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;

K is  , or  ;

R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

each R" is independently H, alkyl, substituted alkyl, or a protecting group, or when more than one R" group is present, two R" optionally form a heterocycloalkyl;

R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

each of R_3 and R_4 is independently H, halogen, lower alkyl, or substituted lower alkyl, or R_3 and R_4 or two R_3 groups optionally form a cycloalkyl or a heterocycloalkyl;

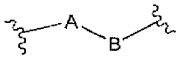
or the –A-B-K-R groups together form a bicyclic or tricyclic cycloalkyl or heterocycloalkyl comprising at least one carbonyl group, including a dicarbonyl group, protected carbonyl group, including a protected dicarbonyl group, or masked carbonyl group, including a masked dicarbonyl group;

or the –K-R group together forms a monocyclic or bicyclic cycloalkyl or heterocycloalkyl comprising at least one carbonyl group, including a dicarbonyl group, protected carbonyl group, including a protected dicarbonyl group, or masked carbonyl group, including a masked dicarbonyl group;

with a proviso that when A is phenylene and each R_3 is H, B is present; and that when A is $-(CH_2)_4-$ and each R_3 is H, B is not $-NHC(O)(CH_2CH_2)-$; and that when A and B are absent and each R_3 is H, R is not methyl. Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[265] In certain embodiments, compounds of Formula (XXXVII) are stable in aqueous solution for at least 1 month under mildly acidic conditions. In certain embodiments, compounds of Formula (XXXVII) are stable for at least 2 weeks under mildly acidic conditions. In certain embodiments, compound of Formula (XXXVII) are stable for at least 5 days under mildly acidic conditions. In certain embodiments, such acidic conditions are pH 2 to 8.

[266] In certain embodiments of compounds of Formula (XXXVII), B is lower alkylene, substituted lower alkylene, -O-(alkylene or substituted alkylene)-, -C(R')=N-N(R')-, -N(R')CO-, -C(O)-, -C(R')=N-, -C(O)-(alkylene or substituted alkylene)-, -CON(R')-(alkylene or substituted alkylene)-, -S(alkylene or substituted alkylene)-, -S(O)(alkylene or substituted alkylene)-, or $-S(O)_2$(alkylene or substituted alkylene)-. In certain embodiments of compounds of Formula (XXXVII), B is $-O(CH_2)-$, -CH=N-, -CH=N-NH-, $-NHCH_2-$, -NHCO-, -C(O)-, $-C(O)-(CH_2)-$, $-CONH-(CH_2)-$, $-SCH_2-$, $-S(=O)CH_2-$, or $-S(O)_2CH_2-$. In certain embodiments of compounds of Formula (XXXVII), R is C_{1-6} alkyl or cycloalkyl. In certain embodiments of compounds of Formula (XXXVII) R is $-CH_3$, $-CH(CH_3)_2$, or cyclopropyl. In certain embodiments of compounds of Formula (XXXVII), R_1 is H, tert-butyloxycarbonyl (Boc), 9- Fluorenylmethoxycarbonyl (Fmoc), N-acetyl, tetrafluoroacetyl (TFA), or benzyloxycarbonyl (Cbz). In certain embodiments of compounds of Formula (XXXVII), R_1 is a resin, amino acid, polypeptide, antibody, or polynucleotide. In certain embodiments of compounds of Formula (XXXVII), R_2 is OH, O-methyl, O-ethyl, or O-*t*-butyl. In certain embodiments of compounds of Formula (XXXVII), R_2 is a resin, amino acid, polypeptide, antibody, or polynucleotide. In certain embodiments of compounds of Formula (XXXVII), R_2 is a polynucleotide. In certain embodiments of compounds of Formula (XXXVII), R_2 is ribonucleic acid (RNA).

[267] In certain embodiments of compounds of Formula (XXXVII), 

is selected from the group consisting of:

(i) A is substituted lower alkylene, C_4-arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

B is optional, and when present is a divalent linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S(O)-, -$S(O)_2$-, -$NS(O)_2$-, -$OS(O)_2$-, -C(O)-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -N(R')-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, -N(R')C(S)-, -S(O)N(R'), -$S(O)_2$N(R'), -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, -N(R')S(O)N(R')-, -N(R')$S(O)_2$N(R')-, -N(R')-N=, -C(R')=N-N(R')-, -C(R')=N-N=, -$C(R')_2$-N=N-, and -$C(R')_2$-N(R')-N(R')-;

(ii) A is optional, and when present is substituted lower alkylene, C_4-arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

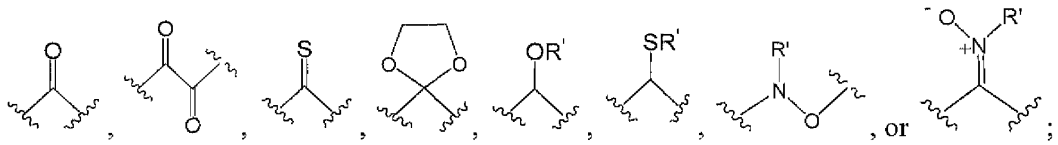
B is a divalent linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S(O)-, -$S(O)_2$-, -$NS(O)_2$-, -$OS(O)_2$-, -C(O)-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -N(R')-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, -N(R')C(S)-, -S(O)N(R'), -$S(O)_2$N(R'), -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, -N(R')S(O)N(R')-, -N(R')$S(O)_2$N(R')-, -N(R')-N=, -C(R')=N-N(R')-, -C(R')=N-N=, -$C(R')_2$-N=N-, and -$C(R')_2$-N(R')-N(R')-;

(iii) A is lower alkylene;

B is optional, and when present is a divalent linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S(O)-, -$S(O)_2$-, -$NS(O)_2$-, -$OS(O)_2$-, -C(O)-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -N(R')-, -C(O)N(R')-, -CSN(R')-, -CON(R')-(alkylene or substituted alkylene)-, -N(R')C(O)O-, -N(R')C(S)-, -S(O)N(R'), -$S(O)_2$N(R'), -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, -N(R')S(O)N(R')-, -N(R')$S(O)_2$N(R')-, -N(R')-N=, -C(R')=N-N(R')-, -C(R')=N-N=, -$C(R')_2$-N=N-, and -$C(R')_2$-N(R')-N(R')-; and

(iv) A is phenylene;

B is a divalent linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S(O)-, -$S(O)_2$-, -$NS(O)_2$-, -$OS(O)_2$-, -C(O)-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -N(R')-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, -N(R')C(S)-, -S(O)N(R'), -$S(O)_2$N(R'), -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, -N(R')S(O)N(R')-, -N(R')$S(O)_2$N(R')-, -N(R')-N=, -C(R')=N-N(R')-, -C(R')=N-N=, -$C(R')_2$-N=N-, and -$C(R')_2$-N(R')-N(R')-;

K is  , or ;

each R' is independently H, alkyl, or substituted alkyl;

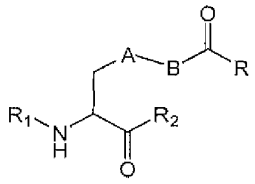
R_1 is optional, and when present, is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is optional, and when present, is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide; and

each R_3 and R_4 is independently H, halogen, lower alkyl, or substituted lower alkyl;

R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

[268] In addition, amino acids having the structure of Formula (XXXVIII) are included:



(XXXVIII),

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted

alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, -$S(O)_k$- where k is 1, 2, or 3, -$S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, -$NS(O)_2$-, -$OS(O)_2$-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, -$S(O)_k$N(R')-, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, -N(R')$S(O)_k$N(R')-, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, -$C(R')_2$-N=N-, and -$C(R')_2$-N(R')-N(R')-, where each R' is independently H, alkyl, or substituted alkyl;

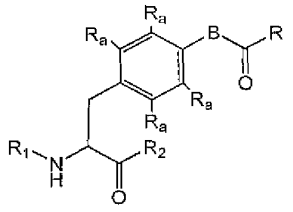
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

R_1 is H, an amino protecting group, resin, at least one amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, at least one amino acid, polypeptide, or polynucleotide;

with a proviso that when A is phenylene, B is present; and that when A is $-(CH_2)_4$-, B is not $-NHC(O)(CH_2CH_2)$-; and that when A and B are absent, R is not methyl. Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[269] In addition, amino acids having the structure of Formula (XXXIX) are included:



(XXXIX),

wherein:

B is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, -$S(O)_k$- where k is 1, 2, or 3, -$S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, -$NS(O)_2$-, -$OS(O)_2$-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-,

-CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, -S(O)$_k$N(R')-, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, -N(R')S(O)$_k$N(R')-, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, -C(R')$_2$-N=N-, and -C(R')$_2$-N(R')-N(R')-, where each R' is independently H, alkyl, or substituted alkyl;

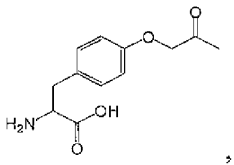
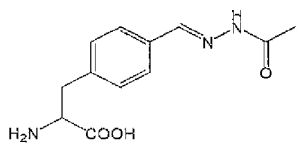
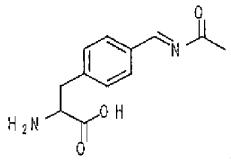
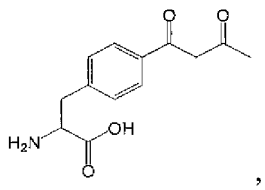
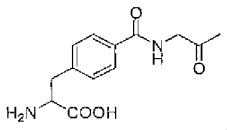
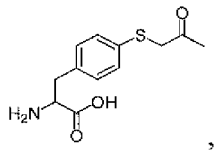
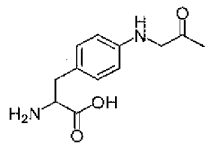
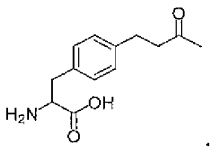
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

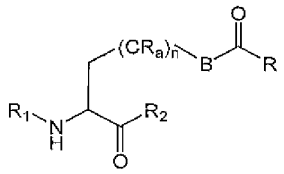
each R_a is independently selected from the group consisting of H, halogen, alkyl, substituted alkyl, -N(R')$_2$, -C(O)$_k$R' where k is 1, 2, or 3, -C(O)N(R')$_2$, -OR', and -S(O)$_k$R', where each R' is independently H, alkyl, or substituted alkyl. Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[270] In addition, the following amino acids are included:


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, and

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Such non-natural amino acids may be are optionally amino protected group, carboxyl protected and/or in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[271] In addition, the following amino acids having the structure of Formula (XXXX) are included:



(XXXX)

wherein

-$NS(O)_2$-, -$OS(O)_2$-, optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, -$S(O)_k$- where k is 1, 2, or 3, -$S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, -$S(O)_k$N(R')-, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, -N(R')$S(O)_k$N(R')-, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, -$C(R')_2$-N=N-, and -$C(R')_2$-N(R')-N(R')-, where each R' is independently H, alkyl, or substituted alkyl;

R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

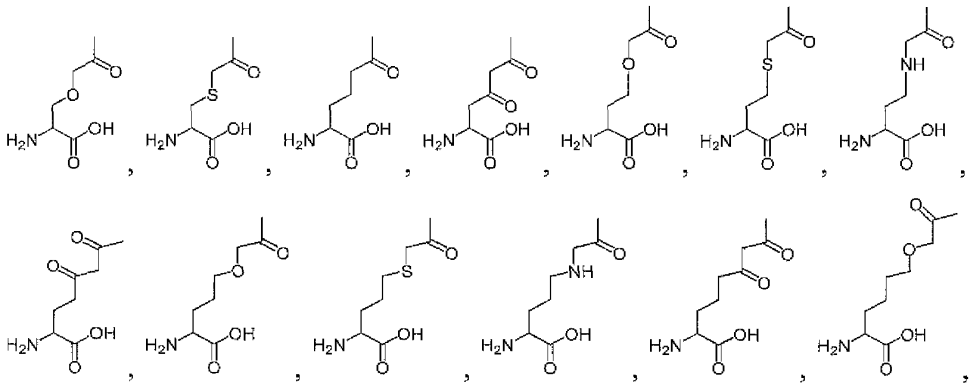
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

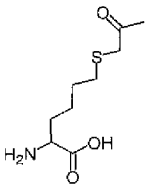
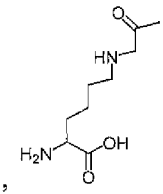
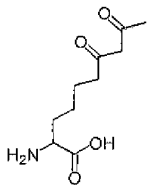
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

each R_a is independently selected from the group consisting of H, halogen, alkyl, substituted alkyl, -$N(R')_2$, -$C(O)_kR'$ where k is 1, 2, or 3, -$C(O)N(R')_2$, -OR', and -$S(O)_kR'$, where each R' is independently H, alkyl, or substituted alkyl; and n is 0 to 8;

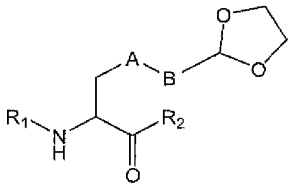
with a proviso that when A is –$(CH_2)_4$-, B is not –$NHC(O)(CH_2CH_2)$-. Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[272] In addition, the following amino acids are included:





, and  , wherein such compounds are optionally amino protected, optionally carboxyl protected, optionally amino protected and carboxyl protected, or a salt thereof, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[273] In addition, the following amino acids having the structure of Formula (XXXXI) are included:


(XXXXI),

wherein,

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

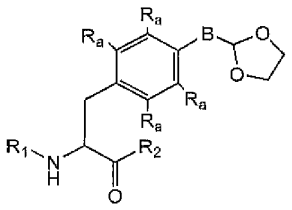
B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, $-NS(O)_2-$, $-OS(O)_2-$, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, $-N(R')C(O)O-$, $-S(O)_kN(R')-$, $-N(R')C(O)N(R')-$, $-N(R')C(S)N(R')-$, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;

R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[274] In addition, the following amino acids having the structure of Formula (XXXXII) are included:



(XXXXII),

wherein,

B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, $-NS(O)_2-$, $-OS(O)_2-$, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, $-S(O)_kN(R')-$, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;

R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

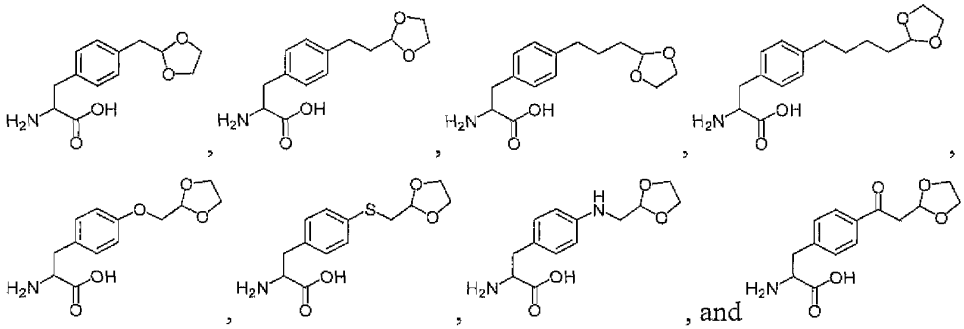
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

wherein each R_a is independently selected from the group consisting of H, halogen, alkyl, substituted alkyl, $-N(R')_2$, $-C(O)_kR'$ where k is 1, 2, or 3, $-C(O)N(R')_2$, -OR', and $-S(O)_kR'$, where each R' is independently H, alkyl, or substituted alkyl.

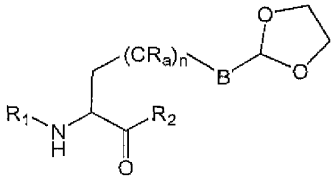
Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[275] In addition, the following amino acids are included:



wherein such compounds are optionally amino protected, optionally carboxyl protected, optionally amino protected and carboxyl protected, or a salt thereof, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[276] In addition, the following amino acids having the structure of Formula (XXXXIV) are included:



(XXXXIV),

wherein,

B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, $-NS(O)_2-$, $-OS(O)_2-$, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, $-N(R')C(O)O-$, $-S(O)_kN(R')-$, $-N(R')C(O)N(R')-$, $-N(R')C(S)N(R')-$, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;

R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

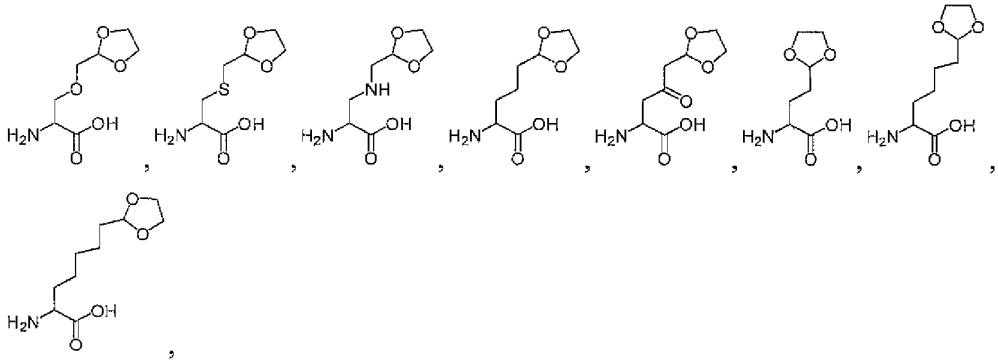
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

each R_a is independently selected from the group consisting of H, halogen, alkyl, substituted alkyl, $-N(R')_2$, $-C(O)_kR'$ where k is 1, 2, or 3, $-C(O)N(R')_2$, $-OR'$, and $-S(O)_kR'$, where each R' is independently H, alkyl, or substituted alkyl; and n is 0 to 8.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

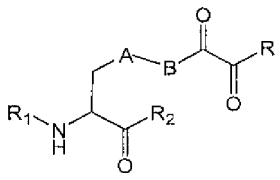
[277] In addition, the following amino acids are included:



wherein such compounds are optionally amino protected, optionally carboxyl protected, optionally amino protected and carboxyl protected, or a salt thereof, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[278] In addition to monocarbonyl structures, the non-natural amino acids described herein may include groups such as dicarbonyl, dicarbonyl like, masked dicarbonyl and protected dicarbonyl groups.

For example, the following amino acids having the structure of Formula (XXXXV) are included:



(XXXXV),

wherein,

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower

heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, $-NS(O)_2-$, $-OS(O)_2-$, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, $-N(R')C(O)O-$, $-S(O)_kN(R')-$, $-N(R')C(O)N(R')-$, $-N(R')C(S)N(R')-$, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;

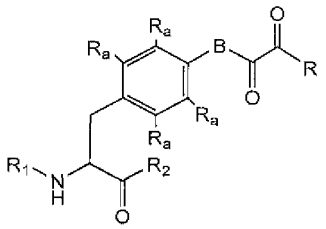
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[279] In addition, the following amino acids having the structure of Formula (XXXXVI) are included:



(XXXXVI),

wherein,

B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, -

$S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, $-NS(O)_2$-, $-OS(O)_2$-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, $-N(R')C(O)O-$, $-S(O)_kN(R')-$, $-N(R')C(O)N(R')-$, $-N(R')C(S)N(R')-$, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;

R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

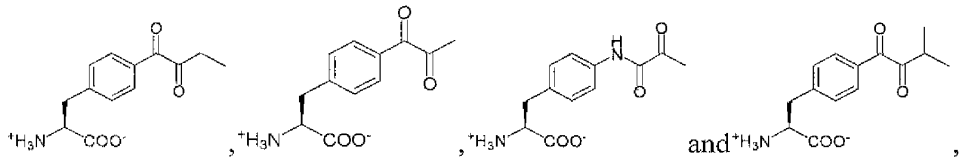
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

wherein each R_a is independently selected from the group consisting of H, halogen, alkyl, substituted alkyl, $-N(R')_2$, $-C(O)_kR'$ where k is 1, 2, or 3, $-C(O)N(R')_2$, -OR', and $-S(O)_kR'$, where each R' is independently H, alkyl, or substituted alkyl.

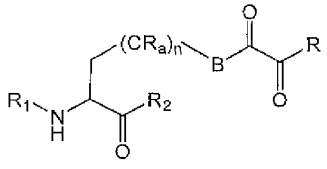
Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[280] In addition, the following amino acids are included:



wherein such compounds are optionally amino protected and carboxyl protected, or a salt thereof. Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[281] In addition, the following amino acids having the structure of Formula (XXXXVII) are included:



(XXXXVII),

wherein,

B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, -$S(O)_k$- where k is 1, 2, or 3, -$S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, -$NS(O)_2$-, -$OS(O)_2$-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, -$S(O)_k$N(R')-, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, -N(R')$S(O)_k$N(R')-, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, -$C(R')_2$-N=N-, and -$C(R')_2$-N(R')-N(R')-, where each R' is independently H, alkyl, or substituted alkyl;

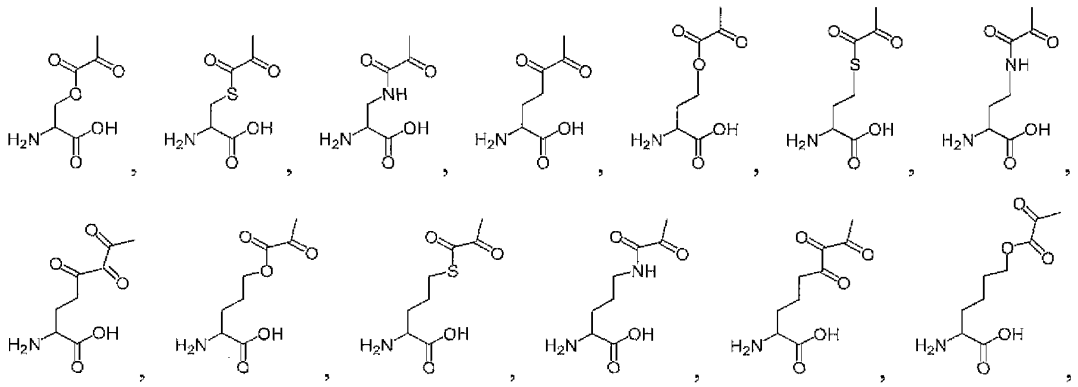
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

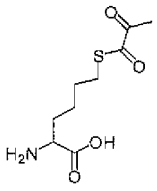
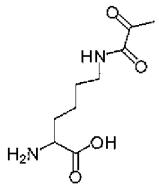
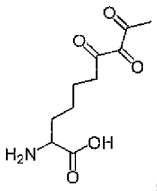
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

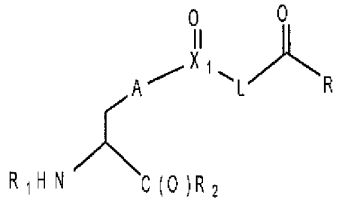
each R_a is independently selected from the group consisting of H, halogen, alkyl, substituted alkyl, -$N(R')_2$, -$C(O)_kR'$ where k is 1, 2, or 3, -$C(O)N(R')_2$, -OR', and -$S(O)_kR'$, where each R' is independently H, alkyl, or substituted alkyl; and n is 0 to 8. Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[282] In addition, the following amino acids are included:





, and  , wherein such compounds are optionally amino protected and carboxyl protected, or a salt thereof, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[283] In addition, the following amino acids having the structure of Formula (XXXXVIII) are included:


(XXXXVIII);

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

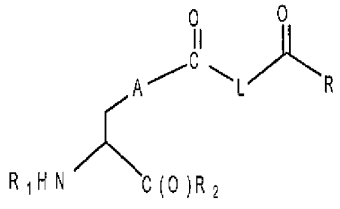
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

X_1 is C, S, or S(O); and L is alkylene, substituted alkylene, N(R')(alkylene) or N(R')(substituted alkylene), where R' is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[284] In addition, the following amino acids having the structure of Formula (XXXXIX) are included:



(XXXXIX)

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

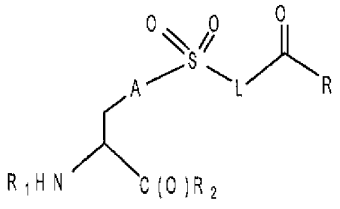
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

L is alkylene, substituted alkylene, N(R')(alkylene) or N(R')(substituted alkylene), where R' is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[285] In addition, the following amino acids having the structure of Formula (XXXXX) are included:



(XXXXX)

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

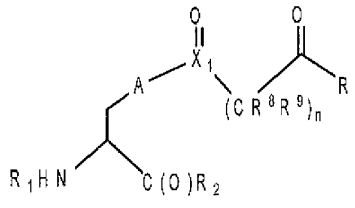
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

L is alkylene, substituted alkylene, N(R')(alkylene) or N(R')(substituted alkylene), where R' is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[286] In addition, the following amino acids having the structure of Formula (XXXXXI) are included:



(XXXXXI);

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

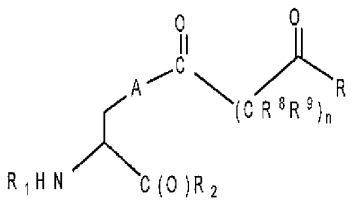
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

X_1 is C, S, or S(O); and n is 0, 1, 2, 3, 4, or 5; and each R^8 and R^9 on each CR^8R^9 group is independently selected from the group consisting of H, alkoxy, alkylamine, halogen, alkyl, aryl, or any R^8 and R^9 can together form =O or a cycloalkyl, or any to adjacent R^8 groups can together form a cycloalkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[287] In addition, the following amino acids having the structure of Formula (XXXXXII) are included:



(XXXXXII)

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

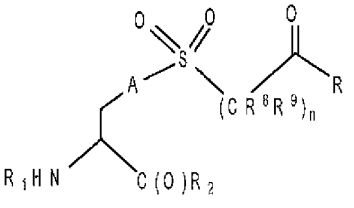
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

n is 0, 1, 2, 3, 4, or 5; and each R^8 and R^9 on each CR^8R^9 group is independently selected from the group consisting of H, alkoxy, alkylamine, halogen, alkyl, aryl, or any R^8 and R^9 can together form =O or a cycloalkyl, or any to adjacent R^8 groups can together form a cycloalkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[288] In addition, the following amino acids having the structure of Formula (XXXXXIII) are included:



(XXXXXIII)

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene,

heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

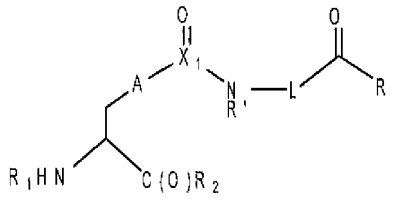
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

n is 0, 1, 2, 3, 4, or 5; and each R^8 and R^9 on each CR^8R^9 group is independently selected from the group consisting of H, alkoxy, alkylamine, halogen, alkyl, aryl, or any R^8 and R^9 can together form =O or a cycloalkyl, or any to adjacent R^8 groups can together form a cycloalkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[289] In addition, the following amino acids having the structure of Formula (XXXXXIV) are included:



(XXXXXIV);

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

X_1 is C, S, or S(O); and L is alkylene, substituted alkylene, N(R')(alkylene) or N(R')(substituted alkylene), where R' is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl.