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(54) Title: TRISPECIFIC ANTAGONISTS

(57) Abstract: Antitumor antagonists that bind specifically to immune checkpoint regulator and/or components of the angiogenesis pathways and/or components of the TGF pathway are disclosed. Also disclosed is a method of treating proliferative disorders with the antitumor antagonists.

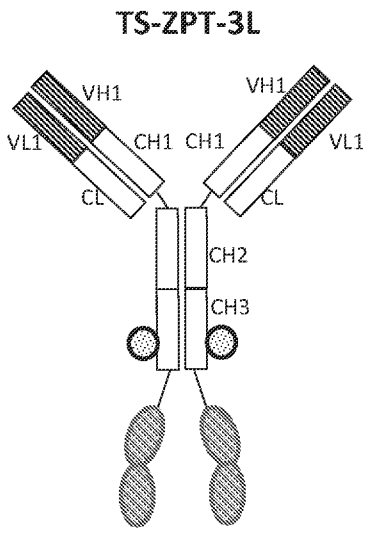

FIG. 3E

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TITLE

TRISPECIFIC ANTAGONISTS

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 62/691,658, filed on June 29, 2018, and U.S. Provisional Patent Application Serial No. 62/823,989, filed on March 26, 2019, the contents of which are expressly incorporated herein by reference for all purposes.

FIELD

[0002] The present application relates generally to cancer treatment and in particular, to bispecific and trispecific antagonists capable of modulating pathways associated with tumorigenesis, tumor immunity, and angiogenesis.

BACKGROUND

[0003] The inability of the host to eliminate cancer cells remains a major problem. Although an increasing number of therapeutic monoclonal antibodies have been approved for treatment of various cancers, emergence of resistance to these antibodies is frequently observed, given the many different molecular pathways underlying cancer growth and progression to metastasis. Although the immune system is the principal mechanism of cancer prevention, cancer cells counteract immune surveillance. Therefore, there exists a need for improved therapeutic binding antagonists or antibodies and methods of treating cancer and chronic viral infections with such reagents.

SUMMARY

[0004] One aspect of the present application relates to a trispecific antagonist, comprising: an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain; a first targeting domain comprising one or more variable domains selected from the group consisting of anti-PD-1 variable domains, anti-PD-L1 variable domains, anti-TIGIT variable domains and anti-LAG-3 variable domains; a second targeting domain that binds specifically to VEGF and comprises one or more peptide domains derived from VEGFR; and a third targeting domain that comprises a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway; wherein the second targeting domain is structurally linked to a carboxy-terminal of the CH3 domain and wherein the third targeting domain is inserted within the CH3 domain.

[0005] Another aspect of the present application relates to a trispecific antagonist, comprising: an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain; a first targeting domain comprising one or more variable domains selected from the group consisting of anti-PD-1 variable domains, anti-PD-L1 variable domains, anti-

TIGIT variable domains and anti-LAG-3 variable domains; a second targeting domain of anti-PD-1 variable domains, anti-PD-L1 variable domains, anti-TIGIT variable domains and anti-LAG-3 variable domains; and a third targeting domain that comprises a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway; wherein the second targeting domain is structurally linked to a carboxy-terminal of the CH3 domain and wherein the third targeting domain is inserted within the CH3 domain.

[0006] Another aspect of the present application relates to a trispecific antagonist, comprising: an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain; a first targeting domain comprising one or more variable domains selected from the group consisting of anti-VEGF variable domains; a second targeting domain comprising a TGF-β pathway inhibitor; and a third targeting domain that comprises a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway; wherein the second targeting domain is structurally linked to a carboxy-terminal of the CH3 domain and wherein the third targeting domain is inserted within the CH3 domain.

[0007] Another aspect of the present application relates to a trispecific antagonist that comprises an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain, a first targeting domain comprising one or more variable domains selected from the group consisting of anti-PD-1 variable domains, anti-PD-L1 variable domains, anti-TIGIT variable domains and anti-LAG-3 variable domains; a second targeting domain comprising a component of VEGFR; and a third targeting domain comprising a TGF-β pathway inhibitor, wherein the second targeting domain is structurally linked to a carboxy-terminal of the CH3 domain, and wherein the third targeting domain is structurally linked to a carboxy-terminal of the second targeting domain.

[0008] Another aspect of the present application relates to a bispecific antagonist that comprises an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain, a first targeting domain comprising one or more variable domains selected from the group consisting of anti-PD-1 variable domains, anti-PD-L1 variable domains, anti-TIGIT variable domains and anti-LAG-3 variable domains; and a second targeting domain comprising a component of VEGFR, and wherein the third targeting domain is structurally linked to a carboxy-terminal of the second targeting domain.

[0009] Another aspect of the present application relates to a method for treating a cell proliferative disorder. The method comprises the step of administering to a subject in need thereof an effective amount of the trispecific antagonists of the present application.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] **FIG. 1** shows HCVR and LCVR sequences of certain checkpoint antagonists,

anti-PD-1, anti-PD-L1, anti-TIGIT, anti-LAG-3, monoclonal antibodies (Mabs).

[0011] FIG. 2 shows HCVR and LCVR and other domain sequences for trebananib, bevacizumab, ranibizumab, Tie2, VEGF, and TGF-B antagonists.

[0012] FIGS. 3A-3H show eight trispecific antitumor antagonists, TS-ZPT-1.1, TS-ZPT-1.2, TS-ZPT-1.3, TS-ZPT-2, TS-ZPT-3, TS-ZPT-4, TS-ZPT-5 and TS-ZPT-6, respectively, with IgG1 or IgG4 backbones comprising: (1) anti-PD-1 variable region (VH1, VL1) domains or other checkpoint antibody variable domains; (2) an aflibercept fusion protein domain: (i) at the amino-terminal end of one or both IgG arms (**FIGS. 3A-3C, 3F**); (ii) at the carboxy-terminal end of each antagonist (**FIGS. 3D, 3E, 3H**); or (iii) between the carboxy-terminal end of the CH3 domain and a trebananib peptide (or other biological peptide) (**FIG. 3G**) (3) a trebananib peptide (or other biological peptide): (i) fused to the carboxy-terminal end of each CH3 region (**FIGS. 3A-3D**); (ii) inserted within each of the two CH3 regions (**FIG. 3E**); (iii) fused to the carboxy-terminal end of each CH1 region (**FIG. 3F**); (iv) fused to the carboxy-terminal end of each aflibercept fusion protein domain (**FIG. 3G**); or (v) fused to the carboxy-terminal end of each CL region (**FIG. 3H**). The aflibercept fusion protein domain comprises vascular endothelial growth factor (VEGF)-binding portions from the extracellular domains of human VEGF receptors 1 and 2, while the trebananib peptide targets and binds to Ang1 and Ang2, thereby preventing the interaction of Ang1 and/or Ang2 with their cognate Tie2 receptors.

[0013] FIGS. 4A-4C show three different trispecific antitumor antagonists, TS-ZPT-7, TS-ZPT-8 and TS-ZPT-9, respectively, comprising (1) an aflibercept fusion protein domain at each carboxy-terminal end; (2) anti-PD-1 or other checkpoint antagonist antibody variable domain (VH1, VL1); and (3) a trebananib peptide (or other biological peptide): (i) inserted within each of the two CH3 regions (**FIG. 4A**); (ii) inserted between the carboxy-terminal end of each CH2 region and an aflibercept fusion protein domain at the carboxy-terminal end of each polypeptide chain in the antagonist (**FIG. 4B**); or (iii) fused to the amino terminal end of each IgG1 arm (**FIG. 4C**).

[0014] FIGS. 5A-5G show seven different trispecific antitumor antagonists, TS-LPT-1, TS-LPT-2, TS-LPT-3, TS-LPT-4, TS-LPT-5, TS-M3, and TS-M4, respectively, comprising: (1) VH2 and VL2 regions corresponding to ranibizumab (Lucentis), bevacizumab (Avastin) or other anti-VEGF variable domains; (2) VH1 and VL1 regions corresponding to anti-PD-1 or other checkpoint antagonist antibody variable domains; and (3) a trebananib peptide (or other biological peptide): (i) inserted within each of the two CH3 regions (**FIG. 5A**); (ii) fused to the carboxy-terminal end of each CL1 region (**FIG. 5B**) (iii) fused to the carboxy-terminal end of the CH1 region in each of the two polypeptide chains

(**FIG. 5C**); (iv) fused to the carboxy-terminal end of the CL region in each of the two polypeptide chains (**FIG. 5D**); (v) fused to the carboxy-terminal end of the CH1 region in each of the two polypeptide chains and fused to the carboxy-terminal end of the CL region in each of the two polypeptide chains (**FIG. 5E**) (vi and vii fused to the carboxy-terminus of the CH3 (**FIG. 5F and 5G**). The ranibizumab variable domains are derived from bevacizumab and both are known to block binding of human VEGF-A to VEGFR 1 and 2. Exemplary sequences of TS-LPT-1, TS-LPT-2, TS-LPT-3, TS-LPT-4, TS-LPT-5 TS-M3, and TS-M4 are shown in **SEQ ID NOS:247-269**.

[0015] FIGS. 6A and 6B show non-reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of trispecific antitumor antagonists depicted in **FIGS. 3A-3E, 3G, 3H and 5A** and expressed in transiently transfected HEK293 cells.

[0016] FIGS. 7A and 7B show IC50 values (nM) calculated from a cell-based binding assay reflecting the ability of the trispecific antitumor antagonists depicted in **FIGS. 3A-3H** to block the interaction between human PD-1 and human PD-L1.

[0017] FIGS. 8A and 8B show IC50 values (nM) calculated from a binding assay reflecting the ability of the trispecific antitumor antagonists depicted in **FIGS. 3D, 3E and 3G** to block the interaction between VEGF and VEGFR-2.

[0018] FIG. 9 shows IC50 values (nM) calculated from a binding assay reflecting the ability of the trispecific antitumor antagonists depicted in **FIGS. 3B, 3D, 3G, 3H and 5A** to block the interaction between Ang2 and Tie2, compared to a bevacizumab-trebananib fusion control molecule.

[0019] FIG. 10 shows the results of a size exclusion chromatography analysis (SEC) of purified trispecific antitumor antagonists TS-ZPT-2(2P17), TS-ZPT-3L(2P17) and TS-ZPT-5(2P17) and anti-PD-1 monospecific antibody 2P17, produced by transient transfection of HEK293 cells. The percentage of high molecular weight (HWM%) species, low molecular weight (LMW%) species and dimerized molecule (Dimer%) are shown. The amino acid sequences of the HCVR and LCVR of these trispecific antitumor antagonists are shown in **SEQ ID NOS:201-204.**

[0020] FIG. 11 shows the results of a size exclusion chromatography analysis (SEC) of purified trispecific antitumor antagonists TS-ZPT-2(2P17), TS-ZPT-3L(2P17) and TS-ZPT-5(2P17) expressed by CHO stable pools. Two transfection pools were assessed for TS-ZPT-3L(2P17). The percentage of high molecular weight (HWM%) species, low molecular weight (LMW%) species and dimerized molecule (Dimer%) are shown.

[0021] FIGS. 12A and 12B show the chromatography traces and results of a size exclusion chromatography analysis (SEC) of purified trispecific antitumor antagonist TS-

ZPT-3L(2P17) purified from the CHO stable pool and stored at 4 degrees C for 112 days. The percentage of high molecular weight (HWM%) species, low molecular weight (LMW%) species and dimerized molecule (Dimer%) are shown.

[0022] FIG. 13A shows the heavy chain (**SEQ ID NO:202**) and light chain amino acid sequences (**SEQ ID NO:201**) for an exemplary trispecific antitumor antagonist with the trebananib long peptide, *i.e.*, TS-ZPT-3L (2P17), comprising (1) anti-PD-1 of another checkpoint antagonist antibody 2P17 variable domain (VH1, VL1), aflibercept VEFG binding domain fused to the carboxyl-terminal end of each antagonist, and connected to the CH3 domain with a 3xG4S linker; and (3) a trebananib peptide (or other biological peptide) inserted within each of the two CH3 regions. **FIG. 13B** depicts an exemplary molecule derived from these sequences.

[0023] FIG. 14A shows the heavy chain (**SEQ ID NO:200**) and light chains amino acid sequences (**SEQ ID NO:201**) for an exemplary trispecific antitumor antagonist with the trebananib short peptide, *i.e.*, TS-ZPT-3S(2P17), comprising (1) anti-PD-1 of another checkpoint antagonist antibody 2P17 variable domain (VH1, VL1), aflibercept VEFG binding domain fused to the carboxyl-terminal end of each antagonist, and connected to the CH3 domain with a 3xG4S linker; and (3) a trebananib peptide (or other biological peptide) inserted within each of the two CH3 regions; (i) a single copy of the trebananib blocking peptide. **FIG. 14B** depicts an exemplary molecule derived from these sequences.

[0024] FIG. 15 show SDS-PAGE of various trispecific antitumor antagonists depicted in **FIGS. 5F** and **5G** and **FIGS. 13A-14B** that were produced by transiently transfected HEK293 cells.

[0025] FIG. 16 shows the results from a size exclusion chromatography analysis of TS-ZPT-3L(2P17), TS-ZPT-3S(2P17), TS-ZPT-3L(2P16), and TS-ZPT-3S(2P16) produced from HEK293 cells.

[0026] FIG. 17 shows the size exclusion chromatography analysis of TS-ZPT-3L(2P17) and TS-ZPT-3S(2P17) over time with storage at 4 degrees C.

[0027] FIG. 18A shows the results of a cell-based binding assay measuring the ability of TS-ZPT-3S(2P16 and 2P17) and TS-ZPT-3L(2P16 and 2P17) trispecific antitumor antagonists compared to parental antibody 2P17 to block the interaction between PD-1 and PD-L1. **FIG. 18B** shows the IC50 values (nM) obtained from this analysis.

[0028] FIG. 19A shows the results of a cell-based bioassay measuring the ability of TS-ZPT-3S(2P16 and 2P17) and TS-ZPT-3L(2P16 and 2P17) trispecific antitumor antagonists compared to the benchmark antibody bevacizumab and a non-specific control antibody to block the interaction between VEGF and VEGFR-2. **FIG. 19B** shows the IC50

values (nM) obtained from this analysis.

[0029] FIG. 20A shows the results of a binding assay measuring the ability of TS-TS-ZPT-3S(2P16 and 2P17) and TS-ZPT-3L(2P16 and 2P17) trispecific antitumor antagonists compared to the benchmark molecule trebananib and a non-specific control antibody to block the interaction between Ang2 and Tie2. **FIG. 20B** shows the IC50 values (nM) obtained from this analysis.

[0030] FIGS. 21A-21E show the results of PD-1 binding to TS-ZPT-3S(2P16 and 2P17) and TS-ZPT-3L(2P16 and 2P17) trispecific antitumor antagonists compared to the benchmark anti-PD-1 antibody as determined by bio-layer interferometry, along with their resultant binding affinity constants (**FIG. 21F**).

[0031] FIGS. 22A-22E show the results of VEGF165 binding to TS-ZPT-3S(2P16 and 2P17) and TS-ZPT-3L(2P16 and 2P17) trispecific antitumor antagonists compared to the benchmark antibody bevacizumab as determined by bio-layer interferometry, along with their resultant binding affinity constants (**FIG. 22F**).

[0032] FIGS. 23A-23E show the results of Ang2 binding to TS-ZPT-3S(2P16 and 2P17) and TS-ZPT-3L(2P16 and 2P17) trispecific antitumor antagonists compared to the benchmark molecule trebananib as determined by bio-layer interferometry, along with their resultant binding affinity constants (**FIG. 23F**).

[0033] FIG.24 shows the use of bio-layer interferometry to characterize the sequential binding of TS-ZPT-3S(2P16 and 2P17) and TS-ZPT-3L(2P16 and 2P17) trispecific antitumor antagonists to each of its three binding partners using the Octet RED96 System (ForteBio).

[0034] FIG. 25 shows pharmacokinetic profiles of the TS-ZPT-3L(2P17) trispecific antitumor antagonists in 4 separate mice.

[0035] FIGS. 26A-26B show certain trispecific antitumor checkpoint antagonist configurations, where: (1) the VH1 and VL1 regions correspond to checkpoint #1, for example anti-PD-1 or anti-PD-L1 variable domains; (2) the VH2 and VL2 regions correspond to checkpoint #2, for example, anti-TIGIT, anti-LAG-3 variable domains; and (3) the circular region corresponds to trebananib (or any other biological peptide) inserted within the CH3 domain.

[0036] FIG. 27 depicts a trispecific antitumor antagonist, TS-A1BT-1, having amino-terminal anti-VEGF variable regions (VH1, VL1) containing two amino acid substitutions in the VH region (E6Q, L11V) from Avastin/bevacizumab in a mutant IgG1 (K447A) scaffold; Trebananib short peptide inserted within the IgG1 CH3 domain; and a carboxy-terminal TGF-β1 RII ECD connected to the CH3 domain with a 4xG4S linker. The amino acid

sequences of the HC and LC of this trispecific antitumor antagonist is listed in **SEQ ID NOS:229 and 230**.

[0037] FIG. 28 shows a non-reducing SDS-PAGE analysis of TS-A1BT-1 produced by HEK293 transiently transfected cells compared to the control transfection antibody, 2P17, showing good expression levels of the trispecific antitumor antagonist. **FIG. 29** shows the size exclusion chromatography analysis of TS-AIBT-1.

[0038] FIGS. 30A-30F show six trispecific antitumor antagonist configurations comprising a TGF-βRII extracellular domain (ECD): TS-ZPB-1, TS-ZPB-2, TS-ZPB-3, TS-ZPB-4, TS-ZPB-5, and TS-ZPB-6, respectively.

[0039] FIG. 31A shows a non-reducing SDS-PAGE analysis of trispecific antitumor antagonists TS-ZPB-1(2P17), TS-ZPB-2(2P17), TS-ZPB-3(2P17), TS-ZPB-5(2P17), and TS-ZPLB-1 transiently expressed by HEK 293 cells, before and after purification.

[0040] FIG. 32A shows exemplary size exclusion chromatograph (SEC) profiles for TS-ZPB-1(2P17), TS-ZPB-2(2P17), TS-ZPB-3(2P17), TS-ZPB-5(2P17), and TS-ZPLB-1 compared to TGF-β-RII-Fc. The percentage of high molecular weight (HWM%) species, low molecular weight (LMW%) species and dimerized molecule (Dimer%) are shown in **FIG. 32B**.

[0041] FIG. 33A shows the results of a cell-based binding assay measuring the ability of TS-ZPB-1(2P17), TS-ZPB-2(2P17), TS-ZPB-3(2P17), and TS-ZPB-5(2P17) to block the interaction between PD-1 and PD-L1. **FIG. 33B** shows the IC50 values (nM) obtained from this analysis.

[0042] FIG. 34A shows the results of a bioassay to measure the ability of TS-ZPB-1(2P17), TS-ZPB-2(2P17), TS-ZPB-3(2P17), TS-ZPB-5(2P17), and TS-ZPLB-1 to block TGFβ1 signaling compared to the benchmark TGF-β1 RII-Fc. **FIG. 34B** shows the IC50 values (nM) obtained from this analysis.

[0043] FIG. 35A shows the results of an ELISA assay measuring the ability of TS-ZPB-1(2P17), TS-ZPB-2(2P17), TS-ZPB-3(2P17), TS-ZPB-5(2P17), and TS-ZPLB-1 to block the interaction between VEGF and VEGFR-2, as compared to the benchmark bevacizumab. **FIG. 35B** shows the IC50 values (nM) obtained from this analysis.

[0044] FIGS. 36A-36C show three trispecific antagonists: TS-ZPB-5, and variants TS-ZPB-5A, with K447A mutation in the CH3 domain, and TS-ZPB-5B, additionally with mutations to eliminate N-linked glycosylation of the aflibercept VEGF binding domain.

[0045] FIG. 37 shows expression levels of the trispecific antitumor antagonists TS-ZPB-5, TS-ZPB-5A and TS-ZPB-5B produced from transiently transfected HEK293 cells.

[0046] FIG. 38 shows the results of a cell-based assay measuring the ability of

trispecific antitumor antagonists TS-ZPB-5, TS-ZPB-5A and TS-ZPB-5B to block the interaction between VEGF and VEGFR-2.

[0047] FIG. 39 shows the results of a bioassay measuring the ability of trispecific antitumor antagonists TS-ZPB-5, TS-ZPB-5A and TS-ZPB-5B to block TGFβ1 signaling.

[0048] FIG. 40 shows the pharmacokinetic profiles of TS-ZPT-5A and the TS-ZPT-5B in two mice each.

[0049] FIGS. 41A-41C show three different bispecific antitumor antagonist configurations, Bi-ZPL-1 (or Bi-ZP-1) and Bi-ZPL-2 (or Bi-ZP-2) and Bi-ZPL-3 (or Bi-ZP-3) each comprising (1) anti-PD-1 or anti-PD-L1 variable regions and (2) an afilibercept VEGF binding domain (i) fused to the amino-terminus of the VH1; (ii) mutated to eliminate the N-linked glycosylation sites and fused to the amino-terminus of the VH1; (iii) mutated to eliminate the N-Linked glycosylation sites and fused to the carboxy-terminus of the CH3 with a 3xG4S linker.

[0050] FIG. 42A shows the results of a cell-based assay measuring the ability of Bi-ZPL-1 molecules compared to the benchmark anti-PD-1 antibody for the inhibition of the interaction between PD-L1 and PD-1. **FIG. 42B** shows the resulting IC50 values (nM) obtained from this analysis.

[0051] FIG. 43A shows the results of a bioassay measuring the ability of Bi-ZPL-1 molecules compared to the benchmark anti-PD-1 antibody for the inhibition of the interaction between VEGF and VEGFR-2. **FIG. 43B** shows the resulting IC50 values (nM).

[0052] FIG. 44 shows the results of a size exclusion chromatography analysis of protein A purified Bi-ZP-2 and Bi-ZPL-3.

[0053] FIG. 45 shows the pharmacokinetic assessment in mice of Bi-ZP-2 and Bi-ZPL-3.

[0054] FIGS. 46A-46C show the sequences of the exemplary framework regions. **FIG. 46A** shows the exemplary framework regions of anti-TIGIT mAbs. **FIG. 46B** shows the exemplary framework regions of anti-PD-1 and anti-PD-L1 mAbs. **FIG. 46C** shows the exemplary framework regions of anti-LAG3 mAbs.

DETAILED DESCRIPTION

Definitions

[0055] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed method and compositions belong. It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a peptide" includes "one or more" peptides or a

"plurality" of such peptides. With respect to the teachings in the present application, any issued patent or patent application publication described in this application is expressly incorporated by reference herein.

[0056] As used herein, the term "PD-1" refers to any form of PD-1 and variants thereof that retain at least part of the activity of PD-1. Unless indicated differently, such as by specific reference to human PD-1, PD-1 includes all mammalian species of native sequence PD-1, *e.g.*, human, canine, feline, equine, and bovine. An exemplary human PD-1 amino acid sequence is listed below:

MQIPQAPWPVVWAVLQLGWRPGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSN TSESFVLNWYRMSPSNQTDKLAAFPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRN DSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPSPRPAGQFQTLVVGVVGG LLGSLVLLVWVLAVICSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTP EPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPL (**SEQ ID NO:180**).

[0057] As used herein, the term "PD-L1" refers to any form of PD-L1 and variants thereof that retain at least part of the activity of PD-L1. Unless indicated differently, such as by specific reference to human PD-L1, PD-L1 includes all mammalian species of native sequence PD-L1, *e.g.*, human, canine, feline, equine, and bovine. An exemplary human PD-L1 amino acid sequence is listed below:

MRIFAVFIFMTYWHLLNAFTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAALIVYWE MEDKNIIQFVHGEEDLKVQHSSYRQRARLLKDQLSLGNAALQITDVKLQDAGVYRCMI SYGGADYKRITVKVNAPYNKINQRILVVDPVTSEHELTCQAEGYPKAEVIWTSSDHQVL SGKTTTTNSKREEKLFNVTSTLRINTTTNEIFYCTFRRLDPEENHTAELVIPELPLAHPPNE RTHLVILGAILLCLGVALTFIFRLRKGRMMDVKKCGIQDTNSKKQSDTHLEET (**SEQ ID NO:181**).

[0058] As used herein, the term "TIGIT" refers to any form of TIGIT and variants thereof that retain at least part of the activity of TIGIT. Unless indicated differently, such as by specific reference to human TIGIT, TIGIT includes all mammalian species of native sequence TIGIT, *e.g.*, human, canine, feline, equine, and bovine. The following is an exemplary human TIGIT amino acid sequence:

MTGTIETTGNISAEKGGSIILQCHLSSTTAQVTQVNWEQQDQLLAICNADLGWHISPSFK DRVAPGPGLGLTLQSLTVNDTGEYFCIYHTYPDGTYTGRIFLEVLESSVAEHGARFQIPL LGAMAATLVVICTAVIVVVALTRKKKALRIHSVEGDLRRKSAGQEEWSPSAPSPPGSCV QAEAAPAGLCGEQRGEDCAELHDYFNVLSYRSLGNCSFFTETG (**SEQ ID NO:179**).

[0059] As used herein, the term "LAG-3" refers to any form of LAG-3 and variants thereof that retain at least part of the activity of lymphocyte-activation gene 3 (LAG-3). Unless indicated differently, such as by specific reference to human LAG-3, LAG-3 includes all mammalian species of native sequence LAG-3, *e.g.*, human, canine, feline, equine, and bovine. The following is an exemplary human LAG-3 amino acid sequence:
MWEAQFLGLLFLQPLWVAPVKPLQPGAEVPVVWAQEGAPAQLPCSPTIPLQDLSLLRR
AGVTWQHQPDSGPPAAAPGHPLAPGPHPAAPSSWGPRPRRYTVLSVGPGGLRSGRLPL
QPRVQLDERGRQRGDFSLWLRPARRADAGEYRAAVHLRDRALSCRLRLRLGQASMTA
SPPGSLRASDWVILNCSFSRPDRPASVHWFRNRGQGRVPVRESPHHHLAESFLFLPQVSP
MDSGPWGCILTYRDGFNVSIMYNLTVLGLEPPTPLTVYAGAGSRVGLPCRLPAGVGTRS
FLTAKWTPPGGGPDLLVTGDNGDFTLRLEDVSQAQAGTYTCHIHLQEQQLNATVTLAII
TVTPKSFGSPGSLGKLLCEVTPVSGQERFVWSSLDTPSQRSFSGPWLEAQEAQLLSQPW
QCQLYQGERLLGAAVYFTELSSPGAQRSGRAPGALPAGHLPAGHLLLFLILGVLSLLLL
VTGAFGFHLWRRQWRPRRFSALEQGIHPPQAQSKIEELEQEPEPEPEPEPEPEPEPEQL
(SEQ ID NO:293).

[0060] The term "agonist" refers to a substance which promotes (*i.e.*, induces, causes, enhances, or increases) the biological activity or effect of another molecule. The term agonist encompasses substances which bind receptor, such as an antibody, and substances which promote receptor function without binding thereto (*e.g.*, by activating an associated protein).

[0061] The term "antagonist" or "inhibitor" refers to a substance that prevents, blocks, inhibits, neutralizes, or reduces a biological activity or effect of another molecule, such as a receptor or ligand. An antagonist can be a mono-specific antibody, a bispecific antibody or a trispecific antibody.

[0062] As used herein, the term "antibody" refers to a polypeptide or a polypeptide complex that specifically recognizes and binds to an antigen through one or more immunoglobulin variable regions. An antibody can be a whole antibody, an antigen binding fragment or a single chain thereof. The term "antibody" encompasses various broad classes of polypeptides that can be distinguished biochemically. Those skilled in the art will appreciate that heavy chains are classified as alpha, delta, epsilon, gamma, and mu, or α, δ, ε, γ and μ) with some subclasses among them (*e.g.*, γ1-γ4). It is the nature of this chain that determines the "class" of the antibody as IgG, IgM, IgA IgG, or IgE, respectively. The immunoglobulin subclasses (isotypes) *e.g.*, IgG1, IgG2, IgG3, IgG4, etc. are well characterized and are known to confer functional specialization. Modified versions of each of these classes and isotypes are readily discernable to the skilled artisan in view of the instant disclosure and, accordingly, are within the scope of the instant disclosure. All immunoglobulin classes are within the scope of

the present disclosure, the following discussion will generally be directed to the IgG class of immunoglobulin molecules.

[0063] Antibodies or antibody antagonists of the present application may include, but are not limited to, polyclonal, monoclonal, monospecific, multispecific, bispecific, trispecific, human, humanized, primatized, chimeric and single chain antibodies. Antibodies disclosed herein may be from any animal origin, including birds and mammals. Preferably, the antibodies are human, murine, donkey, rabbit, goat, guinea pig, camel, llama, horse, or chicken antibodies. In some embodiments, the variable region may be condricthoid in origin (e.g., from sharks).

[0064] The terms "antibody fragment" or "antigen-binding fragment" are used with reference to a portion of an antibody, such as $F(ab')_2$, $F(ab)_2$, Fab', Fab, Fv, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv), fragments comprising either a VL or VH domain, fragments produced by a Fab expression library and anti-idiotypic (anti-Id) antibodies. Regardless of structure, an antibody fragment binds with the same antigen that is recognized by the intact antibody. The term "antibody fragment" includes DARTs and diabodies. The term "antibody fragment" also includes any synthetic or genetically engineered proteins comprising immunoglobulin variable regions that act like an antibody by binding to a specific antigen to form a complex. A "single-chain fragment variable" or "scFv" refers to a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulins. In some aspects, the regions are connected with a short linker peptide of ten to about 25 amino acids. The linker can be rich in glycine for flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the VH with the C-terminus of the VL, or vice versa. This protein retains the specificity of the original immunoglobulin, despite removal of the constant regions and the introduction of the linker. With regard to IgGs, a standard immunoglobulin molecule comprises two identical light chain polypeptides of molecular weight approximately 23,000 Daltons, and two identical heavy chain polypeptides of molecular weight 53,000-70,000. The four chains are typically joined by disulfide bonds in a "Y" configuration where the light chains bracket the heavy chains starting at the mouth of the "Y" and continuing through the variable region.

[0065] Both the light and heavy chains are divided into regions of structural and functional homology. The terms "constant" and "variable" are used functionally. In this regard, it will be appreciated that the variable domains of both the light (VL) and heavy (VH) chain portions determine antigen recognition and specificity. Conversely, the constant domains of the light chain (CL) and the heavy chain (CH1, CH2 or CH3) confer important biological properties such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. By convention, the numbering of the constant region domains in conventional antibodies

increases as they become more distal from the antigen-binding site or amino-terminus of the antibody. In conventional antibodies, the N-terminal portion is a variable region and at the carboxy-terminal portion is a constant region; the CH3 and CL domains actually comprise the carboxy-terminus of the heavy and light chain, respectively. Exemplary CHI-CH2-CH3 sequences described in the current application include the following wild type IgG1 (**SEQ ID NO:274**), IgG1 with a K447A mutation (**SEQ ID NO:275**), IgG1 with the N297A mutation (**SEQ ID NO:276**), wild type IgG2 (**SEQ ID NO:278**), IgG2 with carboxy terminal lysine deleted (**SEQ ID NO:279**), IgG4 with the hinge S231P mutation (**SEQ ID NO:280**), IgG4 with S23P and K447A (**SEQ ID NO:281**), and IgG4 S231P and the carboxy-terminal lysine deleted (**SEQ ID NO:282**). Exemplary CL sequence is **SEQ ID NO:292**.

[0066] The term "Fc fragment" or "Fc" are used with reference to a portion of an antibody contains no antigen-binding activity but was originally observed to crystallize readily, and for this reason was named the Fc fragment, for Fragment crystallizable. This fragment corresponds to the paired CH2 and CH3 domains and is the part of the antibody molecule that interacts with effector molecules and cells. The Fc fragments described herein may be derived from human IgG1, IgG2 and IgG4 antibodies with the modifications of a-glycosylation, hinge mutation and deletion of carboxy-terminal lysine. Exemplary Fc sequences described in the current application include the following: wild type IgG1 Fc (**SEQ ID NO:210**), a-glycosylated IgG1 Fc (**SEQ ID NO:211**), IgG4 Fc with hinge mutation (**SEQ ID NO:212**), wild type IgG2 Fc (**SEQ ID NO:213**), IgG1 Fc with deletion of carboxy-terminal lysine (**SEQ ID NO:214**), a-glycosylated IgG1 Fc with deletion of carboxy-terminal lysine (**SEQ ID NO:215**), IgG4 Fc with hinge mutation and deletion of carboxy-terminal lysine (**SEQ ID NO:216**), and IgG2 Fc with deletion of carboxy-terminal lysine (**SEQ ID NO:217**).

[0067] As indicated above, the variable region allows the antibody to selectively recognize and specifically bind epitopes on antigens. That is, the VL domain and VH domain, or subset of the complementarity determining regions (CDRs), of an antibody combine to form the variable region that defines a three dimensional antigen-binding site. This quaternary antibody structure forms the antigen-binding site present at the end of each arm of the Y. More specifically, the antigen-binding site is defined by three CDRs on each of the VH and VL chains (*i.e.*, HCDR1, HCDR2, HCDR3, LCDR1, LCDR 2 and LCDR 3). In some instances, *e.g.*, certain immunoglobulin molecules are derived from camelid species or engineered based on camelid immunoglobulins. Alternatively, an immunoglobulin molecule may consist of heavy chains only with no light chains or light chains only with no heavy chains.

[0068] In naturally occurring antibodies, the six CDRs present in each antigen-binding domain are short, non-contiguous sequences of amino acids that are specifically positioned to

form the antigen-binding domain as the antibody assumes its three dimensional configuration in an aqueous environment. The remainder of the amino acids in the antigen-binding domains, referred to as "framework" regions, show less inter-molecular variability. The framework regions largely adopt a β-sheet conformation and the CDRs form loops which connect, and in some cases form part of, the β-sheet structure. Thus, framework regions act to form a scaffold that provides for positioning the CDRs in correct orientation by inter-chain, non-covalent interactions. The antigen-binding domain formed by the positioned CDRs defines a surface complementary to the epitope on the immunoreactive antigen. This complementary surface promotes the non-covalent binding of the antibody to its cognate epitope. The amino acids comprising the CDRs and the framework regions, respectively, can be readily identified for any given heavy or light chain variable region by one of ordinary skill in the art, since they have been precisely defined.

[0069] As used herein, the terms "VH1" and "VH2" refer to immunoglobulin heavy chain variable domains corresponding to two different binding specificities. Likewise, the terms "VL1" and "VL2" refer to light chain variable domains corresponding to two different binding specificities. When used together, it is to be understood that VH1 and VL1 regions define a common binding specificity and that VH2 and VL2 domains define a second binding specificity.

[0070] The term "framework region (FR)" as used herein refers to variable domain residues other than the CDR residues. Each variable domain typically has four FRs flanking the corresponding CDRs. For example, a VH domain typically has four HFRs, HFR1, HFR2, HFR3 and HFR4, flanking the three HCDRs in the configuration of HFR1-HCDR1-HFR2-HCDR2-HFR3-HCDR3-HFR4. Similarly, an LH domain typically has four LFR, LFR1, LFR2, LFR3 and LFR4, flanking the three LCDRs in the configuration of: LFR1-LCDR1-LFR2-LCDR2-LFR3-LCDR3-LFR4. Exemplary FRs are summarized in **FIGS. 46A-46C**.

[0071] Light chains are classified as either kappa or lambda (K, λ). Each heavy chain class may be bound with either a kappa or lambda light chain. In general, the light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages or non-covalent linkages when the immunoglobulins are generated either by hybridomas, B cells or genetically engineered host cells. In the heavy chain, the amino acid sequences run from an N-terminus at the forked ends of the Y configuration to the C-terminus at the bottom of each chain.

[0072] As used herein, the term "light chain constant region (CL)" includes amino acid sequences derived from antibody light chain CL **(SEQ ID NO:292)**. Preferably, the light chain constant region comprises at least one of a constant kappa domain or constant lambda domain.

[0073] As used herein, the term "heavy chain constant region (CH)" includes amino acid sequences derived from an immunoglobulin heavy chain. A polypeptide comprising a heavy chain constant region comprises at least one of: a CH1 domain (**SEQ ID NOS:290-291**), a hinge (*e.g.*, upper, middle, and/or lower hinge region) domain, a CH2 domain, a CH3 domain, or a variant or fragment thereof. For example, an antigen-binding polypeptide for use in the disclosure may comprise a polypeptide chain comprising a CH1 domain; a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, and a CH2 domain; a polypeptide chain comprising a CH1 domain and a CH3 domain; a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, and a CH3 domain, or a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, a CH2 domain, and a CH3 domain. In some embodiments, a polypeptide of the disclosure comprises a polypeptide chain comprising a CH3 domain. Further, an antibody for use in the disclosure may lack at least a portion of a CH2 domain (*e.g.*, all or part of a CH2 domain). It should be understood that the heavy chain constant region may be modified such that they vary in amino acid sequence from the naturally occurring immunoglobulin molecule.

[0074] For example, as reflected in the disclosure herein below, Applicant has found that the CH3 domain can tolerate or accommodate significant insertions (*e.g.*, greater than 100 aa) in the Fc loop of the CH3 domain (*see, e.g.*, **FIG. 3E**). Therefore, in the present application, any of the disclosed inhibitor domains may be similarly inserted in the Fc loop in a manner analogous to the insertion of trebananib or the TGFβ1 RII ECD domain in the Fc loop.

[0075] The heavy chain constant region of an antibody disclosed herein may be derived from different immunoglobulin molecules. For example, a heavy chain constant region of a polypeptide may comprise a CH1 domain derived from an IgG1 molecule and a hinge region derived from an IgG3 molecule. In another example, a heavy chain constant region can comprise a hinge region derived, in part, from an IgG1 molecule and, in part, from an IgG3 molecule. In another example, a heavy chain portion can comprise a chimeric hinge derived, in part, from an IgG1 molecule and, in part, from an IgG4 molecule.

[0076] A "light chain-heavy chain pair" refers to the collection of a light chain and heavy chain that can form a dimer through a disulfide bond between the CL domain of the light chain and the CH1 domain of the heavy chain.

[0077] The subunit structures and three dimensional configurations of the constant regions of the various immunoglobulin classes are well known. As used herein, the term "VH domain" includes the amino terminal variable domain of an immunoglobulin heavy chain and the term "CH1 domain" includes the first (most amino terminal) constant region domain of an

immunoglobulin heavy chain. The CH1 domain is adjacent to the VH domain and is amino terminal to the hinge region of an immunoglobulin heavy chain molecule.

[0078] As used herein the term "CH2 domain" includes the portion of a heavy chain molecule that extends, *e.g.*, from about residue 244 to residue 360 of an antibody using conventional numbering schemes (residues 244 to 360, Kabat numbering system; and residues 231-340, EU numbering system). The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. The CH3 domain extends from the CH2 domain to the carboxy-terminal of the IgG molecule and comprises approximately 108 residues.

[0079] As used herein, the term "hinge region" includes the portion of a heavy chain molecule that joins the CH1 domain to the CH2 domain. This hinge region comprises approximately 25 residues and is flexible, thus allowing the two N-terminal antigen-binding regions to move independently. Hinge regions can be subdivided into three distinct domains: upper, middle, and lower hinge domains.

[0080] As used herein the term "disulfide bond" includes a covalent bond formed between two sulfur atoms. The amino acid cysteine comprises a thiol group that can form a disulfide bond or bridge with a second thiol group. In most naturally occurring IgG molecules, the CH1 and CL regions are structurally linked by a disulfide bond and the two heavy chains are structurally linked by two disulfide bonds at positions corresponding to 239 and 242 using the Kabat numbering system (position 226 or 229, EU numbering system).

[0081] As used herein, a "variant" of antibody, antibody fragment or antibody domain refers to antibody, antibody fragment or antibody domain that (1) shares a sequence identity of at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% with the original antibody, antibody fragment or antibody domain, and (2) binds specifically to the same target that the original antibody, antibody fragment or antibody domain binds specifically. It should be understood that where a measure of sequence identity is presented in the form of the phrase "at least x % identical" or "at least x % identity", such an embodiment includes any and all whole number percentages equal to or above the lower limit. Further it should be understood that where an amino acid sequence is presented in the present application, it should be construed as additionally disclosing or embracing amino acid sequences having a sequence identity of at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identity to that amino acid sequence.

[0082] It should be understood that where a sequence homology range is presented herein, as in *e.g.*, the phrase "about 80% to about 100%", such an embodiment includes any and

all sub-ranges defined by any whole numbers within, wherein the lower number can be any whole number between 80 and 100.

[0083] As used herein, the phrase "humanized antibody" refers to an antibody derived from a non-human antibody, typically a mouse monoclonal antibody. Alternatively, a humanized antibody may be derived from a chimeric antibody that retains or substantially retains the antigen binding properties of the parental, non-human, antibody but which exhibits diminished immunogenicity as compared to the parental antibody when administered to humans.

[0084] As used herein, the phrase "chimeric antibody," refers to an antibody where the immunoreactive region or site is obtained or derived from a first species and the constant region (which may be intact, partial or modified in accordance with the instant disclosure) is obtained from a second species. In certain embodiments the target binding region or site will be from a non-human source (*e.g.*, mouse or primate) and the constant region is human.

[0085] Included within the scope of the multispecific antibodies of the present application are various compositions and methodologies, including asymmetric IgG-like antibodies (*e.g.*, triomab/quadroma, Trion Pharma/Fresenius Biotech); knobs-into-holes antibodies (Genentech); Cross MAbs (Roche); electrostatically matched antibodies (AMGEN); LUZ-Y (Genentech); strand exchange engineered domain (SEED) body (EMD Serono; Biolonic, Merus); Fab-exchanged antibodies (Genmab); symmetric IgG-like antibodies (*e.g.* dual targeting (DT)-Ig (GSK/Domantis); two-in-one antibody (Genentech); crosslinked MAbs (Karmanos Cancer Center); mAb^2 (F-star); Cov X-body (Cov X/Pfizer); dual variable domain (DVD)-Ig fusions (Abbott); IgG-like bispecific antibodies (Eli Lilly); Ts2Ab (Medimmune/AZ); BsAb (ZymoGenetics); HERCULES (Biogen Idec,TvAb, Roche); scFv/Fc fusions; SCORPION (Emergent BioSolutions/Trubion, ZymoGenetics/BMS); dual affinity retargeting technology (Fc-DART); MacroGenics; dual $(scFv)_2$-Fabs (National Research Center for Antibody Medicine); $F(ab)_2$ fusions (Medarex/AMGEN); dual-action or Bis-Fab (Genentech); Dock-and-Lock (DNL, ImmunoMedics); Fab-Fv (UCB-Celltech); scFv- and diabody-based antibodies (*e.g.*, bispecific T cell engagers (BiTEs, Micromet); tandem diabodies (Tandab, Affimed); DARTs (MacroGenics); single-chain diabodies; TCR-like antibodies (AIT, Receptor Logics); human serum albumin scFv fusion (Merrimack); COMBODIES (Epigen Biotech); and IgG/non-IgG fusions (*e.g.*, immunocytokines (EMDSerono, Philogen, ImmunGene, ImmunoMedics).

[0086] By "specifically binds" or "has specificity to", it is generally meant that an antibody binds to an epitope via its antigen-binding domain, and that the binding entails some complementarity between the antigen-binding domain and the epitope. According to this definition, an antibody is said to "specifically bind" to an epitope when it binds to that epitope via its antigen-binding domain more readily than it would bind to a random, unrelated epitope.

The term "specificity" is used herein to qualify the relative affinity by which a certain antibody binds to a certain epitope. For example, antibody "A" may be deemed to have a higher specificity for a given epitope than antibody "B," or antibody "A" may be said to bind to epitope "C" with a higher specificity than it has for related epitope "D". In some embodiments, an antibody or an antibody fragment "has specificity to" an antigen if the antibody or antibody fragment forms a complex with the antigen with a dissociation constant (K_d) of 10^{-6}M or less, 10^{-7}M or less, 10^{-8}M or less, 10^{-9}M or less, or 10^{-10}M or less.

[0087] The term "antagonist antibody" refers to an antibody that binds to a target and prevents or reduces the biological effect of that target. In some embodiments, the term can denote an antibody that prevents the target, *e.g.*, TIGIT, to which it is bound from performing a biological function.

[0088] As used herein, an "anti-PD-1 antagonist antibody" refers to an antibody that is able to inhibit PD-1 biological activity and/or downstream events(s) mediated by PD-1. Anti-PD-1 antagonist antibodies encompass antibodies that block, antagonize, suppress or reduce (to any degree including significantly) PD-1 biological activity, including downstream events mediated by PD-1, such as PD-1 binding and downstream signaling, inhibition of T cell proliferation, inhibition of T cell activation, inhibition of IFN secretion, inhibition of IL-2 secretion, inhibition of TNF secretion, induction of IL-10, and inhibition of anti-tumor immune responses. For purposes of the present application, it will be explicitly understood that the term "anti-PD-1 antagonist antibody" (interchangeably termed "antagonist PD-1 antibody", "antagonist anti-PD-1 antibody" or "PD-1 antagonist antibody") encompasses all the previously identified terms, titles, and functional states and characteristics whereby PD-1 itself, a PD-1 biological activity, or the consequences of the biological activity, are substantially nullified, decreased, or neutralized in any meaningful degree. In some embodiments, an anti-PD-1 antagonist antibody binds PD-1 and upregulates an anti-tumor immune response.

[0089] As used herein, an "anti-PD-L1 antagonist antibody" refers to an antibody that is able to inhibit PD-L1 biological activity and/or downstream event(s) mediated by PD-L1. Anti-PD-L1 antagonist antibodies encompass antibodies that block, antagonize, suppress or reduce (to any degree including significantly) PD-L1 biological activity, including downstream events mediated by PD-L1, such as PD-L1 binding and downstream signaling, inhibition of T cell proliferation, inhibition of T cell activation, inhibition of IFN secretion, inhibition of IL-2 secretion, inhibition of TNF secretion, induction of IL-10, and inhibition of anti-tumor immune responses. For purposes of the present application, it will be explicitly understood that the term "anti-PD-L1 antagonist antibody" (interchangeably termed "antagonist PD-L1 antibody", "antagonist anti-PD-L1 antibody" or "PD-L1 antagonist antibody") encompasses all the

previously identified terms, titles, and functional states and characteristics whereby PD-L1 itself, a PD-L1 biological activity, or the consequences of the biological activity, are substantially nullified, decreased, or neutralized in any meaningful degree. In some embodiments, an anti-PD-L1 antagonist antibody binds PD-L1 and upregulates an anti-tumor immune response.

[0090] The phrase "immune checkpoint regulator" refers to a functional class of agents, which inhibit or stimulate signaling through an immune checkpoint. An "immune checkpoint regulator" includes cell surface receptors and their associated ligands, which together provide a means for inhibiting or stimulating signaling pathways associated with T-cell activation. Exemplary immune checkpoint regulators include, but are not limited to PD-1 and its ligands, PD-L1 and PD-L2; TIGIT and its CD155 ligand, PVR; CTLA-4 and its ligands, B7-1 and B7-2; TIM-3 and its ligand, Galectin-9; LAG-3 and its ligands, including liver sinusoidal endothelial cell lectin (LSECtin) and Galectin-3; CD122 and its CD122R ligand; CD70, B7H3, B and T lymphocyte attenuator (BTLA), and VISTA.

[0091] The phrases "checkpoint regulator antagonist", "immune checkpoint binding antagonist" and "immune checkpoint antagonist" are used interchangeably herein with reference to a class of agents that interfere with (or inhibit) the activity of an immune checkpoint regulator so that, as a result of the binding to the checkpoint regulator or its ligand, signaling through the checkpoint regulator receptor is blocked or inhibited. By inhibiting this signaling, immune-suppression can be reversed so that T cell immunity against cancer cells can be re-established or enhanced. Immune checkpoint regulator antagonists include antibody fragments, peptide inhibitors, dominant negative peptides and small molecule drugs, either in isolated forms or as part of a fusion protein or conjugate.

[0092] The phrases "immune checkpoint binding agonist" and "immune checkpoint agonist" are used interchangeably herein with reference to a class of agents that stimulate the activity of an immune checkpoint regulator so that, as a result of the binding to the checkpoint regulator or its ligand, signaling through the checkpoint regulator receptor is stimulated. By stimulating this signaling, T cell immunity against cancer cells can be re-established or enhanced. Exemplary immune checkpoint regulator agonists include, but are not limited to members of the tumor necrosis factor (TNF) receptor superfamily, such as CD27, CD40, OX40 (CD 134), glucocorticoid-induced TNFR family-related protein (GITR), and 4-1BB (CD137) and their ligands. Additional checkpoint regulator agonists belong to the B7-CD28 superfamily, including CD28 and ICOS.

[0093] The phrases "dominant-negative protein" or "dominant-negative peptide" refer to a protein or peptide derived from a wild type protein that has been genetically modified by

mutation and/or deletion so that the modified protein or peptide interferes with the function of the endogenous wild-type protein from which it is derived.

[0094] The phrase "VEGF binding antagonist" refers to a functional class of agents that bind to VEGF-A or its receptor, VEGFR-2, so that, as a result of the binding, activation of VEGFR-2 by VEGF-A is blocked or inhibited. As used herein, the term "VEGF binding antagonists" include antibody fragments, peptide inhibitors, dominant negative peptides and small molecule drugs, either in isolated forms or as part of a fusion protein or conjugate.

[0095] The phrase "Tie2 tyrosine kinase receptor binding antagonist" refers to a functional class of agents that bind to a Tie2 tyrosine kinase receptor or one of its ligands so that, as a result of the binding, activation of the Tie2 tyrosine kinase receptor by one or more of its ligands (*i.e.,* Ang1, Ang2, Ang3 and Ang4) is blocked or inhibited. As used herein, the term "Tie2 tyrosine kinase receptor binding antagonist" include antibody fragments, peptide inhibitors, dominant negative peptides and small molecule drugs, either in isolated forms or as part of a fusion protein or conjugate.

[0096] The phrase "small molecule drug" refers to a molecular entity, often organic or organometallic, that is not a polymer, that has medicinal activity, and that has a molecular weight less than about 2 kDa, less than about 1 kDa, less than about 900Da, less than about 800Da or less than about 700Da. The term encompasses most medicinal compounds termed "drugs" other than protein or nucleic acids, although a small peptide or nucleic acid analog can be considered a small molecule drug. Examples include chemotherapeutic anticancer drugs and enzymatic inhibitors. Small molecules drugs can be derived synthetically, semi-synthetically (*i.e.*, from naturally occurring precursors), or biologically.

[0097] When describing polypeptide domain arrangements with hyphens between individual domains (*e.g.*, CH2-CH3), it should be understood that the order of the listed domains is from the amino terminal end to the carboxy terminal end.

[0098] The term "immunoconjugate" refers to an antibody which is fused by covalent linkage to an inhibitory peptide or small molecule drug. The peptide or small molecule drug can be chemically linked to the C-terminus of a constant heavy chain or to the N-terminus of a variable light and/or heavy chain.

[0099] A "linker" may be used to link the peptide or small molecule drug, such as a maytansinoid, to the antitumor antagonists in a stable, covalent manner. Linkers can be susceptible to or be substantially resistant to acid-induced cleavage, light-induced cleavage, peptidase-induced cleavage, esterase-induced cleavage, and disulfide bond cleavage, at conditions under which the compound or the antibody remains active. Suitable linkers are well known in the art and include, for example, disulfide groups, thioether groups, acid labile groups,

photolabile groups, peptidase labile groups and esterase labile groups. Linkers also include charged linkers, and hydrophilic forms thereof as described herein and know in the art. The immunoconjugate may further include a flexible 3-15 amino acid linker, for example, GGG (**SEQ ID NO:283**) or GGGGS (G4S) repetitive peptide linker (**SEQ ID NOS:284-289**), between an antitumor antagonist and the peptide and/or small molecule drug.

[0100] As used herein, the term "scaffold" refers to any polymer of amino acids that exhibits properties desired to support the function of an antagonist, including addition of antibody specificity, enhancement of antibody function or support of antibody structure and stability. A scaffold can be grafted with binding domains of a donor polypeptide to confer the binding specificity of the donor polypeptide onto the scaffold.

[0101] As used herein, the phrase "multispecific inhibitor" refers to a molecule comprising at least two targeting domains with different binding specificities. In some embodiments, the multispecific inhibitor is a polypeptide comprising a scaffold and two or more immunoglobulin antigen binding domains targeting different antigens or epitopes. In certain embodiments, the multispecific inhibitor is a bispecific antibody or antagonist. In other embodiments, the multispecific inhibitor is a trispecific antibody or antagonist.

[0102] As used herein, the phrase "bispecific" refers to a molecule comprising at least two targeting domains with different binding specificities. Each targeting domain is capable of binding specifically to a target molecule and inhibiting a biological function of the target molecule upon binding to the target molecule. In some embodiments, the bispecific checkpoint regulator antagonist is a polymeric molecule having two or more peptides. In some embodiments, the targeting domain comprises an antigen binding domain or a CDR of an antibody. In some embodiments, the bispecific inhibitor is a bispecific antibody.

[0103] The terms "bispecific antibody," and "bispecific antagonist" are used interchangeably herein with reference to an antibody that can specifically bind two different antigens (or epitopes). In some embodiments, the bispecific antibody is a full-length antibody that binds one antigen (or epitope) on one of its two binding arms (one pair of HC/LC), and binds a different antigen (or epitope) on its second arm (a different pair of HC/LC). In these embodiments, the bispecific antibody has two distinct antigen binding arms (in both specificity and CDR sequences), and is monovalent for each antigen it binds to.

[0104] In other embodiments, the bispecific antibody is a full-length antibody that can bind two different antigens (or epitopes) in each of its two binding arms (two pairs of HC/LC) In these embodiments, the bispecific antibody has two identical antigen binding arms, with identical specificity and identical CDR sequences, and is bivalent for each antigen it binds to.

[0105] The terms "trispecific antibody" and "trispecific antagonist" are used interchangeably herein with reference to a molecule comprising three targeting domains with three different binding specificities. Each targeting domain is capable of binding specifically to a target molecule and inhibiting a biological function of the target molecule upon binding to the target molecule. In some embodiments, the trispecific antagonist is a polymeric molecule having two or more peptides. In some embodiments, the targeting domain comprises an antigen binding domain or a CDR of an antibody. In some embodiments, the trispecific antagonist is a trispecific antibody.

[0106] Exemplary bispecific and trispecific antibodies may include asymmetric IgG-like antibodies (e.g., triomab/quadroma, Trion Pharma/Fresenius Biotech); knobs-into-holes antibodies (Genentech); Cross MAbs (Roche); electrostatically matched antibodies (AMGEN); LUZ-Y (Genentech); strand exchange engineered domain (SEED) body (EMD Serono; biolonic, Merus); Fab-exchanged antibodies (Genmab), symmetric IgG-like antibodies (e.g. dual targeting (DT)-Ig (GSK/Domantis); two-in-one antibody (Genentech); crosslinked MAbs (Karmanos Cancer Center), mAb2 (F-star); Cov X-body (Cov X/Pfizer); dual variable domain (DVD)-Ig fusions (Abbott); IgG-like bispecific antibodies (Eli Lilly); Ts2Ab (Medimmune/AZ); BsAb (ZymoGenetics); HERCULES (Biogen Idec,TvAb, Roche); scFv/Fc fusions; SCORPION (Emergent BioSolutions/Trubion, ZymoGenetics/BMS); dual affinity retargeting technology (Fc-DART), MacroGenics; dual (scFv)2-Fabs (National Research Center for Antibody Medicine); F(ab)2 fusions (Medarex/AMGEN); dual-action or Bis-Fab (Genentech); Dock-and-Lock (DNL, ImmunoMedics); Fab-Fv (UCB-Celltech); scFv- and diabody-based antibodies (e.g., bispecific T cell engagers (BiTEs, Micromet); tandem diabodies (Tandab, Affimed); DARTs (MacroGenics); single-chain diabodies; TCR-like antibodies (AIT, Receptor Logics); human serum albumin scFv fusion (Merrimack); COMBODIES (Epigen Biotech); and IgG/non-IgG fusions (e.g., immunocytokines (EMDSerono, Philogen, ImmunGene, ImmunoMedics).

[0107] The terms "treat" and "treatment" refer to the amelioration of one or more symptoms associated with a cell proliferative disorder; prevention or delay of the onset of one or more symptoms of a cell proliferative disorder; and/or lessening of the severity or frequency of one or more symptoms of cell proliferative disorder.

[0108] The phrases "to a patient in need thereof", "to a patient in need of treatment" or "a subject in need of treatment" includes subjects, such as mammalian subjects, that would benefit from administration of the antitumor antagonist of the present disclosure for treatment of a cell proliferative disorder.

[0109] The terms "therapeutically effective amount", "pharmacologically effective amount", and "physiologically effective amount" are used interchangeably to mean the amount

of an antitumor antagonist that is needed to provide a threshold level of active antagonist agents in the bloodstream or in the target tissue. The precise amount will depend upon numerous factors, *e.g.*, the particular active agent, the components and physical characteristics of the composition, intended patient population, patient considerations, and the like, and can readily be determined by one skilled in the art, based upon the information provided herein or otherwise available in the relevant literature.

[0110] The terms "improve", "increase" or "reduce", as used in this context, indicate values or parameters relative to a baseline measurement, such as a measurement in the same individual prior to initiation of the treatment described herein, or a measurement in a control individual (or multiple control individuals) in the absence of the treatment described herein.

[0111] A "control individual" is an individual afflicted with the same cell proliferative disorder as the individual being treated, who is about the same age as the individual being treated (to ensure that the stages of the disease in the treated individual and the control individual(s) are comparable). The individual (also referred to as "patient" or "subject") being treated may be a fetus, infant, child, adolescent, or adult human with a cell proliferative disorder.

[0112] The term "cell proliferative disorder" refers to a disorder characterized by abnormal proliferation of cells. A proliferative disorder does not imply any limitation with respect to the rate of cell growth, but merely indicates loss of normal controls that affect growth and cell division. Thus, in some embodiments, cells of a proliferative disorder can have the same cell division rates as normal cells but do not respond to signals that limit such growth. Within the ambit of "cell proliferative disorder" is a neoplasm, cancer or tumor.

[0113] The term "cancer" or "tumor" refers to any one of a variety of malignant neoplasms characterized by the proliferation of cells that have the capability to invade surrounding tissue and/or metastasize to new colonization sites, and includes leukemia, lymphoma, carcinoma, melanoma, sarcoma, germ cell tumor and blastoma. Exemplary cancers for treatment with the methods of the instant disclosure include cancer of the brain, bladder, breast, cervix, colon, head and neck, kidney, lung, non-small cell lung, mesothelioma, ovary, prostate, stomach and uterus, leukemia, and medulloblastoma.

[0114] The term "leukemia" refers to progressive, malignant diseases of the blood-forming organs and is generally characterized by a distorted proliferation and development of leukocytes and their precursors in the blood and bone marrow. Exemplary leukemias include, for example, acute nonlymphocytic leukemia, chronic lymphocytic leukemia, acute granulocytic leukemia, chronic granulocytic leukemia, acute promyelocytic leukemia, adult T-cell leukemia, aleukemic leukemia, a leukocythemic leukemia, basophylic leukemia, blast cell leukemia, bovine leukemia, chronic myelocytic leukemia, leukemia cutis, embryonal leukemia,

eosinophilic leukemia, Gross' leukemia, hairy-cell leukemia, hemoblastic leukemia, hemocytoblastic leukemia, histiocytic leukemia, stem cell leukemia, acute monocytic leukemia, leukopenic leukemia, lymphatic leukemia, lymphoblastic leukemia, lymphocytic leukemia, lymphogenous leukemia, lymphoid leukemia, lymphosarcoma cell leukemia, mast cell leukemia, megakaryocytic leukemia, micromyeloblastic leukemia, monocytic leukemia, myeloblastic leukemia, myelocytic leukemia, myeloid granulocytic leukemia, myelomonocytic leukemia, Naegeli leukemia, plasma cell leukemia, plasmacytic leukemia, promyelocytic leukemia, Rieder cell leukemia, Schilling's leukemia, stem cell leukemia, subleukemic leukemia, and undifferentiated cell leukemia.

[0115] The term "carcinoma" refers to the malignant growth of epithelial cells tending to infiltrate the surrounding tissues and give rise to metastases. Exemplary carcinomas include, for example, acinar carcinoma, acinous carcinoma, adenocystic carcinoma, adenoid cystic carcinoma, carcinoma adenomatosum, carcinoma of adrenal cortex, alveolar carcinoma, alveolar cell carcinoma, basal cell carcinoma, carcinoma basocellulare, basaloid carcinoma, basosquamous cell carcinoma, bronchioalveolar carcinoma, bronchiolar carcinoma, bronchogenic carcinoma, cerebriform carcinoma, cholangiocellular carcinoma, chorionic carcinoma, colloid carcinoma, comedo carcinoma, corpus carcinoma, cribriform carcinoma, carcinoma en cuirasse, carcinoma cutaneum, cylindrical carcinoma, cylindrical cell carcinoma, duct carcinoma, ductal carcinoma in situ, invasive ductal carcinoma, lobular carcinoma, invasive lobular carcinoma, carcinoma durum, embryonal carcinoma, encephaloid carcinoma, epiennoid carcinoma, carcinoma epitheliale adenoides, exophytic carcinoma, carcinoma ex ulcere, carcinoma fibrosum, gelatiniform carcinoma, gelatinous carcinoma, giant cell carcinoma, carcinoma gigantocellulare, glandular carcinoma, granulosa cell carcinoma, hair-matrix carcinoma, hematoid carcinoma, hepatocellular carcinoma, Hurthle cell carcinoma, hyaline carcinoma, hypemephroid carcinoma, infantile embryonal carcinoma, carcinoma in situ, intraepidermal carcinoma, intraepithelial carcinoma, Krompecher's carcinoma, Kulchitzky-cell carcinoma, large-cell carcinoma, lenticular carcinoma, carcinoma lenticulare, lipomatous carcinoma, lymphoepithelial carcinoma, carcinoma medullare, medullary carcinoma, melanotic carcinoma, carcinoma molle, mucinous carcinoma, carcinoma muciparum, carcinoma mucocellulare, mucoepidermoid carcinoma, carcinoma mucosum, mucous carcinoma, carcinoma myxomatodes, naspharyngeal carcinoma, oat cell carcinoma, carcinoma ossificans, osteoid carcinoma, papillary carcinoma, periportal carcinoma, preinvasive carcinoma, prickle cell carcinoma, pultaceous carcinoma, renal cell carcinoma of kidney, reserve cell carcinoma, carcinoma sarcomatodes, schneiderian carcinoma, scirrhous carcinoma, carcinoma scroti, signet-ring cell carcinoma, carcinoma simplex, small-cell carcinoma, solanoid carcinoma, spheroidal

cell carcinoma, spindle cell carcinoma, carcinoma spongiosum, squamous carcinoma, squamous cell carcinoma, string carcinoma, carcinoma telangiectaticum, carcinoma telangiectodes, transitional cell carcinoma, carcinoma tuberosum, tuberous carcinoma, verrucous carcinoma, and carcinoma villosum.

[0116] The term "sarcoma" refers to a tumor made up of a substance like the embryonic connective tissue and is generally composed of closely packed cells embedded in a fibrillar or homogeneous substance. Exemplary sarcomas include, for example, chondrosarcoma, fibrosarcoma, lymphosarcoma, melanosarcoma, myxosarcoma, osteosarcoma, Abemethy's sarcoma, adipose sarcoma, liposarcoma, alveolar soft part sarcoma, ameloblastic sarcoma, botryoid sarcoma, chloroma sarcoma, chorio carcinoma, embryonal sarcoma, Wilns' tumor sarcoma, endometrial sarcoma, stromal sarcoma, Ewing's sarcoma, fascial sarcoma, fibroblastic sarcoma, giant cell sarcoma, granulocytic sarcoma, Hodgkin's sarcoma, idiopathic multiple pigmented hemorrhagic sarcoma, immunoblastic sarcoma of B cells, lymphomas (e.g., Non-Hodgkin Lymphoma), immunoblastic sarcoma of T-cells, Jensen's sarcoma, Kaposi's sarcoma, Kupffer cell sarcoma, angiosarcoma, leukosarcoma, malignant mesenchymoma sarcoma, parosteal sarcoma, reticulocytic sarcoma, Rous sarcoma, serocystic sarcoma, synovial sarcoma, and telangiectaltic sarcoma.

[0117] The term "melanoma" refers to a tumor arising from the melanocytic system of the skin and other organs. Melanomas include, for example, acral-lentiginous melanoma, amelanotic melanoma, benign juvenile melanoma, Cloudman's melanoma, S91 melanoma, Harding-Passey melanoma, juvenile melanoma, lentigo maligna melanoma, malignant melanoma, nodular melanoma subungal melanoma, and superficial spreading melanoma.

[0118] Additional cancers include, for example, Hodgkin's Disease, multiple myeloma, neuroblastoma, breast cancer, ovarian cancer, lung cancer, rhabdomyosarcoma, primary thrombocytosis, primary macroglobulinemia, small-cell lung tumors, primary brain tumors, stomach cancer, colon cancer, malignant pancreatic insulanoma, malignant carcinoid, premalignant skin lesions, testicular cancer, thyroid cancer, neuroblastoma, esophageal cancer, genitourinary tract cancer, malignant hypercalcemia, cervical cancer, endometrial cancer, and adrenal cortical cancer.

I. Trispecific Antitumor Antagonists

[0119] One aspect of the present application relates to trispecific antitumor antagonists that comprise: a protein scaffold, a first targeting domain that binds specifically to an immune checkpoint regulator; a second targeting domain that binds specifically to VEGF and comprises one or more peptide domains derived from VEGFR, a third targeting domain comprising an inhibitor of the angiopoietin/Tie-2 signaling pathway, wherein the first targeting domain is

located at an amino terminal of the protein scaffold and the second targeting domain is located at a carboxyl terminal of the protein scaffold. In some embodiments, the third targeting domain is inserted into a CH3 domain in a Fc loop of the protein scaffold.

[0120] Fusion proteins (e.g., Fc-fusion proteins) produced by recombinant DNA technology often face a serious problem of fully or partially degradation in cell culture (clipping) because of the host-cell derived proteases. Clipping generates low molecular weight (LMW) species of the target protein and leads to inactive polypeptides. While not wishing to be bound by any particular theory, it has been hypothesized that the location of the biological peptide affects the degree of degradation in the resulting fusion protein. The inventors of the present application found that insertion of the second targeting domain into the CH3 region of the Fc loop significantly reduces clipping of the resulting trispecific antagonist.

[0121] Another aspect of the present application relates to trispecific antitumor antagonists that comprise: a protein scaffold, a first targeting domain that binds specifically to an immune checkpoint regulator; a second targeting domain that binds specifically to VEGF and comprises one or more peptide domains derived from VEGFR, and a third targeting domain comprising a TGF pathway inhibitor.

[0122] In some embodiments, the second targeting domain is located at the carboxy-terminal of a CH3 domain of the protein scaffold, and the third targeting domain is located at the carboxy-terminal of the second targeting domain. In some embodiments, the second targeting domain is structurally linked to the carboxy-terminal of the CH3 domain of the protein scaffold through a first linker. In some embodiments, the third targeting domain is structurally linked to the carboxy-terminal of the second targeting domain through a second linker. In some embodiments, the second targeting domain is structurally linked to the carboxy-terminal of the CH3 domain of the protein scaffold through a first linker and the third targeting domain is structurally linked to the carboxy-terminal of the second targeting domain through a second linker. The inventors of the present application unexpected found that such configurations confer improved pharmacokinetics to the corresponding antagonists.

[0123] In some embodiments, the second targeting domain comprises a VEGFR component with amino acid sequence of **SEQ ID NO:185** (afilbercept). In further embodiments, the VEGFR component is a-glycosylated by replacing asparagine residues with glutamic acid residues. An exemplary amino acid sequence of a-glycosylated VEGFR component is demonstrated in **SEQ ID NO:207**. The trispecific antitumor antagonist variants with a-glycosylated VEGFR component retain the VEGFR bioactivity while demonstrating improved pharmacokinetics compared to the wild type afilbercept.

[0124] Another aspect of the present application relates to a trispecific antitumor antagonist that comprises a protein scaffold, a first targeting domain that binds specifically to an immune checkpoint regulator; a second targeting domain comprising a TGF-β pathway inhibitor; and a third targeting domain comprising a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway.

[0125] In some embodiments, the first targeting domain comprises bevacizumab or a variant thereof and the third targeting domain is inserted within each of the two CH3 regions of the Fc loop of the protein scaffold. In some embodiments, the first targeting domain comprising one or more anti-PD-1 variable domains and the third targeting domain is inserted within each of the two CH3 regions of the Fc loop of the protein scaffold. In some embodiments, the first targeting domain comprising one or more anti-PD-L1 variable domains and the third targeting domain is inserted within each of the two CH3 regions of the Fc loop of the protein scaffold.

[0126] In some embodiments, the trebananib peptide comprises the sequence: AQQEECEWDPWTCEHMGSGSATGGSGSTASSGSGSATHQEECEWDPWTCEHMLE (**SEQ ID NO:182**). Unless otherwise noted, the "trebananib peptide" and the designation "-long" or "-L" correspond to the sequence of **SEQ ID NO:182**, which comprises two copies of the Ang-1/2 blocking peptide sequence, QEECEWDPWTCEHM (**SEQ ID NO:194**). In certain embodiments, the trispecific antagonists of the present application comprise modified version of the trebananib peptide containing a single copy of the sequence corresponding to **SEQ ID NO:194** with the designation "-short" or "-S".

II. Bispecific Antitumor Antagonists

[0127] Another aspect of the present application relates to a bispecific antitumor antagonist that comprises a protein scaffold, a first targeting domain that binds specifically to an immune checkpoint regulator; and a second targeting domain comprising a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway.

[0128] In some embodiments, the first targeting domain comprises one or more anti-PD-1 or anti-PD-L1 variable domains, and the second targeting domain is inserted within each of the two CH3 regions of the Fc loop of the protein scaffold.

[0129] Another aspect of the present application relates to a bispecific antitumor antagonist that comprises a protein scaffold, a first targeting domain that binds specifically to an immune checkpoint regulator; and a second targeting domain comprising a VEGFR component.

[0130] **FIGS. 41A-40C** depict exemplary bispecific anti-PD-1/VEGFR component and anti-PD-L1/VEGFR component molecules, Bi-ZPL-1 and Bi-ZPL-2, and Bi-ZPL-3, respectively that contain an aflibercept at the N-terminal end (**FIG. 41A**) or the carboxy-terminal end (**FIG. 41B-C**). In one embodiment, Bi-ZPL-1 has an immunoglobulin heavy chain containing the

sequence set forth in **SEQ ID NO:218** and/or an immunoglobulin light chain containing the sequence set forth in **SEQ ID NO:219.** In another embodiment, Bi-ZP-1 has an immunoglobulin heavy chain containing the sequence set forth in **SEQ ID NO:220** and/or an immunoglobulin light chain containing the sequence set forth in **SEQ ID NO:221**.

[0131] In another embodiment, Bi-ZP-2 has an immunoglobulin heavy chain containing the sequence set forth in **SEQ ID NO:222** and/or an immunoglobulin light chain containing the sequence set forth in **SEQ ID NO:223**. In another embodiment, Bi-ZPL-2 has an immunoglobulin heavy chain containing the sequence set forth in **SEQ ID NO:224** and/or an immunoglobulin light chain containing the sequence set forth in **SEQ ID NO:225.**

[0132] In another embodiment, Bi-ZPL-3 has an immunoglobulin heavy chain containing the sequence set forth in **SEQ ID NO:296** and/or an immunoglobulin light chain containing the sequence set forth in **SEQ ID NO:297**.

[0133] Another aspect of the present application relates to bispecific antitumor antagonists that comprise a protein scaffold, a first targeting domain comprising bevacizumab; and a second targeting domain comprising a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway. In some embodiments, the second targeting domain is inserted within the CH3 domain in the Fc loop of the protein scaffold.

[0134] Another aspect of the present application relates to bispecific antitumor antagonists that comprise a protein scaffold comprising an Fc fragment, a first targeting domain comprising a VEGFR component, and a second targeting domain comprising a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway. In some embodiments, the second targeting domain is inserted within the CH3 domain in the Fc loop of the protein scaffold. In some embodiments, the Fc fragment of protein scaffold comprises an amino acid sequence selected from the group consisting of **SEQ ID NOS:210-217** prior to the insertion of the second targeting domain.

[0135] Another aspect of the present application relates to bispecific antitumor antagonists that comprise a protein scaffold comprising an Fc fragment, a first targeting domain comprising a TGFBR2 ECD, and a second targeting domain comprising a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway. In some embodiments, the second targeting domain is inserted within the CH3 domain in the Fc loop of the protein scaffold. In some embodiments, the Fc fragment of protein scaffold comprises an amino acid sequence selected from the group consisting of **SEQ ID NOS:210-217** prior to the insertion of the second targeting domain.

[0136] The bispecific antitumor antagonists of the present application may be constructed with an IgG backbone. More specifically, any of the bispecific antagonists of the present application may be constructed with an IgG1 or IgG4 backbone. Use of an IgG1

backbone is preferable for cancer treatment where a target is present on antigen presenting cells that can mediate antibody-dependent cell-mediated cytotoxicity (ADCC). Use of an IgG4 backbone allows targeting of antigen where antigen binding alone is sufficient to generate the desired therapeutic benefits. IgG4-based antagonists preclude undesirable effector functions associated with *e.g.*, IgG1 antibodies, including FcγR binding and complement activation.

III. Targeting Domains

A. Targeting Domains that bind specifically to Immune Checkpoint Regulators

[0137] Targeting Domains that bind specifically to immune checkpoint regulators include, but are not limited to, (1) anti-PD-1 antibody and anti-PD-1 antibody fragments, (2) anti-PD-L1 antibody and anti-PD-L1 antibody fragments, (3) Anti-TIGIT antibody and anti-TIGIT antibody fragments, and (4) Anti-LAG-3 antibody and anti-LAG-3 antibody fragments.

1. Anti-PD-1 antibody and anti-PD-1 antibody fragments

[0138] In some embodiments, the checkpoint regulator antagonist is an anti-PD-1 antibody or antibody fragment. In a particular embodiment, the PD-1 inhibitor of the present application is an antibody, or an antigen-binding portion thereof, comprising: (1) a heavy chain variable region, wherein the heavy chain variable region comprises three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3, wherein HCDR1 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:48, 51, 54, 56 and 59**, wherein HCDR2 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:49, 52, 57 and 60**, and wherein HCDR3 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:50, 53, 55, 58 and 61**; and (2) a light chain variable region, wherein the light chain variable region comprises three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3, wherein LCDR1 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:62, 65, 68, 69, 70 and 73**, wherein LCDR2 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:63, 66, 71 and 74**, and wherein LCDR3 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:64, 67, 72 and 75**, wherein the antibody, or the antigen-binding portion thereof, binds specifically to human PD-1.

[0139] In some embodiments, the PD-1 inhibitor of the present application is an antibody, or an antigen-binding portion thereof, comprising: (1) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid

sequence selected from the group consisting of **SEQ ID NOS:127, 129, 131, 133, 135 and 137**; and (2) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:128, 130, 132, 134, 136 and 138**, wherein the antibody, or the antigen-binding portion thereof, binds specifically to human PD-1.

[0140] In some embodiments, the anti-PD-1 antibody or antibody fragment comprises a heavy chain variable region that comprises (1) an HCDR1 of **SEQ ID NO:59**, an HCDR2 of **SEQ ID NO:60** and an HCDR 3 of **SEQ ID NO:61** and (2) an HFR1 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:366**, an HFR2 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:360**, an HFR3 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:371**, an HFR4 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:357**, and an immunoglobulin heavy chain variable region that comprises (1) an LCDR1 of **SEQ ID NO:73**, an LCDR2 of **SEQ ID NO:74** and an LCDR 3 of **SEQ ID NO:75** and (2) an LFR1 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:372**, an LFR2 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:373**, an LFR3 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:374**, an LFR4 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:375**.

[0141] In some embodiments, the anti-PD-L1 antibody or antibody fragment comprises a heavy chain variable region that comprises (1) an HCDR1 of **SEQ ID NO:56**, an HCDR2 of **SEQ ID NO:57** and an HCDR 3 of **SEQ ID NO:58** and (2) an HFR1 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:366**, an HFR2 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:360**, an HFR3 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:367**, an HFR4 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:357**, and an immunoglobulin heavy chain variable region that comprises (1) an LCDR1 of **SEQ ID NO:70**, an LCDR2 of **SEQ ID NO:71** and an LCDR 3 of **SEQ ID NO:72** and (2) an LFR1 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:368**, an LFR2 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:369**, an LFR3 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:370**, an LFR4 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:365**.

[0142] The term "2P17" or "PD-06" refers to the PD-1 inhibitor of the present application, comprising: (1) a heavy chain variable region having an amino acid sequence of

SEQ ID NO:137, and (2) a light chain variable region having an amino acid sequence of **SEQ ID NO:138**. The CDR and FR regions of 2P17 (or PD-06) are listed as **SEQ ID NOS:59-61, 73-75, 357, 360, 366 and 371-375**.

[0143] The term "2P16" or "PD-05" refers to the PD-1 inhibitor of the present application, comprising: (1) a heavy chain variable region having an amino acid sequence of **SEQ ID NO:135**, and (2) a light chain variable region having an amino acid sequence of **SEQ ID NO:136**. The CDR and FR regions of 2P16 (or PD-05) are listed as **SEQ ID NOS:56-58, 70-72, 357, 360, 366 and 365-370**.

2. Anti-PD-L1 antibody and anti-PD-L1 antibody fragments

[0144] In one embodiment, the checkpoint regulator antagonist is an anti-PD-L1 antibody or antibody fragment. In a particular embodiment, the PD-L1 inhibitor of the present application is an antibody, or an antigen-binding portion thereof, comprising: (1) a heavy chain variable region, wherein the heavy chain variable region comprises three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3, wherein HCDR1 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:76, 79, 85 and 88**, wherein HCDR2 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:77, 80, 82, 84, 86 and 89**, and wherein HCDR3 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:78, 81, 83, 87 and 90**; and (2) a light chain variable region, wherein the light chain variable region comprises three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3, wherein LCDR1 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:91, 94, 98, 101 and 104**, wherein LCDR2 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:92, 95, 99, 102 and 105**, and wherein LCDR3 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:93, 96, 97, 100 and 106**, wherein the antibody, or the antigen-binding portion thereof, binds specifically to human PD-L1.

[0145] In some embodiments, the anti-PD-L1 antibody or antibody fragment comprises a heavy chain variable region that comprises (1) an HCDR1 of **SEQ ID NO:79**, an HCDR2 of **SEQ ID NO:82** and an HCDR 3 of **SEQ ID NO:83** and (2) an HFR1 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:366**, an HFR2 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:360**, an HFR3 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:383**, an HFR4 having at

least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:357**, and an immunoglobulin heavy chain variable region that comprises (1) an LCDR1 of **SEQ ID NO:91**, an LCDR2 of **SEQ ID NO:92** and an LCDR 3 of **SEQ ID NO:93** and (2) an LFR1 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:377**, an LFR2 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:378**, an LFR3 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:379**, an LFR4 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:365**.

[0146] In some embodiments, the PD-L1 inhibitor of the present application is an antibody, or an antigen-binding portion thereof, comprising: (1) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:139, 141, 143, 145, 147, 149, 151 and 153**; and (2) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:140, 142, 144, 146, 148, 150, 152 and 154**, wherein the antibody, or the antigen-binding portion thereof, binds specifically to human PD-L1. In the preferred embodiment the anti-PD-L1 antibody heavy and light chains consist of **SEQ ID NOs: 153-154.**

[0147] The term "PL-08" refers to the PD-L1 inhibitor of the present application, comprising: (1) a heavy chain variable region having an amino acid sequence of **SEQ ID NO:153**, and (2) a light chain variable region having an amino acid sequence of **SEQ ID NO:154**. The CDR and FR regions of PL-08 are listed as **SEQ ID NOS:79, 82, 83, 91-93, 357, 360, 365, 366, 377-379 and 383**.

3. Anti-TIGIT antibody and anti-TIGIT antibody fragments

[0148] In some embodiments, the checkpoint regulator antagonist is an anti-TIGIT antibody or antibody fragment. In a particular embodiment, the TIGIT inhibitor of the present application is an antibody, or an antigen-binding portion thereof, comprising: (1) a heavy chain variable region, wherein the heavy chain variable region comprises three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3, wherein HCDR1 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:1, 6, 11, 15, 17, 20 and 23**, wherein HCDR2 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:2, 4, 7, 9, 12, 13, 16, 18, 21 and 24**, and wherein HCDR3 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:3, 5, 8, 10, 14, 19, 22 and 25**; and (2) a light chain variable region, wherein the light chain variable region comprises

three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3, wherein LCDR1 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:26, 29, 31, 33, 35, 39, 42 and 45**, wherein LCDR2 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:27, 30, 36, 37, 40, 43 and 46**, and wherein LCDR3 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:28, 32, 34, 38, 41, 44 and 47**, wherein the antibody, or the antigen-binding portion thereof, binds specifically to human TIGIT.

[0149] In some embodiments, the anti-TIGIT antibody or antibody fragment comprises a heavy chain variable region that comprises (1) an HCDR1 of **SEQ ID NO:23**, an HCDR2 of **SEQ ID NO:24** and an HCDR 3 of **SEQ ID NO:25** and (2) an HFR1 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:343**, an HFR2 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:330**, an HFR3 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:344**, an HFR4 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:319**, and an immunoglobulin heavy chain variable region that comprises (1) an LCDR1 of **SEQ ID NO:45**, an LCDR2 of **SEQ ID NO:46** and an LCDR 3 of **SEQ ID NO:47** and (2) an LFR1 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:303**, an LFR2 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:311**, an LFR3 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:317**, an LFR4 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:345**.

[0150] In another embodiment, the TIGIT inhibitor of the present application is an antibody, or an antigen-binding portion thereof, comprising: (1) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:107, 109, 111, 113, 115, 117, 119, 121, 123 and 125**; and (2) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:108, 110, 112, 114, 116, 118, 120, 122, 124 and 126**, wherein the antibody, or the antigen-binding portion thereof, binds specifically to human TIGIT. In the preferred embodiment the anti-TIGIT antibody has heavy and light chain sequences consisting of **SEQ ID NOs: 125 and 126.**

[0151] The term "B21-35" or "T-10" refers to the TIGIT inhibitor of the present application, comprising: (1) a heavy chain variable region having an amino acid sequence of

SEQ ID NO:125, and (2) a light chain variable region having an amino acid sequence of **SEQ ID NO:126**. The CDR and FR regions of B21-35 (or T-10) are listed as **SEQ ID NOS:23-25, 45-47, 303, 311, 317, 319, 330 and 343-345**.

4. Anti-LAG-3 antibody and anti-LAG-3 antibody fragments

[0152] In one embodiment, the checkpoint regulator antagonist is an anti-LAG-3 antibody or antibody fragment. In a particular embodiment, the LAG-3 inhibitor of the present application is an antibody, or an antigen-binding portion thereof, comprising: (1) a heavy chain variable region, wherein the heavy chain variable region comprises three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3, wherein HCDR1 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:155-157**, wherein HCDR2 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:158-160**, and wherein HCDR3 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:161-163**; and (2) a light chain variable region, wherein the light chain variable region comprises three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3, wherein LCDR1 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:164-166**, wherein LCDR2 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:167 and 168**, and wherein LCDR3 has an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:169 and 170**, wherein the antibody, or the antigen-binding portion thereof, binds specifically to human LAG-3.

[0153] In some embodiments, the anti-LAG-3 antibody or antibody fragment comprises a heavy chain variable region that comprises (1) an HCDR1 of **SEQ ID NO:156**, an HCDR2 of **SEQ ID NO:158** and an HCDR 3 of **SEQ ID NO:161** and (2) an HFR1 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:399**, an HFR2 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:400**, an HFR3 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:401**, an HFR4 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:402**, and an immunoglobulin heavy chain variable region that comprises (1) an LCDR1 of **SEQ ID NO:164**, an LCDR2 of **SEQ ID NO:167** and an LCDR 3 of **SEQ ID NO:169** and (2) an LFR1 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:403**, an LFR2 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:404**, an LFR3 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ**

ID NO:405, an LFR4 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:406**.

[0154] In other embodiments, the LAG-3 inhibitor of the present application is an antibody, or an antigen-binding portion thereof, comprising: (1) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:171-174**; and (2) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:175-178**, wherein the antibody, or the antigen-binding portion thereof, binds specifically to human LAG-3. In the preferred embodiment, the anti-LAG-3 antibody has heavy and light chain sequences consisting of **SEQ ID NOS:171 and 175.**

[0155] The term "2L2A.1" refers to the LAG-3 inhibitor of the present application, comprising: (1) a heavy chain variable region having an amino acid sequence of **SEQ ID NO:171**, and (2) a light chain variable region having an amino acid sequence of **SEQ ID NO:175**. The CDR and FR regions of 2L2A.1 are listed as **SEQ ID NOS:156, 158, 161, 164, 167, 169 and 399-406**.

B. Targeting Domains that bind specifically to Angiogenesis Pathway Components

[0156] Angiogenesis, the development of new blood vessels from pre-existing vessels, is essential for tumor growth and metastasis. Angiogenesis inhibition presents a potentially valuable strategy for treating diseases, such as cancer, in which progression (*e.g.*, metastasis) is dependent on neovascularization. Inhibition of angiogenesis leads to tumor cell death, which may feed tumor antigen into host antigen presentation pathways.

[0157] Two important angiogenesis pathways include the vascular endothelial growth factor (VEGF) pathway and the Tie2 pathway.

1. VEGF pathway antagonists and inhibitors

[0158] The principal VEGF pathway is mediated by the transmembrane tyrosine kinase VEGF-R2. Various isoforms of VEGF, particularly VEGF-A, bind to VEGF-R1 and VEGFF-R2, resulting in dimerization and activation through phosphorylation of various downstream tyrosine kinases.

[0159] In some embodiments, the VEGF pathway antagonist binds to VEGF-A or its receptors VEGFR-1 and VEGFR-2 so that, as a result of the binding, activation of VEGFR-1 and VEGFR-2 by VEGF-A is blocked or inhibited. Angiogenesis inhibitors may be in the form of e.g., antibodies, variable domain fragments, or dominant negative fusion protein fragments.

[0160] An exemplary dominant negative anti-VEGFR antagonist is a protein fragment corresponding to the extracellular domain (ECD) of human VEGF receptor 1 or 2. In a

preferred embodiment, the dominant negative anti-VEGFR antagonist is aflibercept (Zaltrap®), a recombinant fusion protein containing VEGF-A binding portions from the extracellular domains of human VEGF receptors 1 and 2 fused to the human IgG1 or IgG4 Fc fragment. VEGFR ECDs, such as aflibercept act as soluble receptor decoys for VEGF-A.

[0161] Aflibercept (also known as Zaltrap®) is a recombinant VEGF fusion protein consisting of vascular endothelial growth factor (VEGF)-binding portions from the extracellular domains of human VEGF receptors 1 and 2 that are fused to the Fc portion of the human IgG1 immunoglobulin. Aflibercept binds multiple ligands involved in angiogenesis, including VEGF-A, VEGF-B, anti-placental growth factor (PIGF)-1 and PIGF-2, including soluble ligands in circulation.

[0162] In a preferred embodiment, the aflibercept domain of the trispecific antitumor antagonists described herein is a-glycosylated by replacing asparagine (Asn, N) residues with glutamic acid (Glu, E) residues. N-linked glycosylation is the attachment of a glycan, to a nitrogen atom (the amide nitrogen) of an Asn residue of a protein. The Asn residue to be glycosylated must be located in a specific consensus sequence in the primary structure (Asn-X-Ser or Asn-X-Thr, where X refers to any amino acid except proline). By replacing the Asn residues with Glu residues (or any other amino acid residues), no glycosylation occurs on these sites. One or more Asn residues locate in the specific consensus sequence described above may be replaced.

[0163] Asialoglycoprotein receptor 1 and 2 (ASGR1 and ASGR2) is transmembrane proteins that play a critical role in serum glycoprotein homeostasis by mediating the endocytosis and lysosomal degradation of glycoproteins with exposed terminal galactose or N-acetylgalactosamine residues. In some cases, glycosylation of proteins can lead to increased clearance by ASGR1 and ASGR2. To improve the pharmacokinetics (i.e., achieve longer half-life) of the trispecific antitumor antagonists with VEGFR component domains, the inventors of the present application created a-glycosylated variant of the VEGFR component for limiting protein clearance by ASGR1 and ASGR2. The trispecific antitumor antagonist variant with a-glycosylated VEGFR component retains the VEGFR bioactivity while improves the alpha phase of pharmacokinetics by about five fold compared to the glycosylated VEGFR component. *See* **FIG. 37.** An exemplary amino acid sequence of aflibercept VEGF binding domain is demonstrated in **SEQ ID NO:185**, *i.e.*,

SDTGRPFVEMYSEIPEIIHMTEGRELVIPCRVTSPNITVTLKKFPLDTLIPDGKRIIWDSRK
GFIISNATYKEIGLLTCEATVNGHLYKTNYLTHRQTNTIIDVVLSPSHGIELSVGEKLVLN
CTARTELNVGIDFNWEYPSSKHQHKKLVNRDLKTQSGSEMKKFLSTLTIDGVTRSDQG

LYTCAASSGLMTKKNSTFVRVHEK, and an exemplary amino acid sequence of a-glycosylated aflibercept VEGF binding domain is demonstrated in **SEQ ID NO:207**, *i.e.*, SDTGRPFVEMYSEIPEIIHMTEGRELVIPCRVTSPEITVTLKKFPLDTLIPDGKRIIWDSRK GFIISEATYKEIGLLTCEATVNGHLYKTNYLTHRQTNTIIDVVLSPSHGIELSVGEKLVLE CTARTELNVGIDFNWEYPSSKHQHKKLVNRDLKTQSGSEMKKFLSTLTIDGVTRSDQG LYTCAASSGLMTKKESTFVRVHEK. The replacement sites are underlined.

[0164] An exemplary VEGF antibody antagonist is bevacizumab (AVASTIN™), a humanized antibody. Bevacizumab comprises mutated human IgG1 framework regions (FRs) and antigen-binding complementarity-determining regions from the murine anti-hVEGF monoclonal antibody A4.6.1 that blocks binding of human VEGF-A to VEGFR-2. Approximately 93% of the amino acid sequence of bevacizumab, including most of the framework regions, is derived from human IgG1, and about 7% of the sequence is derived from the murine antibody A4.6.1. Bevacizumab has a molecular mass of about 149,000 Daltons and is glycosylated. In certain embodiments, amino acid substitutions may be included in a bevacizumab/AVASTIN antibody as described in U.S. Patent No. 7,575,893 (**SEQ ID NOS:226-227**). Exemplary amino acid substitutions include, but are not limited to E1Q, E6Q, L11V, Q13K, L18V, R19K, A23K, or combinations thereof.

[0165] Additional anti-VEGF antibodies include ranibizumab (trade name Lucentis™)(**SEQ ID NOS:294-295**), a monoclonal antibody fragment derived from the same parent murine antibody as bevacizumab; the G6 or B20 series antibodies (*e.g.*, G6-23, G6-31, B20-4.1) described in U.S. Publication No. 2006/0280747, 2007/0141065 and/or 2007/0020267, as well as the antibodies described in U.S. Patent Nos. 7,297,334, 7,060,269, 6,884,879, 6,582,959, 6,703,020, 6,054,297; U.S. Patent Application Publication Nos. U.S. 2007/059312, U.S. 2006/009360, U.S. 2005/0186208, U.S. 2003/0206899, U.S. 2003/0190317, and U.S. 2003/0203409.

[0166] An exemplary anti-VEGFR-2 antibody antagonist is the humanized IgG1 monoclonal antibody, ramucirumab, which binds to the extracellular domain of VEGFR-2, thereby blocking its interaction with VEGF-A. Additional anti-VEGFR-2 antibodies are described in U.S. Patent Nos. 7,498,414, 6,448,077 and 6,365,157.

[0167] Exemplary small molecule antagonists of the VEGF pathway include multikinase inhibitors of VEGFR-2, including sunitinib, sorafenib, cediranib, pazonpanib and nintedanib.

2. Tie2 pathway antagonists and inhibitors

[0168] The Tie2 pathway is another angiogenesis pathway for which therapeutic antibodies and small molecule drugs have been developed. The Tie2 tyrosine kinase receptor activates angiogenesis in response to binding by its angiopoietin (Ang) ligands (i.e., Ang1,

Ang2, Ang3 (mouse) and Ang4). A Tie2 pathway antagonist binds to the Tie2 tyrosine kinase receptor or one of its angiopoietin (Ang) ligands (i.e., Ang-1, Ang-2, Ang-3 and Ang-4) so that, as a result of the binding, activation of the Tie2 receptor by one or more of its ligands is blocked or inhibited.

[0169] In one embodiment, the Tie2 receptor binding antagonist is an inhibitory peptide. In another embodiment, the inhibitory peptide comprises the amino acid sequence in **SEQ ID NO:182**, *i.e.*,
AQQEECEWDPWTCEHMGSGSATGGSGSTASSGSGSATHQEECEWDPWTCEHMLE. In another embodiment, the Tie2 receptor binding antagonist comprises the peptide of **SEQ ID NO:182** fused to the C-terminus of an Fc fragment, *i.e.*,
DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGGGGAQ
QEECEWDPWTCEHMGSGSATGGSGSTASSGSGSATHQEECEWDPWTCEHMLE (**SEQ ID NO:183**). In another embodiment, the inhibitory peptide comprises a single copy of the amino acid sequence of QEECEWDPWTCEHM (**SEQ ID NO:194**).

[0170] In another embodiment, the inhibitory peptide is incorporated within each of the two CH3 regions in the Fc loops. The loop region is an irregular secondary structure in proteins that is not α-helix or β-sheet, while it connects together β-sheets to β-sheets, β-sheets to α-helices, or α-helices to α-helices. The inhibitory peptide may be added by insertion (i.e., between amino acids in the previously existing Fc loop) or by replacement of amino acids in the previously existing Fc loop (i.e., removing amino acids in the previously existing Fc loop and adding peptide amino acids).

[0171] An exemplary Fc fragment comprises the amino acid sequence in **SEQ ID NO:208**, *i.e.*,
DKTHTCPPCPAPELLGGPSVFLF**PP**KPKDTLMISRTPEVTCVVV**DV**<u>**SHE**</u>**DPE**VKFNWYV
DGVE**VHNA**KTKPR**EEQ**<u>**YNS**</u>**T**YRVVSVLT**VLHQDWLNGKE**YKCKVS<u>**NKA**</u>**LPAPIEK**
TISKAKGQPREPQVYTLPPSR**E**<u>**EMTK**</u>NQVSLTCLVKGFYPSDIAVEWES**NGQP**<u>**ENN**</u>Y
K**TTPP**<u>**VLDSD**</u>**GS**FFLYSKLTVD**KSRWQ**<u>**QGN**</u>**V**FSCSVMHEALHNHYTQKSLSLSPGK.
The predicated loop regions are in bold. Any or all of the sites shown in bold may be suitable for full or partial replacement by or insertion of the inhibitory peptide sequences. Specifically preferred replacement and insertion sites are underlined.

[0172] In another embodiment, the inhibitory peptide is inserted into the Fc loop defined as the sequence **EEMTK**, between Fc residues Met and Lys and includes two Gly residues as

linkers flanking either side of the inserted peptide, as shown in **SEQ ID NO: 209**, *i.e.*, SCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI SKAKGQPREPQVYTLPPSREEM*GG*AQQEECEWDPWTCEHMGSGSATGGSGSTASSGSG SATHQEECEWDPWTCEHMLE*GG*TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK. The Gly linkers are in italics, and the inhibitory peptide insertion is underlined. The insertion site is also demonstrated in **FIGS.13A-14B** of TS-ZPT-3. In the heavy chain, the first underlined section corresponds to the anti-PD-1 variable domain, the second underlined section corresponds to the trebananib peptide inserted in the Fc loop, and the third underlined section corresponds to the aflibercept domain fused to the carboxy-terminal end.

[0173] Other peptide inhibitors of Tie2 activation (including Ang-2 inhibitors) include A-11 (Compugen), which comprises the amino acid sequence ETFLSTNKLENQ (**SEQ ID NO:184**); a peptide having the amino acid sequence NSLSNASEFRAPY (**SEQ ID NO:195**); a peptide having the amino acid sequence NLLMAAS (**SEQ ID NO:196**); the CVX-060 peptide (Pfizer); the CVX-037 peptide (Pfizer); and CGEN-25017 (Compugen). Additional peptide inhibitors of Tie2 activation are described in U.S. Patent No. 7,138,370. Exemplary peptide inhibitors of angiopoietin -1 or -2 are described in U.S. Patent Nos. 7,138,370, 7,521,053, 7,658,924, and 8,030,025.

[0174] Antibody inhibitors of Tie2 activation (and/or angiopoietin-2) include AMG-780 (Amgen), MEDI-3617 (MedImmune/AstraZeneca), DX-2240 (Dyax/Sanofi-Aventis), REGN-910 (Sanofi/Regeneron), RG7594 (Roche), LC06 (Roche), TAvi6 (Roche), AT-006 (Roche/Affitech). Additional Tie2 receptor binding antibody antagonists and antibody binding sequences therefrom are described in U.S. Patent Nos. 6,376,653, 7,521,053, 7,658,924, 8,030,025, as well as U.S. Patent Application Publication Nos. 2013/0078248, 2013/0259859, and 2015/0197578.

[0175] Tie2 binding antagonists also include the small molecule inhibitors, CGI-1842 (CGI Pharmaceuticals), LP-590 (Locus Pharmaceuticals), ACTB-1003 (Act Biotech/Bayer AG), CEP-11981 (Cephalon/Teva), MGCD265 (Methylgene), Regorafenib (Bayer), Cabozantinib/XL-184/BMS-907351 (Exelixis), Foretnib (Exelixis), MGCD-265 (MethylGene Inc.).

C. TGF Pathway Inhibitors

[0176] TGF-β includes a multifunctional set of peptides that control cell proliferation and differentiation, migration and adhesion, extracellular matrix modification including tumor stroma and immunosuppression, angiogenesis and desmoplasia, apoptosis, and other functions in

many cell types. TGF-β is a potent inducer of angiogenesis, which provides a critical support system for solid tumors, as well as a mechanism for tumor cell dissemination. Many cells synthesize TGF-β and almost all of them have specific receptors for these peptides. TGF-β1, TGF-β2, and TGF-β3 all function through the same receptor signaling systems. The active form of TGF-β is a dimer that signals through the formation of a membrane bound heterotetramer composed of the serine threonine type 1 and type 2 receptors, TGF-β RI and TGFβ RII, respectively.

[0177] Recently, TGF-β pathway inhibitors have been developed in the form of e.g., antibodies or binding fragments directed against TGF-β1 or TGF-β1 RII, such as dominant negative fusion protein fragments containing the extracellular domain (ECD) of TGF-β1 RII. As further described herein, the TGFβ1 RII ECD has been further combined with additional binding agents targeting additional checkpoint regulator pathways or angiogenesis pathways, which are central to tumor growth and dissemination. An exemplary human TGF-β1 RII ECD (wild-type) has the amino acid sequence set forth in **SEQ ID NO:186**.

[0178] A TGF-β pathway inhibitor may be in the form of e.g., antibodies or variable domain fragments directed against TGF-β1 or a TGF-β1 RII, a TGF binding peptide, or dominant negative fusion protein fragment, such as the extracellular domain (ECD) of TGF-β1 RII. In certain embodiments, a bispecific or trispecific antitumor antagonist comprises a TGF-β1 RII ECD. In certain embodiments, a bispecific or trispecific antitumor antagonist comprises a TGF-β1 RII ECD. In some embodiments, the TGF-β1 RII ECD is fused to the carboxy-terminus of an IgG in a bispecific or trispecific antitumor antagonist, as depicted in *e.g.*, **FIGS. 30A and 30E**. In other embodiments, the TGF-β1 RII ECD is fused to the amino-terminus of an IgG in a bispecific or trispecific antitumor antagonist, as depicted in *e.g.*, **FIGS. 30B and 30D**. In yet other embodiments, the TGF-β1 RII ECD is inserted within the IgG Fc fragments (*i.e.*, CH2 or CH3 regions) of an IgG in a bispecific or trispecific antitumor antagonist, as depicted in *e.g.*, **FIGS. 30C and 30F**.

[0179] Exemplary anti-TGF-β1 antibodies are described in U.S. Patent Nos. 7,067,637, 7,494,651, 7,527,791, and 7,619,069. Exemplary anti-TGF-β1 RII antibodies are described in U.S. Patent No. 7,579,186. An exemplary peptide inhibitor of TGF-β1 is KRIWFIPRSSWYERA (**SEQ ID NO:197**).

D. Overall Configurations of the Targeting Domains

[0180] Any one of the anti-PD-1, anti-PD-L1, anti-TIGIT, anti-LAG-3, anti-VEGF, anti-VEGFR, anti-angiopoietin-1/2, and/or anti-Tie2 receptor antagonists can be in the form of a monoclonal antibody, chimeric antibody, humanized antibody, scFv or multi-specific antagonists, such as bispecific and trispecific antagonists. In addition, any of the antagonists

described herein may include multiple binding specificities targeting PD-1, PD-L1, TIGIT, LAG-3, VEGF, VEGFR, angiopoietin, and/or Tie2 receptor. Moreover, any of the antibody antagonists may be engineered to target multiple epitopes in a given target. Furthermore, in some embodiments, the checkpoint antagonist and/or angiogenesis specificity may be included in the form of a dominant negative fusion protein, such as an extracellular domain (ECD) from a corresponding receptor.

[0181] The HCVRs and LCVRs described herein may be structurally linked to a naturally-occurring CH1-CH2-CH3 region or a non-naturally occurring or mutated Fc (CH2-CH3) region, e.g., an effectorless or mostly effectorless Fc (e.g., human IgG2 or IgG4) or, alternatively, an Fc with enhanced binding to one or more activating Fc receptors (FcγRI, FcγRIIa or FcγRIIIa) so as to enhance Treg depletion in the tumor environment. Accordingly, in certain embodiments the anti-PD-1, anti-PD-L1, anti-TIGIT, anti-LAG-3, anti-VEGF, anti-VEGFR HCVRs and LCVRs described herein may be structurally linked to an CH1-CH2-CH3 comprising one or more modifications, typically to alter one or more functional properties of the antibody, such as serum half-life, complement fixation, Fc receptor binding, and/or antigen-dependent cellular cytotoxicity. Furthermore, an antibody described herein may be chemically modified (e.g., one or more chemical moieties can be attached to the antibody) or it may be modified to alter its glycosylation, to alter one or more functional properties of the antibody. More specifically, in certain embodiments, the antibodies in the present application may include modifications in the Fc region in order to generate an Fc variant with (a) increased or decreased antibody-dependent cell-mediated cytotoxicity (ADCC), (b) increased or decreased complement mediated cytotoxicity (CDC), (c) increased or decreased affinity for C1q and/or (d) increased or decreased affinity for a Fc receptor relative to the parent Fc. Such Fc region variants will generally comprise at least one amino acid modification in the Fc region. Combining amino acid modifications is thought to be particularly desirable. For example, the variant Fc region may include two, three, four, five, etc. substitutions therein, e.g., of the specific Fc region positions identified herein.

[0182] For uses where effector function is to be avoided altogether, e.g., when antigen binding alone is sufficient to generate the desired therapeutic benefit, and effector function only leads to (or increases the risk of) undesired side effects, IgG4 antibodies may be used, or antibodies or fragments lacking the Fc region or a substantial portion thereof can be devised, or the Fc may be mutated to eliminate glycosylation altogether (e.g., N297A). Alternatively, a hybrid construct of human IgG2 (CH1 domain and hinge region) and human IgG4 (CH2 and CH3 domains) may be generated that is devoid of effector function, lacking the ability to bind FcγRs (like IgG2) and activate complement (like IgG4). When using an IgG4 constant domain,

it is usually preferable to include the substitution S228P, which mimics the hinge sequence in IgG1 and thereby stabilizes IgG4 molecules, reducing Fab-arm exchange between the therapeutic antibody and endogenous IgG4 in the patient being treated.

[0183] In certain embodiments, the anti-PD-1, anti-PD-L1, anti-TIGIT, anti-LAG-3, anti-VEGF, anti-VEGFR, anti-angiopoietin, and anti-Tie2R antibodies or fragments thereof may be modified to increase its biological half-life. Various approaches may be employed, including e.g., that increase the binding affinity of the Fc region for FcRn. In one embodiment, the antibody is altered within the CH1 or CL region to contain a salvage receptor binding epitope taken from two loops of a CH2 domain of an Fc region of an IgG, as described in U.S. Patent Nos. 5,869,046 and 6,121,022. The numbering of residues in the Fc region is that of the EU index. Sequence variants disclosed herein are provided with reference to the residue number followed by the amino acid that is substituted in place of the naturally occurring amino acid, optionally preceded by the naturally occurring residue at that position. Where multiple amino acids may be present at a given position, e.g., if sequences differ between naturally occurring isotypes, or if multiple mutations may be substituted at the position, they are separated by slashes (e.g., "X/Y/Z").

[0184] Exemplary Fc variants that increase binding to FcRn and/or improve pharmacokinetic properties include substitutions at positions 259, 308, and 434, including for example 259I, 308F, 428L, 428M, 434S, 434H, 434F, 434Y, and 434M. Other variants that increase Fc binding to FcRn include: 250E, 250Q, 428L, 428F, 250Q/428L (Hinton et al., 2004, J. Biol. Chem. 279(8): 6213-6216, Hinton et al. 2006 Journal of Immunology 176:346-356), 256A, 272A, 305A, 307A, 31 IA, 312A, 378Q, 380A, 382A, 434A (Shields et al. (2001) J. Biol. Chem., 276(9):6591-6604), 252F, 252Y, 252W, 254T, 256Q, 256E, 256D, 433R, 434F, 434Y, 252Y/254T/256E, 433K/434F/436H (Dall'Acqua et al. (2002) J. Immunol., 169:5171-5180, Dall'Acqua et al. (2006) J. Biol. Chem., 281:23514-23524, and U.S. Patent No. 8,367,805.

[0185] Modification of certain conserved residues in IgG Fc (1253, H310, Q311, H433, N434), such as the N434A variant (Yeung et al. (2009) J. Immunol. 182:7663), have been proposed as a way to increase FcRn affinity, thus increasing the half-life of the antibody in circulation (WO 98/023289). The combination Fc variant comprising M428L and N434S has been shown to increase FcRn binding and increase serum half-life up to five-fold (Zalevsky et al. (2010) Nat. Biotechnol. 28:157). The combination Fc variant comprising T307A, E380A and N434A modifications also extends half-life of IgG1 antibodies (Petkova et al. (2006) Int. Immunol. 18:1759). In addition, combination Fc variants comprising M252Y-M428L, M428L-N434H, M428L-N434F, M428L-N434Y, M428L-N434A, M428L-N434M, and M428L-N434S variants have also been shown to extend half-life (U.S. 2006/173170). Further, a combination

Fc variant comprising M252Y, S254T and T256E was reported to increase half-life-nearly 4-fold. Dall'Acqua et al. (2006) J. Biol. Chem. 281:23514.

IV. Preferred Embodiments of Trispecific antagonists

[0186] In one embodiment, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:127, 129, 131, 133, 135 and 137**; and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:128, 130, 132, 134, 136 and 138**; (2) a second targeting domain conferred by one or more anti-VEGF antagonist and/or one or more anti-VEGFR antagonist as further described herein and (3) a third targeting domain comprising a biological peptide that binds specifically to Tie2 tyrosine kinase receptor, Ang-1, Ang-2, Ang-3 or Ang-4 as further described herein. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted within the CH3 domain.

[0187] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:137**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:138**; (2) a second targeting domain conferred by one or more anti-VEGF antagonist and/or one or more anti-VEGFR antagonist as further described herein and (3) a third targeting domain comprising a biological peptide that binds specifically to Tie2 tyrosine kinase receptor, Ang-1, Ang-2, Ang-3 or Ang-4 as further described herein. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted within the CH3 domain.

[0188] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:135**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:136**; (2) a second targeting domain conferred by one or more anti-VEGF antagonist and/or one or more anti-VEGFR antagonist as further described herein and (3) a third targeting domain comprising a biological peptide that binds specifically to Tie2 tyrosine kinase receptor, Ang-1, Ang-2, Ang-3 or Ang-4 as further described herein. In another embodiment, the second targeting domain is inserted downstream of the CH3

domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted within the CH3 domain.

[0189] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region, wherein the heavy chain variable region comprises three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3, wherein HCDR1 has an amino acid sequence that is about 80% or about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:48, 51, 54, 56 and 59**, wherein HCDR2 has an amino acid sequence that is about 80%, about 85%, about 90%, about 95% or about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:49, 52, 57 and 60**, and wherein HCDR3 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:50, 53, 55, 58 and 61**, and (b) a light chain variable region, wherein the light chain variable region comprises three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3, wherein LCDR1 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:62, 65, 68, 69, 70 and 73**, wherein HCDR2 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:63, 66, 71 and 74**, and wherein HCDR3 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:64, 67, 72 and 75**; (2) a second targeting domain conferred by one or more anti-VEGF antagonist and/or one or more anti-VEGFR antagonist as further described herein and (3) a third targeting domain comprising a biological peptide that binds specifically to Tie2 tyrosine kinase receptor, Ang-1, Ang-2, Ang-3 or Ang-4 as further described herein. In a preferred embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted within the CH3 domain.

[0190] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region, wherein the heavy chain variable region comprises three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3, wherein HCDR1 has an amino acid sequence that is about 80% or about 100% identity to an amino acid of **SEQ ID NO:59**, wherein HCDR2 has an amino acid sequence that is about 80%, about 85%, about 90%, about 95% or about 100% identity to an amino acid sequence of **SEQ ID NO:60**, and wherein HCDR3 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:61**, and (b) a light chain variable region, wherein the light chain variable region comprises three complementarity

determining regions (LCDRs): LCDR1, LCDR2 and LCDR3, wherein LCDR1 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:73**, wherein HCDR2 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:74**, and wherein HCDR3 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:75**; (2) a second targeting domain conferred by one or more anti-VEGF antagonist and/or one or more anti-VEGFR antagonist as further described herein and (3) a third targeting domain comprising a biological peptide that binds specifically to Tie2 tyrosine kinase receptor, Ang-1, Ang-2, Ang-3 or Ang-4 as further described herein. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted within the CH3 domain.

[0191] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region, wherein the heavy chain variable region comprises three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3, wherein HCDR1 has an amino acid sequence that is about 80% or about 100% identity to an amino acid of **SEQ ID NO:56**, wherein HCDR2 has an amino acid sequence that is about 80%, about 85%, about 90%, about 95% or about 100% identity to an amino acid sequence of **SEQ ID NO:57**, and wherein HCDR3 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:58**, and (b) a light chain variable region, wherein the light chain variable region comprises three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3, wherein LCDR1 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:70**, wherein HCDR2 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:71**, and wherein HCDR3 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:72**; (2) a second targeting domain conferred by one or more anti-VEGF antagonist and/or one or more anti-VEGFR antagonist as further described herein and (3) a third targeting domain comprising a biological peptide that binds specifically to Tie2 tyrosine kinase receptor, Ang-1, Ang-2, Ang-3 or Ang-4 as further described herein. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted within the CH3 domain.

[0192] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain conferred by one or more variable regions comprising one or more anti-PD-1

variable domains or one or more anti-PD-L1 variable domains; (2) a second targeting domain conferred by an aflibercept (or Zaltrap®) domain with amino acid sequence of **SEQ ID NO:185**; and (3) a third targeting domain comprising a biological peptide that binds specifically to Tie2 tyrosine kinase receptor, Ang-1, Ang-2, Ang-3 or Ang-4 as further described herein. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted within the CH3 domain.

[0193] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:137**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:138**; (2) a second targeting domain conferred by an aflibercept (or Zaltrap®) domain with amino acid sequence of **SEQ ID NO:185**; and (3) a third targeting domain comprising a biological peptide that binds specifically to Tie2 tyrosine kinase receptor, Ang-1, Ang-2, Ang-3 or Ang-4 as further described herein. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted within the CH3 domain.

[0194] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:135**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:136**; (2) a second targeting domain conferred by an aflibercept (or Zaltrap®) domain with amino acid sequence of **SEQ ID NO:185**; and (3) a third targeting domain comprising a biological peptide that binds specifically to Tie2 tyrosine kinase receptor, Ang-1, Ang-2, Ang-3 or Ang-4 as further described herein. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted within the CH3 domain.

[0195] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:137**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:138**; (2) a second targeting domain conferred by an aflibercept (or Zaltrap®) domain with amino acid sequence of **SEQ ID NO:185**; and (3) a

third targeting domain comprises only one of the amino acid sequence of **SEQ ID NO:194**. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted within the CH3 domain.

[0196] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:135**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:136**; (2) a second targeting domain conferred by an aflibercept (or Zaltrap®) domain with amino acid sequence of **SEQ ID NO:185**; and (3) a third targeting domain comprises only one of the amino acid sequence of **SEQ ID NO:194**. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted within the CH3 domain.

[0197] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:137**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:138**; (2) a second targeting domain conferred by an aflibercept (or Zaltrap®) domain with amino acid sequence of **SEQ ID NO:185**; and (3) a third targeting domain comprises the amino acid sequence of **SEQ ID NO:182**. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted within the CH3 domain.

[0198] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:135**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:136**; (2) a second targeting domain conferred by an aflibercept (or Zaltrap®) domain with amino acid sequence of **SEQ ID NO:185**; and (3) a third targeting domain comprises the amino acid sequence of **SEQ ID NO:182**. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted within the CH3 domain.

[0199] In other embodiments, the trispecific antitumor antagonists comprises: a heavy chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:198, 200, 202, 203 and 204**; and a light chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:199 and 201**.

[0200] In other embodiments, the trispecific antitumor antagonists comprises: a heavy chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:198**; and a light chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:199**.

[0201] In other embodiments, the trispecific antitumor antagonists comprises: a heavy chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:200**; and a light chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:201**.

[0202] In other embodiments, the trispecific antitumor antagonists comprises: a heavy chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:202**; and a light chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:201**.

[0203] **FIGS. 3A-FIG. 4C** show a variety of different trispecific antitumor antagonists, where: (1) the VH1 and VL1 regions correspond to anti-PD-1 variable domains or other checkpoint antibodies; (2) the double ovals correspond to aflibercept fusion protein domains; and (3) the circles correspond to trebananib peptide. **FIGS. 5A-5E** show a variety of different trispecific antagonists where (1) the VH1 and VL1 regions correspond to anti-PD-1 variable domains or another checkpoint antibody; (2) VH and VL of bevacizumab or another anti-VEGF-A antibody (3) the circles correspond to trebananib peptide. As shown in these figures, these components can be rearranged in multiple configurations. For example, the trebananib peptides are arranged in the following locations: (i) fused to the carboxy-terminal end of each IgG4 CH3 region (**FIGS. 3A-3D**); (ii) inserted within each of the two CH3 regions (**FIG. 3E, FIG. 4A, and FIG. 5A**); (iii) fused to the carboxy-terminal end of each CH1 region (**FIG. 3F, FIG. 4C, FIG. 5C-5E**); (iv) fused to the carboxy-terminal end of each aflibercept fusion protein domain (**FIG. 3G**); (v) fused to the carboxy-terminal end of each CL region (**FIG. 3H, FIG. 5B**); or (vi) fused to the amino-terminal end of the CH2 domain (**FIG. 4B**).

[0204] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:127, 129, 131, 133, 135 and 137**; and (b) a light chain variable

region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:128, 130, 132, 134, 136 and 138**; (2) a second targeting domain comprising an anti-VEGF antagonist or an anti-VEGFR antagonist; and (3) a third targeting domain comprising a TGF-β pathway inhibitor. In one embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted downstream of the second targeting domain at the carboxy-terminal end.

[0205] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:137**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:138**; (2) a second targeting domain comprising an anti-VEGF antagonist or an anti-VEGFR antagonist; and (3) a third targeting domain comprising a TGF-β pathway inhibitor. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted downstream of the second targeting domain at the carboxy-terminal end.

[0206] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:135**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:136**; (2) a second targeting domain comprising an anti-VEGF antagonist or an anti-VEGFR antagonist; and (3) a third targeting domain comprising a TGF-β pathway inhibitor. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted downstream of the second targeting domain at the carboxy-terminal end.

[0207] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region, wherein the heavy chain variable region comprises three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3, wherein HCDR1 has an amino acid sequence that is about 80% or about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:48, 51, 54, 56 and 59**, wherein HCDR2 has an amino acid sequence that is about 80%, about 85%, about 90%, about 95% or about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:49, 52, 57 and 60**, and wherein HCDR3 has an amino acid sequence that is

about 80%, about 90% or about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:50, 53, 55, 58 and 61**, and (b) a light chain variable region, wherein the light chain variable region comprises three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3, wherein LCDR1 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:62, 65, 68, 69, 70 and 73**, wherein HCDR2 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:63, 66, 71 and 74**, and wherein HCDR3 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:64, 67, 72 and 75**; (2) a second targeting domain comprising an anti-VEGF antagonist or an anti-VEGFR antagonist; and (3) a third targeting domain comprising a TGF-β pathway inhibitor. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted downstream of the second targeting domain at the carboxy-terminal end.

[0208] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region, wherein the heavy chain variable region comprises three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3, wherein HCDR1 has an amino acid sequence that is about 80% or about 100% identity to an amino acid of **SEQ ID NO:59**, wherein HCDR2 has an amino acid sequence that is about 80%, about 85%, about 90%, about 95% or about 100% identity to an amino acid sequence of **SEQ ID NO:60**, and wherein HCDR3 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:61**, and (b) a light chain variable region, wherein the light chain variable region comprises three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3, wherein LCDR1 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:73**, wherein HCDR2 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:74**, and wherein HCDR3 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:75**; (2) a second targeting domain conferred by one or more anti-VEGF antagonist and/or one or more anti-VEGFR antagonist as further described herein and (3) a third targeting domain comprising a TGF-β pathway inhibitor. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted downstream of the second targeting domain at the carboxy-terminal end.

[0209] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region, wherein the heavy chain variable region comprises three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3, wherein HCDR1 has an amino acid sequence that is about 80% or about 100% identity to an amino acid of **SEQ ID NO:56**, wherein HCDR2 has an amino acid sequence that is about 80%, about 85%, about 90%, about 95% or about 100% identity to an amino acid sequence of **SEQ ID NO:57**, and wherein HCDR3 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:58**, and (b) a light chain variable region, wherein the light chain variable region comprises three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3, wherein LCDR1 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:70**, wherein HCDR2 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:71**, and wherein HCDR3 has an amino acid sequence that is about 80%, about 90% or about 100% identity to an amino acid sequence of **SEQ ID NO:72**; (2) a second targeting domain conferred by one or more anti-VEGF antagonist and/or one or more anti-VEGFR antagonist as further described herein and (3) a third targeting domain comprising a TGF-β pathway inhibitor. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted downstream of the second targeting domain at the carboxy-terminal end.

[0210] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:137**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:138**; (2) a second targeting domain conferred by an aflibercept (or Zaltrap®) domain with amino acid sequence of **SEQ ID NO:185**; and (3) a third targeting domain comprising a TGF-β pathway inhibitor. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted downstream of the second targeting domain at the carboxy-terminal end.

[0211] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:135**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:136**; (2) a second targeting domain conferred

by an aflibercept (or Zaltrap®) domain with amino acid sequence of **SEQ ID NO:185**; and (3) a third targeting domain comprising a TGF-β pathway inhibitor. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted downstream of the second targeting domain at the carboxy-terminal end.

[0212] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:137**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:138**; (2) a second targeting domain conferred by an a-glycosylated aflibercept (or Zaltrap®) domain with amino acid sequence of **SEQ ID NO:185**; and (3) a third targeting domain comprising a TGF-β pathway inhibitor. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted downstream of the second targeting domain at the carboxy-terminal end.

[0213] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:135**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:136**; (2) a second targeting domain conferred by an a-glycosylated aflibercept (or Zaltrap®) domain with amino acid sequence of **SEQ ID NO:185**; and (3) a third targeting domain comprising a TGF-β pathway inhibitor. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted downstream of the second targeting domain at the carboxy-terminal end.

[0214] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:137**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:138**; (2) a second targeting domain conferred by an aflibercept (or Zaltrap®) domain with amino acid sequence of **SEQ ID NO:185**; and (3) a third targeting domain comprises a TGFβ1 RII extracellular domain (EAD) with amino acid sequence of **SEQ ID NO:186**. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third

targeting domain is inserted downstream of the second targeting domain at the carboxy-terminal end.

[0215] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:135**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:136**; (2) a second targeting domain conferred by an aflibercept (or Zaltrap®) domain with amino acid sequence of **SEQ ID NO:185**; and (3) a third targeting domain comprises a TGFβ1 RII extracellular domain (ECD) with amino acid sequence of **SEQ ID NO:186**. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted downstream of the second targeting domain at the carboxy-terminal end.

[0216] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:137**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:138**; (2) a second targeting domain conferred by an a-glycosylated aflibercept (or Zaltrap®) domain with amino acid sequence of **SEQ ID NO:185**; and (3) a third targeting domain comprises a TGFβ1 RII extracellular domain (EAD) with amino acid sequence of **SEQ ID NO:186**. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted downstream of the second targeting domain at the carboxy-terminal end.

[0217] In other embodiments, the trispecific antitumor antagonist comprises: (1) a first targeting domain comprises: (a) a heavy chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:135**, and (b) a light chain variable region having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:136**; (2) a second targeting domain conferred by an a-glycosylated aflibercept (or Zaltrap®) domain with amino acid sequence of **SEQ ID NO:185**; and (3) a third targeting domain comprises a TGFβ1 RII extracellular domain (ECD) with amino acid sequence of **SEQ ID NO:186**. In another embodiment, the second targeting domain is inserted downstream of the CH3 domain at the carboxy-terminal end of the antagonist, and the third targeting domain is inserted downstream of the second targeting domain at the carboxy-terminal end.

[0218] In other embodiments, the trispecific antitumor antagonists comprise: a heavy chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:187, 189, 190, 192, 205 and 206**; and a light chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence selected from the group consisting of **SEQ ID NOS:188, 191 and 193**.

[0219] In other embodiments, the trispecific antitumor antagonists comprise: a heavy chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:192**; and a light chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:193**.

[0220] In other embodiments, the trispecific antitumor antagonists comprise: a heavy chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:205**; and a light chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:193**.

[0221] In other embodiments, the trispecific antitumor antagonists comprise: a heavy chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:206**; and a light chain having an amino acid sequence that is about 80% to about 100% identity to an amino acid sequence of **SEQ ID NO:193**.

[0222] The trispecific antitumor antagonists of the present application may be constructed with an IgG backbone. More specifically, any of the trispecific antagonists of the present application may be constructed with an IgG1 or IgG4 backbone. Use of an IgG1 backbone is preferable for cancer treatment where a target is present on antigen presenting cells that can mediate antibody-dependent cell-mediated cytotoxicity (ADCC). Use of an IgG4 backbone allows targeting of antigen where antigen binding alone is sufficient to generate the desired therapeutic benefits. IgG4-based antagonists preclude undesirable effector functions associated with *e.g.*, IgG1 antibodies, including FcγR binding and complement activation.

V. Methods of Using the Antitumor Antagonists

[0223] The antitumor antagonists of the present application, such as anti-PD-1 antagonists, anti-PD-1 antibody fragments, anti-PD-L1 antibodies, anti-PD-L1 antibody fragments, anti-TIGIT antibodies, anti-TIGIT antibody fragments, anti-LAG-3 antibodies, anti-LAG-3 antibody fragments, angiogenesis pathway inhibitors, TGFβ pathway inhibitors, as well as bispecific antitumor antagonists and trispecific antitumor antagonists that bind specifically to PD-1, PD-L1, TIGIT, LAG-3, VEGF/VEGFR, TGFβ/TGFβ receptor, have numerous *in vitro* and *in vivo* utilities including, for example, enhancement of immune responses and treatment of cancers, infectious diseases or autoimmune diseases.

A. Antitumor Therapy

[0224] In some embodiments, the antitumor antagonists of the present application are administered to cells in culture, in vitro or ex vivo, or to human subjects, e.g., in vivo, to enhance immunity in a variety of diseases. Accordingly, provided herein are methods of modifying an immune response in a subject comprising administering to the subject an antibody or antigen-binding fragment thereof as described herein such that the immune response in the subject is enhanced, stimulated or up-regulated. Preferred subjects include human patients in whom enhancement of an immune response would be desirable. The methods are particularly suitable for treating human patients having a disorder that can be treated by augmenting an immune response (e.g., the T-cell mediated immune response). The methods are particularly suitable for treatment of cancer or chronic infections in vivo. For example, the anti-PD-1, anti-PD-L1, anti-TIGIT or anti-LAG-3 compositions may be administered together with an antigen of interest or the antigen may already be present in the subject to be treated (e.g., a tumor-bearing or virus-bearing subject) to enhance antigen-specific immunity. When anti-TIGIT antibodies are administered together with another agent, the two can be administered separately or simultaneously.

[0225] In some embodiments, the checkpoint regulator antagonist used in the above-described method is an anti-PD-1 antibody, anti-PD-L1 antibody, anti-TIGIT antibody, anti-LAG-3 antibody, a fragment thereof, or combination thereof. In some embodiments, the checkpoint regulator antagonist is a bispecific or trispecific antibody of the present application.

[0226] In some embodiments, the checkpoint regulator antagonist or antitumor antagonist is in the form of an antibody or antibody fragment. In some embodiments, the antibodies described herein are human or humanized antibodies.

[0227] Also encompassed are methods for detecting and/or measuring the presence of human PD-1, human PD-L1, human TIGIT or human LAG-3, in a sample comprising contacting the sample and a control sample, with a human monoclonal antibody thereof, or an antigen binding fragment thereof, which specifically binds to human PD-1, human PD-L1, human TIGIT or human LAG-3 under conditions that allow for formation of a complex between the antibody or fragment thereof and human PD-1, human PD-L1, human TIGIT or human LAG-3. The formation of a complex is then detected, wherein a difference in complex formation between the sample compared to the control sample is indicative the presence of human PD-1, human PD-L1, human TIGIT or human LAG-3 antigen in the sample.

[0228] Given the ability of anti-PD-1, anti-PD-L1, anti-TIGIT and anti-LAG-3 antibodies to block inhibition or co-inhibition of T cell responses, provided herein are in vitro and in vivo methods of using the antibodies described herein to stimulate, enhance or upregulate antigen-specific T cell responses, e.g., anti-tumor T cell responses. In certain embodiments,

CD3 stimulation is also provided (e.g., by co-incubation with a cell expressing membrane CD3), which stimulation can be provided at the same time, before, or after treatment with an anti-PD-1, anti-PD-L1, anti-TIGIT or anti-LAG-3 antibody. For example, provided herein are methods of enhancing an antigen-specific T cell response comprising contacting said T cell with an anti-PD-1, anti-PD-L1, anti-TIGIT or anti-LAG-3 antibody described herein, and optionally with CD3, such that an antigen-specific T cell response is enhanced, e.g., by removal of a PD-1, PD-L1, TIGIT or LAG-3 mediated inhibitory effect. Any suitable indicator of an antigen-specific T cell response can be used to measure the antigen-specific T cell response. Non-limiting examples of such suitable indicators include increased T cell proliferation in the presence of the antibody and/or increase cytokine production in the presence of the antibody. In a preferred embodiment, interleukin-2 and/or interferon-gamma production by the antigen-specific T cell is enhanced.

[0229] Further encompassed are methods for enhancing an immune response (e.g., an antigen-specific T cell response) in a subject comprising administering an anti-PD-1 antibody, an anti-PD-L1 antibody, an anti-TIGIT antibody, an anti-LAG-3 antibody, or a bispecific or trispecific antitumor antagonist described herein to the subject such that an immune response (e.g., an antigen-specific T cell response) in the subject is enhanced. In a preferred embodiment, the subject is a tumor-bearing subject and an immune response against the tumor is enhanced. A tumor may be a solid tumor or a liquid tumor, e.g., a hematological malignancy. In certain embodiments, a tumor is an immunogenic tumor. In other embodiments, a tumor is non-immunogenic. In certain embodiments, a tumor is PD-L1 positive. In other embodiments a tumor is PD-L1 negative. A subject may also be a virus-bearing subject in whom an immune response against the virus is enhanced as a consequence of administering an anti-PD-1 antibody, anti-PD-L1 antibody, anti-TIGIT antibody, anti-LAG-3 antibody, bispecific antitumor antagonist or trispecific antitumor antagonist as described herein.

[0230] In one embodiment, a method for inhibiting the growth of tumor cells in a subject comprises administering to the subject an anti-PD-1 antibody, anti-PD-L1 antibody, anti-TIGIT antibody, anti-LAG-3 antibody, bispecific antitumor antagonist or trispecific antitumor antagonist described herein such that growth of the tumor is inhibited in the subject. Also provided are methods of treating chronic viral infection in a subject comprising administering to the subject an anti-PD-1 antibody, anti-PD-L1 antibody, anti-TIGIT antibody, anti-LAG-3 antibody, bispecific antitumor antagonist or trispecific antitumor antagonist as described herein such that the chronic viral infection is treated in the subject.

[0231] Also encompassed herein are methods for depleting Treg cells from the tumor microenvironment of a subject with a tumor, e.g., cancerous tumor, comprising administering to the subject a therapeutically effective amount of an anti-PD-1 antibody, anti-PD-L1 antibody,

anti-TIGIT antibody, anti-LAG-3 antibody, bispecific antitumor antagonist or trispecific antitumor antagonist described herein that comprises an Fc that stimulates depletion of Treg cells in the tumor microenvironment. An Fc may, e.g., be an Fc with effector function or enhanced effector function, such as binding or having enhanced binding to one or more activating Fc receptors.

[0232] In a preferred embodiment, Treg depletion occurs without significant depletion or inhibition of Teff in the tumor microenvironment, and without significant depletion or inhibition of Teff cells and Treg cells outside of the tumor microenvironment. In certain embodiments, the subject has higher levels of TIGIT on Treg cells than on Teff cells, e.g., in the tumor microenvironment. In certain embodiments, anti-TIGIT antibodies or antagonists may deplete Tregs in tumors and/or Tregs in tumor infiltrating lymphocytes (TILs). For example, in the CT26 tumor model, an anti-mouse TIGIT antibody formatted as a mouse IgG2a (which exhibits effector function) partially depleted both Treg and CD8+ T cells, but did not deplete CD4+ T cells. An effectorless counterpart anti-TIGIT antibody, formatted as a mouse IgG1 D265A, did not deplete T cells.

[0233] When considering whether or not to employ Fc effector function or an effectorless anti-TIGIT antibody, due consideration must be given to the tradeoff between depletion of Tregs, which may enhance anti-tumor immune response, and depletion of CD8+ T cells, which would eliminate some of the cells needed to actually kill tumor cells. Although depletion of Tregs might be expected to enhance anti-tumor activity, recent studies have demonstrated that ligation of TIGIT on TIGIT+ Tregs promotes Treg cell-mediated suppression of Teff cell proliferation (Joller et al. (2014) Immunity 40:569), suggesting that blocking of TIGIT signaling (e.g., using an antagonist anti-TIGIT antibody of the present invention) might also enhance anti-tumor activity. Accordingly, it may be most efficacious to use an antagonist anti-TIGIT antibody lacking effector function, which: i) blocks TIGIT signaling in Tregs thus reducing their immunesuppresive activity; ii) activates anti-tumor CD8+ T cells by blocking TIGIT's inhibitory effects, while at the same time avoiding their effector-function-mediated depletion; and iii) enhances DNAM-mediated activation by allowing DNAM to bind to PVR (CD155, the TIGIT ligand) that would otherwise have been bound by TIGIT (and by reducing direct TIGIT-DNAM interactions) (Johnston et al. (2014) Cancer Cell 26:923). The same is applicable to use of anti-PD-1 antibodies, anti-PD-L1 antibodies, bispecific antitumor antagonists, or trispecific antitumor antagonists.

[0234] In certain embodiments, an anti-PD-1 antibody, anti-PD-L1 antibody, anti-TIGIT antibody, anti-LAG-3 antibody, bispecific antitumor antagonist or trispecific antitumor antagonist described herein is given to a subject as an adjunctive therapy. Treatment of cancer

patient with an anti-PD-1 antibody, anti-PD-L1 antibody, anti-TIGIT antibody, anti-LAG-3 antibody, bispecific antitumor antagonist or trispecific antitumor antagonist according to the present application may lead to a long-term durable response relative to the current standard of care; long term survival of at least 1, 2, 3, 4, 5, 10 or more years, recurrence free survival of at least 1, 2, 3, 4, 5, or 10 or more years. In certain embodiments, treatment of a cancer patient with an anti-PD-1 antibody, anti-PD-L1 antibody, anti-TIGIT antibody, anti-LAG-3 antibody, bispecific antitumor antagonist or trispecific antitumor antagonist prevents recurrence of cancer or delays recurrence of cancer by, e.g., 1, 2, 3, 4, 5, or 10 or more years. An anti-PD-1, anti-PD-L1, anti-TIGIT, and/or anti-LAG-3 treatment can be used as a primary or secondary line of treatment.

[0235] In certain preferred embodiments, the subject has a cell proliferative disease or cancer. Blocking of PVR/Nectin-2 signaling through TIGIT by anti-TIGIT antibodies can enhance the immune response to cancerous cells in the patient. Similarly, blocking of Provided herein are methods for treating a subject having cancer, comprising administering to the subject an anti-PD-1, anti-PD-L1, anti-TIGIT, anti-LAG-3, bispecific antitumor antagonist or trispecific antitumor antagonist thereof as described herein, such that the subject is treated, e.g., such that growth of cancerous tumors is inhibited or reduced and/or that the tumors regress. An anti-PD-1, anti-PD-L1, anti-TIGIT, anti-LAG-3, bispecific antitumor antagonist or trispecific antitumor antagonist thereof as described herein can be used alone to inhibit the growth of cancerous tumors. Alternatively, any of these antitumor antagonists can be used in conjunction with another agent, e.g., other anti-cancer targets, immunogenic agents, standard cancer treatments, or other antibodies, as described below.

[0236] Accordingly, provided herein are methods of treating cancer, e.g., by inhibiting growth of tumor cells, in a subject, comprising administering to the subject a therapeutically effective amount of an anti-PD-1, anti-PD-L1, anti-TIGIT, anti-LAG-3, bispecific antitumor antagonist or trispecific antitumor antagonist as described herein. Preferably, the antibody is a human anti-PD-1, anti-PD-L1, anti-TIGIT or anti-LAG-3 antibody comprising the anti-PD-1, anti-PD-L1, anti-TIGIT or anti-LAG-3 HCVRs and LCVR described herein, or it may be a chimeric or humanized non-human anti-hu PD-1, anti-PD-L1 antibody, anti-hu TIGIT or anti-LAG-3 antibody, e.g., a chimeric or humanized anti-PD-1, anti-PD-L1, anti-TIGIT or anti-LAG-3 antibody that competes for binding with, or binds to the same epitope as, at least one of the anti-PD-1, anti-PD-L1, anti-TIGIT or anti-LAG-3 antibodies described herein.

[0237] Cancers whose growth may be inhibited using the antibodies of the application include cancers typically responsive to immunotherapy. Non-limiting examples of cancers for treatment include squamous cell carcinoma, small-cell lung cancer, non-small cell lung cancer,

squamous non-small cell lung cancer (NSCLC), non NSCLC, glioma, gastrointestinal cancer, renal cancer (e.g. clear cell carcinoma), ovarian cancer, liver cancer, colorectal cancer, endometrial cancer, kidney cancer (e.g., renal cell carcinoma (RCC)), prostate cancer (e.g. hormone refractory prostate adenocarcinoma), thyroid cancer, neuroblastoma, pancreatic cancer, glioblastoma (glioblastoma multiforme), cervical cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon carcinoma, and head and neck cancer (or carcinoma), gastric cancer, germ cell tumor, pediatric sarcoma, sinonasal natural killer, melanoma (e.g., metastatic malignant melanoma, such as cutaneous or intraocular malignant melanoma), bone cancer, skin cancer, uterine cancer, cancer of the anal region, testicular cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, solid tumors of childhood, cancer of the ureter, carcinoma of the renal pelvis, neoplasm of the central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, Kaposi's sarcoma, epidermoid cancer, squamous cell cancer, T-cell lymphoma, environmentally-induced cancers including those induced by asbestos, virus-related cancers (e.g., human papilloma virus (HPV)-related tumor), and hematologic malignancies derived from either of the two major blood cell lineages, i.e., the myeloid cell line (which produces granulocytes, erythrocytes, thrombocytes, macrophages and mast cells) or lymphoid cell line (which produces B, T, NK and plasma cells), such as all types of leukemias, lymphomas, and myelomas, e.g., acute, chronic, lymphocytic and/or myelogenous leukemias, such as acute leukemia (ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), and chronic myelogenous leukemia (CML), undifferentiated AML (MO), myeloblastic leukemia (M1), myeloblastic leukemia (M2; with cell maturation), promyelocytic leukemia (M3 or M3 variant [M3V]), myelomonocytic leukemia (M4 or M4 variant with eosinophilia [M4E]), monocytic leukemia (M5), erythroleukemia (M6), megakaryoblastic leukemia (M7), isolated granulocytic sarcoma, and chloroma; lymphomas, such as Hodgkin's lymphoma (HL), non-Hodgkin's lymphoma (NEIL), B-cell lymphomas, T-cell lymphomas, lymphoplasmacytoid lymphoma, monocytoid B-cell lymphoma, mucosa-associated lymphoid tissue (MALT) lymphoma, anaplastic (e.g., Ki 1+) large-cell lymphoma, adult T-cell lymphoma/leukemia, mantle cell lymphoma, angio immunoblastic T-cell lymphoma, angiocentric lymphoma, intestinal T-cell lymphoma, primary mediastinal B-cell lymphoma, precursor T-lymphoblastic lymphoma, T-lymphoblastic; and lymphoma/leukemia (T-Lbly/T-ALL), peripheral T-cell lymphoma, lymphoblastic lymphoma, post-transplantation lymphoproliferative disorder, true

histiocytic lymphoma, primary central nervous system lymphoma, primary effusion lymphoma, lymphoblastic lymphoma (LBL), hematopoietic tumors of lymphoid lineage, acute lymphoblastic leukemia, diffuse large B-cell lymphoma, Burkitt's lymphoma, follicular lymphoma, diffuse histiocytic lymphoma (DHL), immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, cutaneous T-cell lymphoma (CTLC) (also called mycosis fungoides or Sezary syndrome), and lymphoplasmacytoid lymphoma (LPL) with Waldenstrom's macroglobulinemia; myelomas, such as IgG myeloma, light chain myeloma, nonsecretory myeloma, smoldering myeloma (also called indolent myeloma), solitary plasmocytoma, and multiple myelomas, chronic lymphocytic leukemia (CLL), hairy cell lymphoma; hematopoietic tumors of myeloid lineage, tumors of mesenchymal origin, including fibrosarcoma and rhabdomyoscarcoma; seminoma, teratocarcinoma, tumors of the central and peripheral nervous, including astrocytoma, schwannomas; tumors of mesenchymal origin, including fibrosarcoma, rhabdomyoscaroma, and osteosarcoma; and other tumors, including melanoma, xeroderma pigmentosum, keratoacanthoma, seminoma, thyroid follicular cancer and teratocarcinoma, hematopoietic tumors of lymphoid lineage, for example T-cell and B-cell tumors, including but not limited to T-cell disorders such as T-prolymphocytic leukemia (T-PLL), including of the small cell and cerebriform cell type; large granular lymphocyte leukemia (LGL) preferably of the T-cell type; a/d T-NHL hepatosplenic lymphoma; peripheral/post-thymic T cell lymphoma (pleomorphic and immunoblastic subtypes); angiocentric (nasal) T-cell lymphoma; cancer of the head or neck, renal cancer, rectal cancer, cancer of the thyroid gland; acute myeloid lymphoma, as well as any combinations of said cancers. The methods described herein may also be used for treatment of metastatic cancers, refractory cancers (e.g., cancers refractory to previous immunotherapy, e.g., with a blocking CTLA-4 or PD-1 antibody), and recurrent cancers.

[0238] An anti-PD-1 antibody, anti-PD-L1 antibody, anti-TIGIT, anti-LAG-3 antibody, bispecific antitumor antagonist or trispecific antitumor antagonist can be administered alone, in combination with another antitumor antagonist, or concurrently with another antitumor antagonist. An anti-PD-1 antibody, anti-TIGIT, anti-LAG-3 antibody, bispecific antitumor antagonist or trispecific antitumor antagonist can also be administered in combination, or concurrently with, an immunogenic agent, such as cancerous cells, tumor vaccines, purified tumor antigens (including recombinant proteins, peptides, and carbohydrate molecules), cells transfected with genes encoding immune stimulating cytokines, in a cancer vaccine strategy (He et al. (2004) J. Immunol. 173:4919-28), or an oncolytic virus.

[0239] Many experimental strategies for vaccination against tumors have been devised. In one of these strategies, a vaccine is prepared using autologous or allogeneic tumor cells. Some of these cellular vaccines have been shown to be most effective when the tumor cells are

transduced to express GM-CSF. GM-CSF has been shown to be a potent activator of antigen presentation for tumor vaccination (Dranoff et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90: 3539-43). Cancer vaccines have been shown to enhance effector T-cell infiltration into the tumors in preclinical models. The major types of cancer vaccines include peptide vaccines, vector-based antigen specific vaccines, whole-cell vaccines, and dendritic cell vaccines. All vaccine-based therapies are designed to deliver either single or multiple antigenic epitopes or antigens from the whole cells to the patients and induce tumor-specific effector T cells. Thus, a vaccine-based therapy may be the most efficient way to induce T-cell infiltration into the tumor.

[0240] The study of gene expression and large scale gene expression patterns in various tumors has led to the definition of so called tumor specific antigens (Rosenberg, S A (1999) Immunity 10: 281-7). In many cases, these tumor specific antigens are differentiation antigens expressed in the tumors and in the cell from which the tumor arose, for example melanocyte antigens gp100, MAGE antigens, and Trp-2. More importantly, many of these antigens can be shown to be the targets of tumor specific T cells found in the host.

[0241] PD-1, PD-L1, TIGIT and/or LAG-3 inhibition may be used in conjunction with a collection of recombinant proteins and/or peptides expressed in a tumor in order to generate an immune response to these proteins. Such proteins may be viewed by the immune system as self-antigens and are therefore tolerant to them. The tumor antigen can include the protein telomerase, which is required for the synthesis of telomeres of chromosomes and which is expressed in more than 85% of human cancers and in only a limited number of somatic tissues (Kim et al. (1994) Science 266: 2011-2013). Tumor antigens can also be "neo-antigens" expressed in cancer cells because of somatic mutations that alter protein sequence or create fusion proteins between two unrelated sequences (i.e., bcr-abl in the Philadelphia chromosome), or idiotype from B cell tumors.

[0242] Non-limiting examples of tumor vaccines include sipuleucel-T (Provenge®), an FDA-approved tumor vaccine for metastatic prostate cancer; tumor cells transfected to express the cytokine granulocyte macrophage colony-stimulating factor (GM-CSF), such as the whole cell GM-CSF-secreting irradiated, allogeneic pancreatic cancer vaccine (GVAX; Johns Hopkins); a multi-peptide vaccine consisting of immunogenic peptides derived from breast cancer antigens, neu, legumain, and β-catenin, which prolonged the vaccine-induced progression-free survival of breast tumor-bearing mice when administered in combination with anti-PD-1 antibody (Karyampudi L. et al. (2014) Cancer Res 74:2974-2985); peptides of melanoma antigens, such as peptides of gp100, MAGE antigens, Trp-2, MARTI and/or tyrosinase. Other tumor vaccines include proteins from viruses implicated in human cancers such as human papilloma viruses (HPV)(e.g., Gardasil®, Gardasil 9®, and Cervarix®; hepatitis

B virus (e.g., Engerix-B and Recombivax HB); hepatitis C virus (HCV), Kaposi's sarcoma associated herpes sarcoma virus (KSHV). Another form of tumor specific antigen that can be used in conjunction with TIGIT inhibition is purified heat shock proteins (HSP) isolated from the tumor tissue itself. These heat shock proteins contain fragments of proteins from the tumor cells and these HSPs are highly efficient at delivery to antigen presenting cells for eliciting tumor immunity. Talimogene laherparepvec (T-VEC, or Imlygic®) is an FDA-approved oncolytic virus for the treatment of some patients with metastatic melanoma that cannot be surgically removed.

[0243] Dendritic cells (DC) are potent antigen presenting cells that can be used to prime antigen-specific responses. DC's can be produced ex vivo and loaded with various protein and peptide antigens, as well as tumor cell extracts (Nestle et al. (1998) Nature Medicine 4: 328-332). DCs can also be transduced by genetic means to express these tumor antigens as well. DCs have also been fused directly to tumor cells for the purposes of immunization (Kugler et al. (2000) Nature Medicine 6:332-336). As a method of vaccination, DC immunization may be effectively combined with TIGIT blocking to activate (unleash) more potent anti-tumor responses.

[0244] PD-1, PD-L1, TIGIT and/or LAG-3 inhibition can also be combined with standard cancer treatments (e.g., surgery, radiation, and chemotherapy). In particular, PD-1, PD-L1, TIGIT and/or LAG-3 inhibition can be effectively combined with chemotherapeutic regimes. In these instances, it may be possible to reduce the dose of chemotherapeutic reagent administered (Mokyr et al. (1998) Cancer Research 58: 5301-5304). An example of such a combination is an antitumor antagonist in combination with decarbazine for the treatment of melanoma. Another example of such a combination is a checkpoint regulator antagonist or antitumor antagonist in combination with interleukin-2 (IL-2) for the treatment of melanoma. For example, the scientific rationale behind the combined use of PD-1, PD-L1, TIGIT and/or LAG-3 inhibition and chemotherapy to promote cell death is a consequence of the cytotoxic action of most chemotherapeutic compounds, should result in increased levels of tumor antigen in the antigen presentation pathway. Other combination therapies that may result in synergy with TIGIT, PD-1, PD-L1 and/or LAG-3 inhibition through cell death are radiation, surgery, and hormone deprivation. Each of these protocols creates a source of tumor antigen in the host. Angiogenesis inhibitors can also be combined with TIGIT, PD-1, PD-L1 and/or LAG-3 inhibition. Inhibition of angiogenesis leads to tumor cell death, which may feed tumor antigen into host antigen presentation pathways.

[0245] The anti-TIGIT antibodies, anti-PD-1 antibodies, anti-PD-L1 antibodies, bispecific antitumor antagonists and trispecific antitumor antagonists described herein may also

be used in combination with bispecific antibodies that target Fcα or Fcγ receptor-expressing effectors cells to tumor cells (see, e.g., U.S. Patent Nos. 5,922,845 and 5,837,243). Bispecific antibodies can be used to target two separate antigens. For example anti-Fc receptor/antitumor antigen (e.g., Her-2/neu) bispecific antibodies have been used to target macrophages to sites of tumor. This targeting may more effectively activate tumor specific responses. The T cell arm of these responses would be augmented by the inhibition of TIGIT, PD-1, PD-L1 and/or LAG-3. Alternatively, antigen may be delivered directly to DCs by the use of bispecific antibodies that bind to tumor antigen and a dendritic cell specific cell surface marker.

[0246] Tumors evade host immune surveillance by a large variety of mechanisms. Many of these mechanisms may be overcome by the inactivation of immunosuppressive proteins expressed by the tumors. These include among others TGF-β, IL-10, and Fas ligand. Antibodies to each of these entities can be used in combination with the antitumor antagonists described herein to counteract the effects of the immunosuppressive agent and favor tumor immune responses by the host.

[0247] Other antibodies that activate host immune responsiveness can be used in combination with the antitumor antagonists described herein. These include molecules on the surface of dendritic cells that activate DC function and antigen presentation. Anti-CD40 antibodies are able to substitute effectively for T cell helper activity (Ridge et al. (1998) Nature 393: 474-478) and can be used in conjunction with anti-TIGIT antibodies. Activating antibodies to T cell costimulatory molecules, such as OX-40 (Weinberg et al. (2000) Immunol 164: 2160-2169), CD137/4-1BB (Melero et al. (1997) Nature Medicine 3: 682-685 (1997), and ICOS (Hutloff et al. (1999) Nature 397: 262-266) may also provide for increased levels of T cell activation. In addition, inhibitors of other immune checkpoint regulators may also be used in conjunction with other antitumor antagonists described herein, as further described below.

[0248] Bone marrow transplantation is currently being used to treat a variety of tumors of hematopoietic origin. While graft versus host disease is a consequence of this treatment, TIGIT inhibition may be used to increase the effectiveness of the donor engrafted tumor specific T cells by reducing graft vs. tumor responses.

[0249] Ex vivo activation and expansion of antigen specific T cells and adoptive transfer of these cells into recipients in order to stimulate antigen-specific T cells against cancers or viral infections in the presence of anti-TIGIT antibodies can increase the frequency and activity of the adoptively transferred T cells.

[0250] There are also several experimental treatment protocols that involve ex vivo activation and expansion of antigen specific T cells and adoptive transfer of these cells into recipients in order to stimulate antigen-specific T cells against tumor (Greenberg & Riddell

(1999) Science 285: 546-51). These methods can also be used to activate T cell responses to infectious agents such as CMV. Ex vivo activation in the presence of anti-TIGIT antibodies can increase the frequency and activity of the adoptively transferred T cells.

[0251] In certain embodiments, an antitumor antagonist described herein may be administered to a subject with an infectious disease, especially chronic infections. In this case, similar to its application to cancer, antibody-mediated PD-1, PD-L1, TIGIT and/or LAG-3 inhibition can be used alone, or as an adjuvant, in combination with vaccines, to enhance immune responsiveness to pathogens, toxins, and self-antigens. Exemplary pathogens for which this therapeutic approach can be applied include, but are not limited to, HIV, Hepatitis (A, B, & C), Influenza, Herpes, Giardia, Malaria, Leishmania, Staphylococcus aureus, and Pseudomonas aeruginosa. PD-1, PD-L1, TIGIT and/or LAG-3 inhibition is particularly useful against established infections by agents such as HIV that present novel or altered antigens over the course of the infections. Administration of the anti-PD-1 antibodies, anti-PD-L1 antibodies, anti-TIGIT antibodies, bispecific antitumor antagonists and trispecific antitumor antagonists can allow for recognition of these antigens as foreign so as to provoke an appropriate T cell response.

[0252] Other pathogenic viruses causing infections treatable by the methods described herein include HIV, hepatitis (A, B, or C), herpesvirus infections (e.g., VZV, HSV-1, HAV-6, HSV-II, and CMV, Epstein Barr virus), and infections caused by an adenovirus, influenza virus, flavivirus, echoviruses, rhinoviruses, coxsackie viruses, coronaviruses, respiratory syncytial viruses, mumps viruses, rotavirus, measles virus, rubella virus, parvovirus, vaccinia virus, HTLV virus, dengue virus, papillomavirus, molluscum virus, poliovirus, rabies virus, JC virus, arboviral encephalitis virus, or combination thereof.

[0253] Exemplary pathogenic bacteria or diseases caused therefrom which may be treatable by the methods described herein include Chlamydia, Rickettsia, Mycobacteria, Staphylococci, Streptococci, Pneumonococci, Meningococci and Gonococci, Klebsiella, Proteus, Serratia, Pseudomonas, Legionella, Diphtheria, Salmonella, Bacilli, Cholera, Leptospirosis tetanus, botulism, anthrax, plague, and Lyme disease.

[0254] Exemplary pathogenic fungi causing infections treatable by the methods described herein include Candida (e.g., albicans, krusei, glabrata, tropicalis, etc.), Cryptococcus neoformans, Aspergillus (e.g., fumigatus, niger, etc.), Mucorales (e.g., mucor, absidia, rhizopus), Sporothrix schenkii, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Coccidioides immitis and Histoplasma capsulatum.

[0255] Exemplary pathogenic parasites causing infections treatable by the methods described herein include Entamoeba histolytica, Balantidium coli, Naegleriafowleri,

Acanthamoeba sp., Giardia Zambia, Cryptosporidium sp., Pneumocystis carinii, Plasmodium vivax, Babesia microti, Trypanosoma brucei, Trypanosoma cruzi, Leishmania donovani, Toxoplasma gondii, and Nippostrongylus brasiliensis.

[0256] In all of the above methods, PD-1, PD-L1, TIGIT and/or LAG-3 inhibition can be combined with other forms of immunotherapy such as cytokine treatment (e.g., interferons, GM-CSF, G-CSF, IL-2), or bispecific antibody therapy using two different binding specificities to provide enhanced presentation of tumor antigens.

[0257] Anti-PD-1 antibodies, anti-PD-L1 antibodies, anti-TIGIT antibodies, anti-LAG-3 antibodies, bispecific antitumor antagonists and trispecific antitumor antagonists described herein can be used to enhance antigen-specific immune responses by co-administration of one or more of any of these antibodies with an antigen of interest (e.g., a vaccine). Accordingly, provided herein are methods of enhancing an immune response to an antigen in a subject, comprising administering to the subject: (i) the antigen; and (ii) anti-PD-1 antibody, anti-PD-L1 antibody, an anti-TIGIT antibody, anti-LAG-3 antibody, bispecific antitumor antagonists, trispecific antitumor antagonist, or combination thereof, such that an immune response to the antigen in the subject is enhanced. The antigen can be, for example, a tumor antigen, a viral antigen, a bacterial antigen or an antigen from a pathogen. Non-limiting examples of such antigens include those discussed in the sections above, such as the tumor antigens (or tumor vaccines) discussed above, or antigens from the viruses, bacteria or other pathogens described above.

[0258] In certain embodiments, a peptide or fusion protein comprising the epitope to which an anti-PD-1 antibody, anti-PD-L1 antibody, anti-TIGIT antibody, LAG-3 antibody, bispecific antitumor antagonists, trispecific antitumor antagonist binds may be used as a vaccine instead of, or in addition to, the antitumor antagonist(s).

[0259] Suitable routes of administering the antibody compositions (e.g., human monoclonal antibodies, multi-specific antibodies or antagonists and immunoconjugates) described herein in vivo and in vitro are well known in the art and can be selected by those of ordinary skill. For example, the antibody compositions can be administered by injection (*e.g.*, intravenous or subcutaneous). Suitable dosages of the molecules used will depend on the age and weight of the subject and the concentration and/or formulation of the antibody composition.

B. Pharmaceutical Compositions and Methods of Treatment

[0260] Another aspect of the present application relates to pharmaceutical compositions and methods for treating a cell proliferative disorder, such as cancer, chronic infections, or immunologically compromised disease states. In one embodiment, the pharmaceutical composition comprises one or more antitumor antagonists of the present application. In some

embodiments, the antitumor antagonist(s) comprise one or more checkpoint regulator antagonists, such as PD-1 inhibitors, PD-L1 inhibitors, anti-**TIGIT** inhibitors, LAG-3 inhibitors; one or more angiogenesis inhibitors, such as VEGF inhibitors, VEGFR2 inhibitors, angiopoietin-1/2 inhibitors, and Tie2R inhibitors; one or more antitumor inhibitors, such as TGF-β1 inhibitors and TGF-β1 RII inhibitors; or bispecific and trispecific antitumor antagonists thereof. The antagonist(s) are formulated together with a pharmaceutically acceptable carrier. Pharmaceutical composition of the present application may include one or more different antibodies, one or more multispecific antibodies, one or more immunoconjugates, or a combination thereof as described herein.

[0261] As described above, methods for using the pharmaceutical compositions described herein comprise administering to a subject in need thereof an effective amount of the pharmaceutical composition according to the present disclosure.

[0262] Any suitable route or mode of administration can be employed for providing the patient with a therapeutically or prophylactically effective dose of the antibody or antagonist. Exemplary routes or modes of administration include parenteral (e.g., intravenous, intraarterial, intramuscular, subcutaneous, intratumoral), oral, topical (nasal, transdermal, intradermal or intraocular), mucosal (e.g., nasal, sublingual, buccal, rectal, vaginal), inhalation, intralymphatic, intraspinal, intracranial, intraperitoneal, intratracheal, intravesical, intrathecal, enteral, intrapulmonary, intralymphatic, intracavital, intraorbital, intracapsular and transurethral, as well as local delivery by catheter or stent.

[0263] A pharmaceutical composition comprising an antibody or antagonist in accordance with the present disclosure may be formulated in any pharmaceutically acceptable carrier(s) or excipient(s). As used herein, the term "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Pharmaceutical compositions may comprise suitable solid or gel phase carriers or excipients. Exemplary carriers or excipients include but are not limited to, calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols. Exemplary pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the therapeutic agents.

[0264] The antitumor antagonist can be incorporated into a pharmaceutical composition suitable for parenteral administration. Suitable buffers include but are not limited to, sodium succinate, sodium citrate, sodium phosphate or potassium phosphate. Sodium chloride can be used to modify the toxicity of the solution at a concentration of 0-300 mM (optimally 150 mM for a liquid dosage form). Cryoprotectants can be included for a lyophilized dosage form, principally 0-10% sucrose (optimally 0.5-1.0%). Other suitable cryoprotectants include trehalose and lactose. Bulking agents can be included for a lyophilized dosage form, principally 1-10% mannitol (optimally 2-4%). Stabilizers can be used in both liquid and lyophilized dosage forms, principally 1-50 mM L-Methionine (optimally 5-10 mM). Other suitable bulking agents include glycine, arginine, can be included as 0-0.05% polysorbate-80 (optimally 0.005-0.01%). Additional surfactants include but are not limited to polysorbate 20 and BRIJ surfactants.

[0265] Therapeutic antitumor antagonist preparations can be lyophilized and stored as sterile powders, preferably under vacuum, and then reconstituted in bacteriostatic water (containing, for example, benzyl alcohol preservative) or in sterile water prior to injection. Pharmaceutical composition may be formulated for parenteral administration by injection e.g., by bolus injection or continuous infusion.

[0266] The therapeutic agents in the pharmaceutical compositions may be formulated in a "therapeutically effective amount" or a "prophylactically effective amount". A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the recombinant vector may vary depending on the condition to be treated, the severity and course of the condition, the mode of administration, whether the antibody or agent is administered for preventive or therapeutic purposes, the bioavailability of the particular agent(s), the ability of the antitumor antagonist to elicit a desired response in the individual, previous therapy, the age, weight and sex of the patient, the patient's clinical history and response to the antibody, the type of the antitumor antagonist used, discretion of the attending physician, etc. A therapeutically effective amount is also one in which any toxic or detrimental effect of the recombinant vector is outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result.

[0267] Preferably, the polypeptide domains in the antitumor antagonist are derived from the same host in which they are to be administered in order to reduce inflammatory responses against the administered therapeutic agents.

[0268] The antitumor antagonist is suitably administered to the patent at one time or over a series of treatments and may be administered to the patient at any time from diagnosis

onwards. The antitumor antagonist may be administered as the sole treatment or in conjunction with other drugs or therapies useful in treating the condition in question.

[0269] As a general proposition, a therapeutically effective amount or prophylactically effective amount of the antitumor antagonist will be administered in a range from about 1 ng/kg body weight/day to about 100 mg/kg body weight/day whether by one or more administrations. In a particular embodiment, each antitumor antagonist is administered in the range of from about 1 ng/kg body weight/day to about 10 mg/kg body weight/day, about 1 ng/kg body weight/day to about 1 mg/kg body weight/day, about 1 ng/kg body weight/day to about 100 μg/kg body weight/day, about 1 ng/kg body weight/day to about 10 μg/kg body weight/day, about 1 ng/kg body weight/day to about 1 μg/kg body weight/day, about 1 ng/kg body weight/day to about 100 ng/kg body weight/day, about 1 ng/kg body weight/day to about 10 ng/kg body weight/day, about 10 ng/kg body weight/day to about 100 mg/kg body weight/day, about 10 ng/kg body weight/day to about 10 mg/kg body weight/day, about 10 ng/kg body weight/day to about 1 mg/kg body weight/day, about 10 ng/kg body weight/day to about 100 μg/kg body weight/day, about 10 ng/kg body weight/day to about 10 μg/kg body weight/day, about 10 ng/kg body weight/day to about 1 μg/kg body weight/day, 10 ng/kg body weight/day to about 100 ng/kg body weight/day, about 100 ng/kg body weight/day to about 100 mg/kg body weight/day, about 100 ng/kg body weight/day to about 10 mg/kg body weight/day, about 100 ng/kg body weight/day to about 1 mg/kg body weight/day, about 100 ng/kg body weight/day to about 100 μg/kg body weight/day, about 100 ng/kg body weight/day to about 10 μg/kg body weight/day, about 100 ng/kg body weight/day to about 1 μg/kg body weight/day, about 1 μg/kg body weight/day to about 100 mg/kg body weight/day, about 1 μg /kg body weight/day to about 10 mg/kg body weight/day, about 1 μg /kg body weight/day to about 1 mg/kg body weight/day, about 1 μg /kg body weight/day to about 100 μg/kg body weight/day, about 1 μg /kg body weight/day to about 10 μg/kg body weight/day, about 10 μg/kg body weight/day to about 100 mg/kg body weight/day, about 10 μg /kg body weight/day to about 10 mg/kg body weight/day, about 10 μg /kg body weight/day to about 1 mg/kg body weight/day, about 10 μg /kg body weight/day to about 100 μg/kg body weight/day, about 100 μg/kg body weight/day to about 100 mg/kg body weight/day, about 100 μg /kg body weight/day to about 10 mg/kg body weight/day, about 100 μg /kg body weight/day to about 1 mg/kg body weight/day, about 1 mg/kg body weight/day to about 100 mg/kg body weight/day, about 1 mg/kg body weight/day to about 10 mg/kg body weight/day, about 10 mg/kg body weight/day to about 100 mg/kg body weight/day.

[0270] In other embodiments, the antitumor antagonist is administered at a dose of 500 μg to 20 g every three days, or 25 mg/kg body weight every three days.

[0271] In other embodiments, each antitumor antagonist is administered in the range of about 10 ng to about 100 ng per individual administration, about 10 ng to about 1 μg per individual administration, about 10 ng to about 10 μg per individual administration, about 10 ng to about 100 μg per individual administration, about 10 ng to about 1 mg per individual administration, about 10 ng to about 10 mg per individual administration, about 10 ng to about 100 mg per individual administration, about 10 ng to about 1000 mg per injection, about 10 ng to about 10,000 mg per individual administration, about 100 ng to about 1 μg per individual administration, about 100 ng to about 10 μg per individual administration, about 100 ng to about 100 μg per individual administration, about 100 ng to about 1 mg per individual administration, about 100 ng to about 10 mg per individual administration, about 100 ng to about 100 mg per individual administration, about 100 ng to about 1000 mg per injection, about 100 ng to about 10,000 mg per individual administration, about 1 μg to about 10 μg per individual administration, about 1 μg to about 100 μg per individual administration, about 1 μg to about 1 mg per individual administration, about 1 μg to about 10 mg per individual administration, about 1 μg to about 100 mg per individual administration, about 1 μg to about 1000 mg per injection, about 1 μg to about 10,000 mg per individual administration, about 10 μg to about 100 μg per individual administration, about 10 μg to about 1 mg per individual administration, about 10 μg to about 10 mg per individual administration, about 10 μg to about 100 mg per individual administration, about 10 μg to about 1000 mg per injection, about 10 μg to about 10,000 mg per individual administration, about 100 μg to about 1 mg per individual administration, about 100 μg to about 10 mg per individual administration, about 100 μg to about 100 mg per individual administration, about 100 μg to about 1000 mg per injection, about 100 μg to about 10,000 mg per individual administration, about 1 mg to about 10 mg per individual administration, about 1 mg to about 100 mg per individual administration, about 1 mg to about 1000 mg per injection, about 1 mg to about 10,000 mg per individual administration, about 10 mg to about 100 mg per individual administration, about 10 mg to about 1000 mg per injection, about 10 mg to about 10,000 mg per individual administration, about 100 mg to about 1000 mg per injection, about 100 mg to about 10,000 mg per individual administration and about 1000 mg to about 10,000 mg per individual administration. The antitumor antagonist may be administered daily, every 2, 3, 4, 5, 6 or 7 days, or every 1, 2, 3 or 4 weeks.

[0272] In other particular embodiments, the amount of the antitumor antagonist may be administered at a dose of about 0.0006 mg/day, 0.001 mg/day, 0.003 mg/day, 0.006 mg/day, 0.01 mg/day, 0.03 mg/day, 0.06 mg/day, 0.1 mg/day, 0.3 mg/day, 0.6 mg/day, 1 mg/day, 3 mg/day, 6 mg/day, 10 mg/day, 30 mg/day, 60 mg/day, 100 mg/day, 300 mg/day, 600 mg/day,

1000 mg/day, 2000 mg/day, 5000 mg/day or 10,000 mg/day. As expected, the dosage will be dependent on the condition, size, age and condition of the patient.

[0273] In certain embodiments, the coding sequences for an antitumor antagonist are incorporated into a suitable expression vector (e.g., viral or non-viral vector) for expressing an effective amount of the antitumor antagonist in patient with a cell proliferative disorder. In certain embodiments comprising administration of e.g., one or more recombinant AAV (rAAV) viruses, the pharmaceutical composition may comprise the rAAVs in an amount comprising at least 10^{10}, at least 10^{11}, at least 10^{12}, at least 10^{13}, or at least 10^{14} genome copies (GC) or recombinant viral particles per kg, or any range thereof. In certain embodiments, the pharmaceutical composition comprises an effective amount of the recombinant virus, such as rAAV, in an amount comprising at least 1010, at least 1011, at least 1012, at least 1013, at least 1014, at least 1015 genome copies or recombinant viral particles genome copies per subject, or any range thereof.

[0274] Dosages can be tested in several art-accepted animal models suitable for any particular cell proliferative disorder.

[0275] Delivery methodologies may also include the use of polycationic condensed DNA linked or unlinked to killed viruses, ligand linked DNA, liposomes, eukaryotic cell delivery vehicles cells, deposition of photopolymerized hydrogel materials, use of a handheld gene transfer particle gun, ionizing radiation, nucleic charge neutralization or fusion with cell membranes, particle mediated gene transfer and the like.

C. Combination Therapies

[0276] In another aspect, the present application provides combination therapies for enhancing an antigen-specific T cell response in a subject. In one embodiment, the method includes contacting a T cell with an anti-PD-1 antibody, anti-PD-L1 antibody, anti-TIGIT antibody, anti-LAG-3 antibody, antibody fragment thereof, bispecific antitumor antagonist or trispecific antitumor antagonist in combination with a second antibody, antibody fragment, antagonist or drug such that an antigen-specific T cell response or apoptotic pathway is enhanced.

[0277] In a related aspect, a method of reducing or depleting regulatory T cells in a tumor of a subject in need thereof includes administering an effective amount of an antibody or antibody fragment in combination with a second antibody, antibody fragment, antagonist or drug such that the number of regulatory T cells in the subject is reduced.

[0278] In some embodiments, the subject has a cell proliferative disease or cancer as described herein.

[0279] In other embodiments, the subject has a chronic viral infection, inflammatory disease or autoimmune disease as described herein.

[0280] The provision of two distinct signals to T-cells is a widely accepted model for lymphocyte activation of resting T lymphocytes by antigen-presenting cells (APCs). This model further provides for the discrimination of self from non-self and immune tolerance. The primary signal, or antigen specific signal, is transduced through the T-cell receptor (TCR) following recognition of foreign antigen peptide presented in the context of the major histocompatibility-complex (MHC). The second or co-stimulatory signal is delivered to T-cells by co-stimulatory molecules expressed on antigen-presenting cells (APCs). This induces T-cells to promote clonal expansion, cytokine secretion and effector function. In the absence of co-stimulation, T-cells can become refractory to antigen stimulation, which results in a tolerogenic response to either foreign or endogenous antigens.

[0281] In the two-signal model, T-cells receive both positive co-stimulatory and negative co-inhibitory signals. The regulation of such positive and negative signals is critical to maximize the host's protective immune responses, while maintaining immune tolerance and preventing autoimmunity. Negative signals seem necessary for induction of T-cell tolerance, while positive signals promote T-cell activation. Both co-stimulatory and co-inhibitory signals are provided to antigen-exposed T cells, and the interplay between co-stimulatory and co-inhibitory signals is essential to controlling the magnitude of an immune response. Further, the signals provided to the T cells change as an infection or immune provocation is cleared, worsens, or persists, and these changes powerfully affect the responding T cells and re-shape the immune response.

[0282] The mechanism of co-stimulation is of therapeutic interest because the manipulation of co-stimulatory signals has shown to provide a means to either enhance or terminate cell-based immune response. Recently, it has been discovered that T cell dysfunction or energy can occur concurrently with an induced and sustained expression of immune checkpoint regulators, such as PD-1 and its ligands, PD-L1 and PD-L2. PD-L1 is overexpressed in many cancers and is often associated with poor prognosis (Thompson R H et al., Cancer Res 2006, 66(7):3381). Further, the majority of tumor infiltrating T lymphocytes predominantly express PD-1, in contrast to T lymphocytes in normal tissues and peripheral blood T lymphocytes indicating that up-regulation of PD-1 on tumor-reactive T cells can contribute to impaired antitumor immune responses (Blood 2009 114(8):1537). This may be due to exploitation of PD-L1 signaling mediated by PD-L1 expressing tumor cells interacting with PD-1 expressing T cells to result in attenuation of T cell activation and evasion of immune surveillance. Inhibition of the PD-L1/PD-1 interaction provides a means to enhance T cell immunity, including CD8+ T cell-mediated killing of cancer cells and tumors. Similar

enhancements to T cell immunity have been observed by inhibiting the binding of PD-L1 to the binding partner B7-1. Consequently, therapeutic targeting of PD-1 and other immune checkpoint regulators are an area of intense interest.

[0283] Combining inhibition of PD-1, PD-L1, TIGIT and/or LAG-3 signaling with other signaling pathways deregulated in tumor cells can provide a means for enhance treatment efficacy. In recent years, a number of immune checkpoint regulators in the form of receptors and their ligands have been identified. One important family of membrane-bound ligands that bind to co-stimulatory or co-inhibitory receptors is the B7 family, which includes CTLA-4 and its ligands, B7-1 and B7-2; PD-1 and its ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC); B7-H2 (ICOS-L), B7-H3, B7-H4, B7-H5 (VISTA), and B7-H6. Additional immune checkpoint antagonists include, but are not limited to TIM-3 and its ligand, Galectin-9; LAG-3 and its ligands, including liver sinusoidal endothelial cell lectin (LSECtin) and Galectin-3; CD122 and its CD122R ligand; CD70, B7H3, B and T lymphocyte attenuator (BTLA), and VISTA (Le Mercier et al. (2015) Front. Immunol., (6), Article 418). In addition, a number of checkpoint regulator antagonists have been identified and tested in various clinical and pre-clinical models and/or approved by the FDA (Kyi et al., FEBS Letters, 588:368-376 (2014). The concept of inhibitory receptor blockade, also known as immune checkpoint blockade, has been validated by virtue of e.g., the FDA approval of the PD-1 inhibitors, nivolumab and pembrolizumab, as well as the anti-CTLA-4 antibody, ipilimumab for metastatic melanoma.

[0284] An immune checkpoint antagonist modulates or interferes with the activity of the immune checkpoint regulator so that, as a result of the binding to the checkpoint regulator or its ligand, signaling through the checkpoint regulator receptor is blocked or inhibited. By inhibiting this signaling, immune-suppression can be reversed so that T cell immunity against cancer cells can be re-established or enhanced. In contrast, an immune checkpoint agonist (of e.g., a costimulatory molecule) stimulates the activity of an immune checkpoint regulator so that, as a result of the binding to the checkpoint regulator or its ligand, signaling through the checkpoint regulator receptor is stimulated. By stimulating this signaling, T cell immunity against cancer cells can be re-established or enhanced.

[0285] Accordingly, in one embodiment, a method for stimulating an immune response in a subject comprises administering to the subject an anti-PD-1 antibody, anti-PD-L1 antibody, anti-TIGIT antibody, anti-LAG-3 antibody, antibody fragment(s) thereof (e.g., anti-TIGIT HCVR and/LCVRs), bispecific antitumor antagonist or trispecific antitumor antagonist described herein in combination with another immune checkpoint regulator described herein above, such that an immune response is stimulated in the subject, for example to inhibit tumor growth or to stimulate an anti-viral response.

[0286] In one embodiment, an anti-PD-1 antibody, anti-PD-L1 antibody, anti-TIGIT antibody, anti-LAG-3 antibody, antibody fragment(s) thereof, bispecific antitumor antagonist or trispecific antitumor antagonist according to the present application is administered in combination with another immune checkpoint regulator, either as separate antibodies or in multi-specific antibody comprising binding specificities to multiple products. Generally, an anti-PD-1 antibody, anti-PD-L1 antibody, anti-TIGIT antibody, anti-LAG-3 antibody, bispecific antitumor antagonist or trispecific antitumor antagonist described herein can be combined to stimulate an immune response with (i) an antagonist of the IgSF family protein, B7 family or TNF family that inhibit T cell activation, or antagonist of a cytokine that inhibits T cell activation (e.g., IL-6, IL-10, TGF-β, VEGF, or other immunosuppressive cytokines) and/or (ii) an agonist of a stimulatory receptors of the IgSF family, B7 family or TNF family or of cytokines to stimulate T cell activation, for stimulating an immune response.

[0287] In certain embodiments, only subjects with a cancer exhibiting high expression of a ligand for an immune checkpoint regulator are selected for combination treatment with the anti-PD-1, anti-PD-L1, anti-TIGIT, and/or anti-LAG-3 antibody, fragment thereof, or any of the bispecific or trispecific antagonists of the present application. By way of example, in one embodiment, a subject with a cancer exhibiting high expression of PVR (CD155) and/or Nectin-2 (CD112) and/or low expression PD-L1 may be selected for monotherapy with anti-TIGIT antibodies, fragments thereof, or TIGIT antagonists of the present application, or combination therapy with a PD-1 antagonist or other immune checkpoint regulator.

[0288] The anti-PD-1 antibody, anti-PD-L1 antibody, anti-TIGIT antibody may be administered separately from the second antibody, antibody fragment or antagonist, or a multispecific antibody or antagonist may be administered comprising at least one binding specificity for TIGIT and a second binding specificity for the other targeted product. Further, the anti-PD-1 antibody, anti-PD-L1 antibody, anti-TIGIT or bispecific or trispecific antagonists in accordance with the present application may be co-administered with one or more additional agents, e.g., antibodies, antagonists, or drugs in amount(s) effective in stimulating an immune response and/or apoptosis so as to further enhance, stimulate or up-regulate an immune response and/or apoptosis in a subject.

[0289] In some embodiments, the anti-PD-1, anti-PD-L1, anti-TIGIT or anti-LAG-3 antibody or fragment(s) thereof is administered subsequent to treatment with a different antitumor antagonist. For example, in one embodiment, anti-PD-1, anti-PD-L1, anti-TIGIT or anti-LAG-3 antibodies may be administered only after treatment with a PD-1/PD-L1 antagonist has failed, has led to incomplete therapeutic response, or there has been recurrence of the tumor or relapse (or "PD-1 failure"). In some embodiments, cancers exhibiting such failures may be

screened for expression of e.g., PVR and/or Nectin-2 and only those having high level expression are treated with an anti-PD-1, anti-PD-L1, anti-TIGIT or anti-LAG-3 antibody, fragment or antagonist of the present application.

[0290] Other anti-PD-1 antibodies include, but are not limited to, nivolumab (BMS-936558, MDX-1106, OPDIVO™), a humanized immunoglobulin G4 (IgG4) mAb (Bristol-Myers Squibb); pembrolizumab (MK-3475, lambrolizumab, Keytruda™)(Merck); pidilizumab (CT-011)(Medivation); and AMP-224 (Merck). Anti-PD-1 antibodies are commercially available, for example from ABCAM (AB137132), BIOLEGEND™ (EH12.2H7, RMP1-14) and AFFYMETRIX EBIOSCIENCE (J105, J116, MIH4).

[0291] Other anti-PD-L1 antibodies include atezolizumab (MPDL3280A, RG7446), a fully human IgG4 mAb Genentech/Roche); BMS-936559 (MDX-1105), a fully humanized IgG4 mAb (Bristol-Myers Squibb); MEDI4736, a humanized IgG antibody (Medimmune/AstraZeneca); and MSB0010718C, a fully human IgG4 monoclonal antibody (Merck, EMD Serono).

[0292] Exemplary anti-CTLA-4 antibodies for use in accordance with the present methods include ipilimumab, trevilizumab and tremelimumab.

[0293] In certain embodiments, the antitumor antagonist is a dominant negative protein of the immune checkpoint regulator. In particular embodiments, the dominant negative protein comprises an extracellular domain derived from a member selected from the group consisting of PD-L1, PD-L2, PD-1, B7-1, B7-2, B7H3, CTLA-4, LAG-3, TIM-3, TIGIT, BTLA, VISTA, CD70, and combinations thereof. In certain particular embodiments, these extracellular domains are fused to an immunoglobulin constant region or Fc receptor in the presently described antibodies. Such mutants can bind to the endogenous receptor so as to form a complex that is deficient in signaling. In certain embodiments, the extracellular domain is fused to an immunoglobulin constant region or Fc fragment or to a monomer in the oligomeric protein complex.

[0294] In certain embodiments, a dominant negative PD-L1 antagonist comprises the extracellular domain of PD-L1, PD-L2 or PD-1. In another embodiment, a dominant-negative PD-1 antagonist is employed, which has a mutation so that it is no longer able to bind PD-L1. An exemplary dominant negative protein is AMP-224 (co-developed by Glaxo Smith Kline and Amplimmune), a recombinant fusion protein comprising the extracellular domain of PD-L2 and the Fc region of human IgG.

[0295] Exemplary immune checkpoint regulator agonists include, but are not limited to members of the tumor necrosis factor (TNF) receptor superfamily, such as CD27, CD40, OX40, GITR and 4-1BB (CD137) and their ligands, or members of the B7-CD28 superfamily,

including CD28 and ICOS (CD278). Additional checkpoint regulator agonists include CD2, CDS, ICAM-1, LFA-1 (CD11a/CD18), CD30, BAFFR, HVEM, CD7, LIGHT, NKG2C, SLAMF7, NKp80, CD160, B7-H3, CD83 ligand. Immune checkpoint antagonists can include antibodies or soluble fusion protein agonists comprising one or more co-stimulatory domains. Agonist antibodies include, but are not limited to anti-CD40 mAbs, such as CP-870,893, lucatumumab, and dacetuzumab; anti-CD137 mAbs, such as BMS-663513 urelumab, and PF-05082566; anti-OX40 mAbs; anti-GITR mAbs, such as TRX518; anti-CD27 mAbs, such as CDX-1127; and anti-ICOS mAbs.

[0296] Exemplary GITR agonists include, e.g., GITR fusion proteins and anti-GITR antibodies (e.g., bivalent anti-GITR antibodies) such as, e.g., a GITR fusion protein described in U.S. Patent Nos. 6,111,090 and 8,586,023; European Patent No.: 090505B1, U.S. Patent No. PCT Publication Nos.: WO 2010/003118 and 2011/090754. Anti-GITR antibodies are described in, e.g., in U.S. Patent Nos. 7,025,962, 7,618,632, 7,812,135, 8,388,967, and 8,591,886; European Patent Nos.: 1947183B1 and 1866339; PCT Publication Nos.: WO 2011/028683, WO 2013/039954, WO2005/007190, WO 2007/133822, WO2005/055808, WO 99/40196, WO 2001/03720, WO99/20758, WO2006/083289, WO 2005/115451, WO 2011/051726. An exemplary anti-GITR antibody is TRX518.

[0297] Another family of membrane bound ligands that bind to co-stimulatory or co-inhibitory receptors is the TNF family of molecules that bind to cognate TNF receptor family members, which include CD40 and CD40L, OX-40, OX-40L, CD70, CD27L, CD30, CD30L, 4-1BBL, CD137/4-1BB, TRAIL/Apo2-L, TRAILR1/DR4, TRAILR2/DR5, TRAILR3, TRAILR4, OPG, RANK, RANKL, TWEAKR/Fn14, TWEAK, BAFFR, EDAR, XEDAR, TACI, APRIL, BCMA, LTβR, LIGHT, DcR3, HVEM, VEGI/TL1A, TRAMP/DR3, EDAR, EDA1, XEDAR, EDA2, TNFR1, Lymphotoxin α/TNF γ, TNFR2, TNFα, LTβR, Lymphotoxin α 1(32, FAS, FASL, RELT, DR6, TROY, NGFR (see, e.g., Tansey, M.G. et al. (2009) Drug Discovery Today, 14(23-24):1082-1088).

[0298] Immune checkpoint agonists or co-stimulatory molecules include cell surface molecules other than antigen receptors or their ligands that are required for an efficient immune response, and include, but are not limited to MHC class I molecules, MHC class II molecules, TNF receptor proteins, immunoglobulin-like proteins, cytokine receptors, integrins, signaling lymphocytic activation molecules (SLAM proteins), activating NK cell receptors, BTLA, Toll ligand receptor, OX40, CD2, CD7, CD27, CD28, CD30, CD40, CDS, ICAM-1, LFA-1 (CD11a/CD18), 4-1BB (CD137), B7-H3, CDS, ICAM-1, ICOS (CD278), GITR, BAFFR, LIGHT, HVEM (LIGHTR), KIRDS2, SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a,

ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, NKG2C, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, and a ligand that specifically binds with CD83.

[0299] In one aspect, T cell responses can be stimulated by a combination of the anti-PD-1, anti-PD-L1, anti-TIGIT or anti-LAG-3 mAbs of the present application and one or more of (i) an antagonist of a protein that inhibits T cell activation (e.g., immune checkpoint inhibitors), such as CTLA-4, PD-1, PD-L1, PD-L2, LAG-3, TIM-3, Galectin 9, CEACAM-1, BTLA, CD69, Galectin-1, CD113, GPR56, VISTA, 2B4, CD48, GARP, PD-1H, LAIR1, TIM-1, CD96 and TIM-4, and (ii) an agonist of a protein that stimulates T cell activation such as B7-1, B7-2, CD28, 4-1BB (CD137), 4-1BBL, ICOS, CD40, ICOS-L, OX40, OX40L, GITR, GITRL, CD70, CD27, CD40, DR3 and CD28H.

[0300] Exemplary agents that modulate one of the above proteins and may be combined with the anti-PD-1 antibodies, anti-PD-L1 antibodies, anti-TIGIT antibodies and/or anti-LAG-3 antibodies of the present application for treating cancer, include: YERVOY™/ipilimumab or tremelimumab (to CTLA-4), galiximab (to B7.1), OPDIVO™/nivolumab/BMS-936558 (to PD-1), pidilizumab/CT-011 (to PD-1), KEYTRUDA™/pembrolizumab/MK-3475 (to PD-1), AMP224 (to B7-DC/PD-L2), BMS-936559 (to B7-H1), MPDL3280A (to B7-H1), MEDI-570 (to ICOS), AMG557 (to B7H2), MGA271 (to B7H3), IMP321 (to LAG-3), urelumab/BMS-663513 and PF-05082566 (to CD137/4-1BB), CDX-1127 (to CD27), MEDI-6383 and MEDI-6469 (to OX40), RG-7888 (to OX40L), Atacicept (to TACI), CP-870893 (to CD40), lucatumumab (to CD40), dacetuzumab (to CD40), and muromonab-CD3 (to CD3).

[0301] Other molecules that can be combined with the antitumor antagonists described herein for the treatment of cancer include antagonists of inhibitory receptors on NK cells or agonists of activating receptors on NK cells. For example, antagonist anti-PD-1, anti-PD-L1 and/or anti-TIGIT antibodies can be combined with antagonists of KIR (e.g., lirilumab), CSF-1R antagonists, such as RG7155.

[0302] Tumors evade host immune surveillance by a large variety of mechanisms. Many of these mechanisms may be overcome by the inactivation of immunosuppressive proteins expressed by the tumors. These include among others TGF-β, IL-10, and Fas ligand. Antibodies to each of these entities can be used in combination with the antitumor antagonists

described herein to counteract the effects of the immunosuppressive agent and favor tumor immune responses by the host.

[0303] Other antibodies that activate host immune responsiveness can be used in combination with the antitumor antagonists described herein. These include molecules on the surface of dendritic cells that activate DC function and antigen presentation. Anti-CD40 antibodies are able to substitute effectively for T cell helper activity and can be used in conjunction with the antitumor antagonists described herein. Activating antibodies to T cell costimulatory molecules such as OX-40, CD137/4-1BB, and ICOS may also provide for increased levels of T cell activation.

[0304] In certain embodiments, the antitumor antagonists described herein can be co-administered with one or other more therapeutic agents, e.g., anti-cancer agents, radiotoxic agents or an immunosuppressive agent. Such co-administration can solve problems due to development of resistance to drugs, changes in the antigenicity of the tumor cells that would render them unreactive to the antibody, and toxicities (by administering lower doses of one or more agents).

[0305] The antitumor antagonists described herein can be chemically linked to the agent (as an immuno-complex) or can be administered separate from the agent. In the latter case (separate administration), the antibody can be administered before, after or concurrently with the agent or can be co-administered with other known therapies, e.g., an anti-cancer therapy, e.g., radiation. The antitumor antagonists described herein may be co-administered with one or more anti-cancer agents so as to provide two anti-cancer agents operating synergistically via different mechanisms to yield a cytotoxic effect in human cancer cells.

[0306] The antitumor antagonists described herein may be combined with an anti-cancer agent, such an alkylating agent; an anthracycline antibiotic; an anti-metabolite; a detoxifying agent; an interferon; a polyclonal or monoclonal antibody; an EGFR inhibitor; a HER2 inhibitor; a histone deacetylase inhibitor; a hormone; a mitotic inhibitor; a phosphatidylinositol-3-kinase (PI3K) inhibitor; an Akt inhibitor; a mammalian target of rapamycin (mTOR) inhibitor; a proteasomal inhibitor; a poly(ADP-ribose) polymerase (PARP) inhibitor; a Ras/MAPK pathway inhibitor; a centrosome declustering agent; a multi-kinase inhibitor; a serine/threonine kinase inhibitor; a tyrosine kinase inhibitor; a VEGF/VEGFR inhibitor; a taxane or taxane derivative, an aromatase inhibitor, an anthracycline, a microtubule targeting drug, a topoisomerase poison drug, an inhibitor of a molecular target or enzyme (e.g., a kinase or a protein methyltransferase), a cytidine analogue or combination thereof.

[0307] Exemplary alkylating agents include, but are not limited to, cyclophosphamide (Cytoxan; Neosar); chlorambucil (Leukeran); melphalan (Alkeran); carmustine (BiCNU);

busulfan (Busulfex); lomustine (CeeNU); dacarbazine (DTIC-Dome); oxaliplatin (Eloxatin); carmustine (Gliadel); ifosfamide (Ifex); mechlorethamine (Mustargen); busulfan (Myleran); carboplatin (Paraplatin); cisplatin (CDDP; Platinol); temozolomide (Temodar); thiotepa (Thioplex); bendamustine (Treanda); or streptozocin (Zanosar).

[0308] Exemplary anthracycline antibiotics include, but are not limited to, doxorubicin (Adriamycin); doxorubicin liposomal (Doxil); mitoxantrone (Novantrone); bleomycin (Blenoxane); daunorubicin (Cerubidine); daunorubicin liposomal (DaunoXome); dactinomycin (Cosmegen); epirubicin (Ellence); idarubicin (Idamycin); plicamycin (Mithracin); mitomycin (Mutamycin); pentostatin (Nipent); or valrubicin (Valstar).

[0309] Exemplary anti-metabolites include, but are not limited to, fluorouracil (Adrucil); capecitabine (Xeloda); hydroxyurea (Hydrea); mercaptopurine (Purinethol); pemetrexed (Alimta); fludarabine (Fludara); nelarabine (Arranon); cladribine (Cladribine Novaplus); clofarabine (Clolar); cytarabine (Cytosar-U); decitabine (Dacogen); cytarabine liposomal (DepoCyt); hydroxyurea (Droxia); pralatrexate (Folotyn); floxuridine (FUDR); gemcitabine (Gemzar); cladribine (Leustatin); fludarabine (Oforta); methotrexate (MTX; Rheumatrex); methotrexate (Trexall); thioguanine (Tabloid); TS-1 or cytarabine (Tarabine PFS).

[0310] Exemplary detoxifying agents include, but are not limited to, amifostine (Ethyol) or mesna (Mesnex).

[0311] Exemplary interferons include, but are not limited to, interferon alfa-2b (Intron A) or interferon alfa-2a (Roferon-A).

[0312] Exemplary polyclonal or monoclonal antibodies include, but are not limited to, trastuzumab (Herceptin); ofatumumab (Arzerra); bevacizumab (Avastin); rituximab (Rituxan); cetuximab (Erbitux); panitumumab (Vectibix); tositumomab/iodine131 tositumomab (Bexxar); alemtuzumab (Campath); ibritumomab (Zevalin; In-111; Y-90 Zevalin); gemtuzumab (Mylotarg); eculizumab (Soliris) ordenosumab.

[0313] Exemplary EGFR inhibitors include, but are not limited to, gefitinib (Iressa); lapatinib (Tykerb); cetuximab (Erbitux); erlotinib (Tarceva); panitumumab (Vectibix); PKI-166; canertinib (CI-1033); matuzumab (Emd7200) or EKB-569.

[0314] Exemplary HER2 inhibitors include, but are not limited to, trastuzumab (Herceptin); lapatinib (Tykerb) or AC-480.

[0315] Exemplary histone deacetylase inhibitors include, but are not limited to, vorinostat (Zolinza), valproic acid, romidepsin, entinostat abexinostat, givinostat, and mocetinostat.

[0316] Exemplary hormones include, but are not limited to, tamoxifen (Soltamox; Nolvadex); raloxifene (Evista); megestrol (Megace); leuprolide (Lupron; Lupron Depot; Eligard;

Viadur); fulvestrant (Faslodex); letrozole (Femara); triptorelin (Trelstar LA; Trelstar Depot); exemestane (Aromasin); goserelin (Zoladex); bicalutamide (Casodex); anastrozole (Arimidex); fluoxymesterone (Androxy; Halotestin); medroxyprogesterone (Provera; Depo-Provera); estramustine (Emcyt); flutamide (Eulexin); toremifene (Fareston); degarelix (Firmagon); nilutamide (Nilandron); abarelix (Plenaxis); or testolactone (Teslac).

[0317] Exemplary mitotic inhibitors include, but are not limited to, paclitaxel (Taxol; Onxol; Abraxane); docetaxel (Taxotere); vincristine (Oncovin; Vincasar PFS); vinblastine (Velban); etoposide (Toposar; Etopophos; VePesid); teniposide (Vumon); ixabepilone (Ixempra); nocodazole; epothilone; vinorelbine (Navelbine); camptothecin (CPT); irinotecan (Camptosar); topotecan (Hycamtin); amsacrine or lamellarin D (LAM-D).

[0318] Exemplary phosphatidyl-inositol-3 kinase (PI3K) inhibitors include wortmannin an irreversible inhibitor of PI3K, demethoxyviridin a derivative of wortmannin, LY294002, a reversible inhibitor of PI3K; BKM120 (Buparlisib); Idelalisib (a PI3K Delta inhibitor); duvelisib (IPI-145, an inhibitor of PI3K delta and gamma); alpelisib (BYL719), an alpha-specific PI3K inhibitor; TGR 1202 (previously known as RP5264), an oral PI3K delta inhibitor; and copanlisib (BAY 80-6946), an inhibitor PI3Kα,δ isoforms predominantly.

[0319] Exemplary Akt inhibitors include, but are not limited to miltefosine, AZD5363, GDC-0068, MK2206, Perifosine, RX-0201, PBI-05204, GSK2141795, and SR13668.

[0320] Exemplary MTOR inhibitors include, but are not limited to, everolimus (Afinitor) or temsirolimus (Torisel); rapamune, ridaforolimus; deforolimus (AP23573), AZD8055 (AstraZeneca), OSI-027 (OSI), INK-128, BEZ235, PI-103, Torin1, PP242, PP30, Ku-0063794, WAY-600, WYE-687, WYE-354, and CC-223.

[0321] Exemplary proteasomal inhibitors include, but are not limited to, bortezomib (PS-341), ixazomib (MLN 2238), MLN 9708, delanzomib (CEP-18770), carfilzomib (PR-171), YU101, oprozomib (ONX-0912), marizomib (NPI-0052), and disufiram.

[0322] Exemplary PARP inhibitors include, but are not limited to, olaparib, iniparib, velaparib, BMN-673, BSI-201, AG014699, ABT-888, GPI21016, MK4827, INO-1001, CEP-9722, PJ-34, Tiq-A, Phen, PF-01367338 and combinations thereof.

[0323] Exemplary Ras/MAPK pathway inhibitors include, but are not limited to, trametinib, selumetinib, cobimetinib, CI-1040, PD0325901, AS703026, RO4987655, RO5068760, AZD6244, GSK1120212, TAK-733, U0126, MEK162, and GDC-0973.

[0324] Exemplary centrosome declustering agents include, but are not limited to, griseofulvin; noscapine, noscapine derivatives, such as brominated noscapine (e.g., 9-bromonoscapine), reduced bromonoscapine (RBN), N-(3-brormobenzyl) noscapine, aminonoscapine and water-soluble derivatives thereof; CW069; the phenanthridene-derived

poly(ADP-ribose) polymerase inhibitor, PJ-34; N2-(3-pyridylmethyl)-5-nitro-2-furamide, N2-(2-thienylmethyl)-5-nitro-2-furamide, and N2-benzyl-5-nitro-2-furamide.

[0325] Exemplary multi-kinase inhibitors include, but are not limited to, regorafenib; sorafenib (Nexavar); sunitinib (Sutent); BIBW 2992; E7080; Zd6474; PKC-412; motesanib; or AP24534.

[0326] Exemplary serine/threonine kinase inhibitors include, but are not limited to, ruboxistaurin; eril/easudil hydrochloride; flavopiridol; seliciclib (CYC202; Roscovitrine); SNS-032 (BMS-387032); Pkc412; bryostatin; KAI-9803; SF1126; VX-680; Azd1152; Arry-142886 (AZD-6244); SCIO-469; GW681323; CC-401; CEP-1347 or PD 332991.

[0327] Exemplary tyrosine kinase inhibitors include, but are not limited to, erlotinib (Tarceva); gefitinib (Iressa); imatinib (Gleevec); sorafenib (Nexavar); sunitinib (Sutent); trastuzumab (Herceptin); bevacizumab (Avastin); rituximab (Rituxan); lapatinib (Tykerb); cetuximab (Erbitux); panitumumab (Vectibix); everolimus (Afinitor); alemtuzumab (Campath); gemtuzumab (Mylotarg); temsirolimus (Torisel); pazopanib (Votrient); dasatinib (Sprycel); nilotinib (Tasigna); vatalanib (Ptk787; ZK222584); CEP-701; SU5614; MLN518; XL999; VX-322; Azd0530; BMS-354825; SKI-606 CP-690; AG-490; WHI-P154; WHI-P131; AC-220; or AMG888.

[0328] Exemplary VEGF/VEGFR inhibitors include, but are not limited to, bevacizumab (Avastin); sorafenib (Nexavar); sunitinib (Sutent); ranibizumab; pegaptanib; or vandetinib.

[0329] Exemplary microtubule targeting drugs include, but are not limited to, paclitaxel, docetaxel, vincristin, vinblastin, nocodazole, epothilones and navelbine.

[0330] Exemplary topoisomerase poison drugs include, but are not limited to, teniposide, etoposide, adriamycin, camptothecin, daunorubicin, dactinomycin, mitoxantrone, amsacrine, epirubicin and idarubicin.

[0331] Exemplary taxanes or taxane derivatives include, but are not limited to, paclitaxel and docetaxol.

[0332] Exemplary general chemotherapeutic, anti-neoplastic, anti-proliferative agents include, but are not limited to, altretamine (Hexalen); isotretinoin (Accutane; Amnesteem; Claravis; Sotret); tretinoin (Vesanoid); azacitidine (Vidaza); bortezomib (Velcade) asparaginase (Elspar); levamisole (Ergamisol); mitotane (Lysodren); procarbazine (Matulane); pegaspargase (Oncaspar); denileukin diftitox (Ontak); porfimer (Photofrin); aldesleukin (Proleukin); lenalidomide (Revlimid); bexarotene (Targretin); thalidomide (Thalomid); temsirolimus (Torisel); arsenic trioxide (Trisenox); verteporfin (Visudyne); mimosine (Leucenol); (1M tegafur-0.4 M 5-chloro-2,4-dihydroxypyrimidine-1 M potassium oxonate) or lovastatin.

[0333] In certain embodiments, the antitumor antagonists described herein are administered at a subtherapeutic dose, another anti-immune checkpoint regulator antibody or antagonist is administered at a subtherapeutic dose, the angiogenesis antagonist is administered at a subtherapeutic dose, or any antagonist in a combination thereof is each administered at a subtherapeutic dose.

[0334] In certain embodiments, PD-1, PD-L1, TIGIT and/or LAG-3 inhibition is combined with standard cancer treatments (e.g., surgery, radiation, and chemotherapy). PD-1, PD-L1, TIGIT and/or LAG-3 inhibition can be effectively combined with chemotherapeutic regimes. In these instances, it may be possible to reduce the dose of chemotherapeutic reagent administered. An example of such a combination is an anti-TIGIT, anti-PD-1, anti-PD-L1 or anti-LAG-3 antibody in combination with decarbazine for the treatment of melanoma. Another example of such a combination is an anti-PD-1, anti-PD-L1, anti-TIGIT or anti-LAG-3 antibody in combination with interleukin-2 (IL-2) for the treatment of melanoma. It is believed that the combined use of PD-1, PD-L1, TIGIT and/or LAG-3 inhibition and chemotherapy can enhance apoptosis and increase tumor antigen presentation for cytotoxic immunity. Other synergistic combination therapies include PD-1, PD-L1, TIGIT and/or LAG-3 inhibition through cell death when used in combination with radiation, surgery or hormone deprivation. Each of these protocols creates a source of tumor antigen in the host.

[0335] In certain embodiments, the checkpoint regulator antagonists described herein can be used in multi-specific antagonists or in combination with bispecific antibodies targeting Fcα or Fcγ receptor-expressing effector cells to tumor cells (see, e.g., U.S. Patent Nos. 5,922,845 and 5,837,243). Bispecific antibodies can be used to target two separate antigens. For example anti-Fc receptor/anti-tumor antigen (e.g., Her-2/neu) bispecific antibodies have been used to target macrophages to cancer cells or tumors. This targeting may more effectively activate tumor specific responses. The T cell arm of these responses would be augmented by the inhibition of TIGIT, PD-1, PD-L1 and/or LAG-3. Alternatively, antigen may be delivered directly to DCs by the use of bispecific antibodies that bind to tumor antigen and a dendritic cell specific cell surface marker.

VI. Methods of Making the Antitumor Antagonists

A. Nucleic Acids and Host Cells for Expressing Antagonists

[0336] In one aspect, the present application provides nucleic acids encoding the antitumor antagonist of the present application, and expression vectors comprising such nucleic acids. In some embodiments, nucleic acids encodes an HCVR and/or LCVR fragment of an antibody or fragment in accordance with the embodiments described herein, or any of the other antibodies and antibody fragments described herein.

[0337] DNA encoding an antigen binding site in a monoclonal antibody can be isolated and sequenced from the hybridoma cells using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). Alternatively, amino acid sequences from immunoglobulins of interest may be determined by direct protein sequencing, and suitable encoding nucleotide sequences can be designed according to a universal codon table. In other cases, nucleotide and amino acid sequences of antigen binding sites or other immunoglobulin sequences, including constant regions, hinge regions and the like may be obtained from published sources well known in the art.

[0338] Expression vectors encoding a particular monospecific, bispecific or trispecific antitumor antagonist may be used to synthesize the antitumor antagonists of the present disclosure in cultured cells in vitro or they may be directly administered to a patient to express the antitumor antagonist in vivo or ex vivo. As used herein, an "expression vector" refers to a viral or non-viral vector comprising a polynucleotide encoding one or more polypeptide chains corresponding to the monospecific, bispecific or trispecific antitumor antagonists of the present disclosure in a form suitable for expression from the polynucleotide(s) in a host cell for antibody preparation purposes or for direct administration as a therapeutic agent.

[0339] A nucleic acid sequence is "operably linked" to another nucleic acid sequence when the former is placed into a functional relationship with the latter. For example, a DNA for a presequence or signal peptide is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a signal peptide, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, synthetic oligonucleotide adaptors or linkers may be used in accordance with conventional practice.

[0340] Nucleic acid sequences for expressing the antitumor antagonists typically include an amino terminal signal peptide sequence, which is removed from the mature protein. Since the signal peptide sequences can affect the levels of expression, the polynucleotides may encode any one of a variety of different N-terminal signal peptide sequences. It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like.

[0341] The above described "regulatory sequences" refer to DNA sequences necessary for the expression of an operably linked coding sequence in one or more host organisms. The term "regulatory sequences" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cells or those which direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). Expression vectors generally contain sequences for transcriptional termination, and may additionally contain one or more elements positively affecting mRNA stability.

[0342] The expression vector contains one or more transcriptional regulatory elements, including promoters and/or enhancers, for directing the expression of antitumor antagonists. A promoter comprises a DNA sequence that functions to initiate transcription from a relatively fixed location in regard to the transcription start site. A promoter contains core elements required for basic interaction of RNA polymerase and transcription factors, and may operate in conjunction with other upstream elements and response elements.

[0343] As used herein, the term "promoter" is to be taken in its broadest context and includes transcriptional regulatory elements (TREs) from genomic genes or chimeric TREs therefrom, including the TATA box or initiator element for accurate transcription initiation, with or without additional TREs (i.e., upstream activating sequences, transcription factor binding sites, enhancers, and silencers) which regulate activation or repression of genes operably linked thereto in response to developmental and/or external stimuli, and trans-acting regulatory proteins or nucleic acids. A promoter may contain a genomic fragment or it may contain a chimera of one or more TREs combined together.

[0344] Preferred promoters are those capable of directing high-level expression in a target cell of interest. The promoters may include constitutive promoters (e.g., HCMV, SV40, elongation factor-1α (EF-1α)) or those exhibiting preferential expression in a particular cell type of interest. Enhancers generally refer to DNA sequences that function away from the transcription start site and can be either 5' or 3' to the transcription unit. Furthermore, enhancers can be within an intron as well as within the coding sequence. They are usually between 10 and 300 bp in length, and they function in cis. Enhancers function to increase and/or regulate transcription from nearby promoters. Preferred enhancers are those directing high-level expression in the antibody producing cell. Cell or tissue-specific transcriptional regulatory elements (TREs) can be incorporated into expression vectors to restrict expression to desired cell types. Pol III promoters (H1 or U6) are particularly useful for expressing shRNAs from which

siRNAs are expressed. An expression vector may be designed to facilitate expression of the antitumor antagonist in one or more cell types.

[0345] In certain embodiments, one or more expression vectors may be engineered to express both the antitumor antagonist and one or more siRNA targeting the Tie2 pathway, the VEGF pathway or an immune checkpoint regulator.

[0346] An siRNA is a double-stranded RNA that can be engineered to induce sequence-specific post-transcriptional gene silencing of mRNAs. Synthetically produced siRNAs structurally mimic the types of siRNAs normally processed in cells by the enzyme Dicer. When expressed from an expression vector, the expression vector is engineered to transcribe a short double-stranded hairpin-like RNA (shRNA) that is processed into a targeted siRNA inside the cell. Synthetic siRNAs and shRNAs may be designed using well known algorithms and synthesized using a conventional DNA/RNA synthesizer.

[0347] To co-express the individual chains of the antitumor antagonist, a suitable splice donor and splice acceptor sequences may be incorporated for expressing both products. Alternatively, an internal ribosome binding sequence (IRES) or a 2A peptide sequence, may be employed for expressing multiple products from one promoter. An IRES provides a structure to which the ribosome can bind that does not need to be at the 5' end of the mRNA. It can therefore direct a ribosome to initiate translation at a second initiation codon within a mRNA, allowing more than one polypeptide to be produced from a single mRNA. A 2A peptide contains short sequences mediating co-translational self-cleavage of the peptides upstream and downstream from the 2A site, allowing production of two different proteins from a single transcript in equimolar amounts. CHYSEL is a non-limiting example of a 2A peptide, which causes a translating eukaryotic ribosome to release the growing polypeptide chain that it is synthesizing without dissociating from the mRNA. The ribosome continues translating, thereby producing a second polypeptide.

[0348] An expression vector may comprise a viral vector or a non-viral vector. A viral vectors may be derived from an adeno-associated virus (AAV), adenovirus, herpesvirus, vaccinia virus, poliovirus, poxvirus, a retrovirus (including a lentivirus, such as HIV-1 and HIV-2), Sindbis and other RNA viruses, alphavirus, astrovirus, coronavirus, orthomyxovirus, papovavirus, paramyxovirus, parvovirus, picornavirus, togaviruses and the like. A non-viral vector is simply a "naked" expression vector that is not packaged with virally derived components (e.g., capsids and/or envelopes).

[0349] In certain cases, these vectors may be engineered to target certain diseases or cell populations by using the targeting characteristics inherent to the virus vector or engineered into the virus vector. Specific cells may be "targeted" for delivery of polynucleotides, as well as

expression. Thus, the term "targeting", in this case, may be based on the use of endogenous or heterologous binding agents in the form of capsids, envelope proteins, antibodies for delivery to specific cells, the use of tissue-specific regulatory elements for restricting expression to specific subset(s) of cells, or both.

[0350] In some embodiments, expression of the antibody chains is under the control of the regulatory element such as a tissue specific or ubiquitous promoter. In some embodiments, a ubiquitous promoter such as a CMV promoter, CMV-chicken beta-actin hybrid (CAG) promoter, a tissue specific or tumor-specific promoter to control the expression of a particular antibody heavy or light chain or single-chain derivative therefrom.

[0351] Non-viral expression vectors can be utilized for non-viral gene transfer, either by direct injection of naked DNA or by encapsulating the antitumor antagonist-encoding polynucleotides in liposomes, microparticles, microcapsules, virus-like particles, or erythrocyte ghosts. Such compositions can be further linked by chemical conjugation to targeting domains to facilitate targeted delivery and/or entry of nucleic acids into desired cells of interest. In addition, plasmid vectors may be incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, and chemically linked to cell targeting ligands such as asialoorosomucoid, insulin, galactose, lactose or transferrin.

[0352] Alternatively, naked DNA may be employed. Uptake efficiency of naked DNA may be improved by compaction or by using biodegradable latex beads. Such delivery may be improved further by treating the beads to increase hydrophobicity and thereby facilitate disruption of the endosome and release of the DNA into the cytoplasm.

B. Methods for Producing Monospecific or Multispecific Antibodies

[0353] In one aspect, the present application provides host cells transformed with the anti-PD-1, anti-PD-L1, anti-TIGIT, and/or anti-LAG-3 HCVRs and/or LCVRs, encoding nucleic acids or expression vectors, or nucleic acids/expression vectors encoding the bi-specific and/or trispecific antitumor antagonist of the present application. The host cells can be any bacterial or eukaryotic cell capable of expressing the anti-PD-1, anti-PD-L1, anti-TIGIT and/or anti-LAG-3 HCVRs and/or LCVRs encoding nucleic acids or expression vectors or any of the other co-administered antibodies or antagonists described herein.

[0354] In another aspect, a method of producing an antitumor antagonist comprises culturing a host cell transformed with one or more anti-PD-1, anti-PD-L1 anti-TIGIT, and/or anti-LAG-3 HCVRs and/or LCVRs encoding nucleic acids or expression vectors under conditions that allows production of the antibody or fragment, and purifying the antibody from the cell.

[0355] In a further aspect, the present application provides a method for producing an antibody comprising culturing a cell transiently or stably expressing one or more constructs encoding one or more polypeptide chains in the antibody; and purifying the antibody from the cultured cells. Any cell capable of producing a functional antibody may be used. In preferred embodiments, the antibody-expressing cell is of eukaryotic or mammalian origin, preferably a human cell. Cells from various tissue cell types may be used to express the antibodies. In other embodiments, the cell is a yeast cell, an insect cell or a bacterial cell. Preferably, the antibody-producing cell is stably transformed with a vector expressing the antibody.

[0356] One or more expression vectors encoding the antibody heavy or light chains can be introduced into a cell by any conventional method, such as by naked DNA technique, cationic lipid-mediated transfection, polymer-mediated transfection, peptide-mediated transfection, virus-mediated infection, physical or chemical agents or treatments, electroporation, etc. In addition, cells may be transfected with one or more expression vectors for expressing the antibody along with a selectable marker facilitating selection of stably transformed clones expressing the antibody. The antibodies produced by such cells may be collected and/or purified according to techniques known in the art, such as by centrifugation, chromatography, etc.

[0357] Examples of suitable selectable markers for mammalian cells include dihydrofolate reductase (DHFR), thymidine kinase, neomycin, neomycin analog G418, hydromycin, and puromycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented media. Two examples are CHO DHFR- cells and mouse LTK- cells. These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented media. An alternative to supplementing the media is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in non-supplemented media.

[0358] The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin, mycophenolic acid, or hygromycin. The three examples

employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively. Others include the neomycin analog G418 and puromycin.

[0359] Exemplary antibody-expressing cells include human Jurkat, human embryonic kidney (HEK) 293, Chinese hamster ovary (CHO) cells, mouse WEHI fibrosarcoma cells, as well as unicellular protozoan species, such as Leishmania tarentolae. In addition, stably transformed, antibody producing cell lines may be produced using primary cells immortalized with c-myc or other immortalizing agents.

[0360] In one embodiment, the cell line comprises a stably transformed Leishmania cell line, such as Leishmania tarentolae. Leishmania are known to provide a robust, fast-growing unicellular host for high level expression of eukaryotic proteins exhibiting mammalian-type glycosylation patterns. A commercially available Leishmania eukaryotic expression kit is available (Jena Bioscience GmbH, Jena, Germany).

[0361] In some embodiments, the cell line expresses at least 1 mg, at least 2 mg, at least 5 mg, at least 10 mg, at least 20 mg, at least 50 mg, at least 100 mg, at least 200 mg, at least 300 mg, at least 400 mg, or at least 500 mg of the antibody/liter of culture.

[0362] The antibodies in the present application may be isolated from antibody expressing cells following culture and maintenance in any appropriate culture medium, such as RPMI, DMEM, and AIM V®. The antibodies can be purified using conventional protein purification methodologies (e.g., affinity purification, chromatography, etc.), including the use of Protein-A or Protein-G immunoaffinity purification. In some embodiments, antibodies are engineered for secretion into culture supernatants for isolation therefrom.

[0363] The present application is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures and Tables are incorporated herein by reference.

C. Homodimers and Heterodimers

[0364] One of the challenges for efficiently producing bispecific and trispecific antibody preparations concerns mispairing of heavy and light chains, when co-expressing chains of different binding specificities. Table 1 lists several amino acid substitution options for overcoming mispairing between heavy chains of different binding specificities, which "enforce" or preferentially promote correct association between desired heavy chains. Any approach to prevent or reduce mispairing between heavy chains may be used to make the bispecific trispecific antitumor antagonists according to the present disclosure.

[0365] The "knobs-into-hole" (KiH) approach relies on modifications of the interface between the two CH3 domains where most interactions occur. Typically, a bulky residue is introduced into the CH3 domain of one antibody heavy chain and acts similarly to a key. In the other heavy chain, a "hole" is formed that is able to accommodate this bulky residue, mimicking a lock. The resulting heterodimeric Fc-part can be further stabilized by artificial disulfide bridges.

[0366] An alternative approach is based on charged residues with ionic interactions or steric complementarity. This includes altering the charge polarity in the CH3 interface so that co-expression of electrostatically matched Fc domains support favorable attractive interactions and heterodimer formation while retaining the hydrophobic core, whereas unfavorable repulsive charge interactions suppress homodimerization. See Table 1. The amino acid numbering in Table 1 follows the Kabat numbering scheme and can be applied to heavy chain amino acid sequences of the antibodies described herein.

[0367] In a further approach, leucine zipper (LZ) domains may be incorporated into a protein scaffold. A leucine zipper is a common three-dimensional structural motif in proteins, typically as part of a DNA-binding domain in various transcription factors. A single LZ typically contains 4-5 leucine residues at approximately 7-residue intervals, which forms an amphipathic alpha helix with a hydrophobic region running along one side. In a particular embodiment, a heterodimeric protein scaffold comprises a LZ from the c-jun transcription factor associated with a LZ from the c-fos transcription factor. Although c-jun is known to form jun-jun homodimers and c-fos does not form homodimers, the formation of jun-fos heterodimers is greatly favored over jun-jun homodimers.

[0368] A leucine zipper domain may be incorporated in place of CH2-CH3 sequences in the protein scaffold or it may be placed at the carboxy terminal end of the two heavy chains in the bispecific or trispecific antitumor antagonist. In the case of the latter, a furin cleavage site may be introduced between the carboxy terminal end of CH3 and the amino terminal end of the leucine zipper. This can facilitate furin-mediated cleavage of the leucine zipper following the heterodimerization step when co-expressing the heavy and light chains of the bispecific or trispecific antitumor antagonist in an appropriate mammalian cell expression system (*see* Wranik et al., J. Biol. Chem., 287(5):43331-43339, 2012).

Table 1

Type	HC1	HC2
Knobs-into-holes	Y349C, T366S, L368A, Y407V	S354C, T366W
Ionic, electrostatic	S183E, E356K, E357K, D399K	S183K, K370E, K409D, K439E
Ionic, electrostatic	K392D, K409D	E356K, D399K
HA-TF substitutions	S364H, F405A	Y349T, T394F
HF-TA substitutions	S364H, T394F	Y349T, F405A
Leucine zipper heterodimer	human c-Jun leucine zipper	human c-fos leucine zipper

[0369] The amino acid numbering in Table 1 follows the Kabat numbering scheme and can be applied to heavy chain amino acid sequences of the antibodies described herein. The mutations described in Table 1 may be applied to the sequence (published or otherwise) of any immunoglobulin IgG1 heavy chain, as well as other immunoglobulin classes, and subclasses (or isotypes) therein.

[0370] When co-expressing heavy and light chains of bispecific or trispecific antibodies, the light chains of one binding specificity can also mispair with heavy chains of a different binding specificity. Therefore, in certain embodiments, portions of the heavy chain, light chain or both may be modified relative to the "wild-type" antibody chains from which they are derived to prevent or reduce mispairing of both heavy chain constant regions to one another, as well mispairing of light chain constant regions to their heavy chain counterparts.

[0371] The light chain mispairing problem can be addressed in several ways. In some embodiments, sterically complementary mutations and/or disulfide bridges may be incorporated into the two VL/VH interfaces. In other embodiments, mutations can be incorporated based on ionic or electrostatic interactions. In some embodiments, light chain mispairing may be prevented or reduced by employing a first arm with an S183E mutation in the CH1 domain of the heavy chain and an S176K mutation in the CL domain of the light chain. A second arm may include an S183K mutation in the in the CH1 domain of the heavy chain and an S176E mutation in the CL domain of the light chain. In other embodiments, a "CrossMab" approach is employed, where one arm in the bispecific or trispecific antitumor antagonist (e.g., Fab) is left untouched, but in the other arm containing the other binding specificity, one or more domains in the light chain are swapped with one or more domains in the heavy chain at the heavy chain:light chain interface.

[0372] Methods, immunoglobulin domain sequences, including specific mutations for preventing mispairing of heavy and light chains as disclosed above are further described in U.S. Patent Application Publication Nos. 2014/0243505, 2013/0022601.

D. Conjugates

[0373] In certain embodiments, the antitumor antagonists of the present application are chemically conjugated to one or more peptides and/or small molecule drugs. The peptides or small molecule drug can be the same or different. The peptides or small molecule drugs can be attached, for example to reduced SH groups and/or to carbohydrate side chains. Methods for making covalent or non-covalent conjugates of peptides or small molecule drugs with antibodies are known in the art and any such known method may be utilized.

[0374] In some embodiments the peptide or small molecule drug is attached to the hinge region of a reduced antibody component via disulfide bond formation. Alternatively, such agents can be attached using a heterobifunctional cross-linkers, such as N-succinyl 3-(2-pyridyldithio)propionate (SPDP). General techniques for such conjugation are well-known in the art. In some embodiments, the peptide or small molecule drug is conjugated via a carbohydrate moiety in the Fc region of the antibody. The carbohydrate group can be used to increase the loading of the same agent that is bound to a thiol group, or the carbohydrate moiety can be used to bind a different therapeutic or diagnostic agent. Methods for conjugating peptide inhibitors or small molecule drugs to antibodies via antibody carbohydrate moieties are well-known to those of skill in the art. For example, in one embodiment, the method involves reacting an antibody component having an oxidized carbohydrate portion with a carrier polymer that has at least one free amine function. This reaction results in an initial Schiff base (imine) linkage, which can be stabilized by reduction to a secondary amine to form the final conjugate. Exemplary methods for conjugating small molecule drugs and peptides to antibodies are described in U.S. Patent Application Publication No. 2014/0356385.

[0375] Preferably, the antitumor antagonists in the present disclosure retain certain desirable characteristics and pharmacokinetic properties of antibodies, including a desirable in vitro and in vivo stability (e.g., lone half-life and shelf-life stability), efficient delivery into desired target cells, increased affinity for binding partners, desirable antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity, and reduced renal clearance or excretion. Accordingly, careful attention to size and need for particular constant region effector functions may be considered in the design of the antitumor antagonists.

[0376] The anti-PD-1, anti-PD-L1 and anti-TIGIT, inhibitors, including monospecific, bispecific and trispecific antitumor antagonists therefrom, may range in size from 50 kD to 300 kD, from 50 kD to 250 kD, from 60 kD to 250 kD, from 80 kD to 250 kD, from 100 kD to 250

kD, from 125 kD to 250 kD, from 150 kD to 250 kD, from 60 kD to 225 kD, from 75 kD to 225 kD, from 100 kD to 225 kD, from 125 kD to 225 kD, from 150 kD to 225 kD, from 60 kD to 200 kD, from 75 kD to 200 kD, from 100 kD to 125 kD to 200 kD, from 150 kD to 200 kD, from 60 kD to 150 kD, from 75 kD to 150 kD, from 100 kD to 150 kD, from 60 kD to 125 kD, from 75 kD to 125 kD, from 75 kD to 100 kD, or any range encompassed by any combination of whole numbers listed in the above cited ranges or any ranges specified by any combination of whole numbers between any of the above cited ranges.

EXAMPLES

Example 1: Generation of Monoclonal Antibodies

[0377] Monoclonal antibodies (mAbs) of the present application are generated and screened using techniques well known in the art, *see, e.g.*, Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York. The antigen specific hybridoma Mabs are cloned, sequenced and engineered using techniques well known in the art, *see, e.g.*, Lo. B.K.C Methods in Molecular Biology™. Volume 248 2004. Antibody Engineering.

Example 2: Trispecific antagonists having a PD-1 targeting domain and multiple angiogenesis targeting moieties

[0378] **FIG. 1** shows VH and VL sequences of anti-PD-1, anti-PD-L1, anti-LAG-3 and anti-TIGIT mAbs. **FIG. 2** shows other functional domains including VH and VL of ranibizumab, bevacizumab and a mutant variant of the VH, the VEGF binding domain of aflibercept, trebananib-short peptide and the trebananib-long peptide, and extracellular domain of TGFβR-II (TGFβR-II ECD).

[0379] The aflibercept fusion protein domain comprises vascular endothelial growth factor (VEGF)-binding portions from the extracellular domains of human VEGF receptors 1 and 2 and prevents the binding of VEGF-A, VEGF-B and PLGF to their receptors, VEGFR-1 and VEGFR-2. The trebananib peptide targets and binds to Ang1 and Ang2, thereby preventing the interaction of Ang1 and/or Ang2 with their cognate Tie2 receptors. The antitumor antagonists may be constructed with an IgG1, IgG1agly, or IgG4 backbone.

[0380] A variety of trispecific antitumor antagonists were constructed, each comprising (1) one or more anti-PD-1, or other checkpoint antibody variable region domains; an aflibercept peptide; and a trebananib peptide or peptide sequences thereof. **FIGS. 3A-3H** show eight different trispecific antitumor antagonists, TS-ZPT-1.1, -1.2, -1.3 and -2 to -6, respectively (1) anti-PD-1 variable region (VH1, VL1) (**FIGS. 3A-3H**); (2) an aflibercept fusion protein domain: (i) at the amino terminal end of one or both IgG arms (**FIGS. 3A-3C, 3F**); (ii) at the carboxy-terminal end of each antagonist (**FIGS. 3D, 3E, 3H**); or (iii) between the carboxy-terminal end

of the CH3 domain and a trebananib peptide (or other biological peptide) (**FIG. 3G**) (3) a trebananib peptide (or other biological peptide): (i) fused to the carboxy-terminal end of each IgG4 CH3 region (**FIGS. 3A-3D**); (ii) inserted within each of the two CH3 regions (**FIG. 3E**); (iii) fused to the carboxy-terminal end of each CH1 region (**FIG. 3F**); (iv) fused to the carboxy-terminal end of each aflibercept fusion protein domain (**FIG. 3G**); or (v) fused to the carboxy-terminal end of each CL region (**FIG. 3H**).

[0381] FIGS. 4A-4C show five different trispecific antitumor antagonists, TS-ZPT-7, TS-ZPT-8, TS-ZPT-9, respectively: (1) anti-PD-1 or other checkpoint antibody Fab domains (VH1-CH1, VL1-CL1) fused to the carboxy-terminus of the CH3 domain; (2) an aflibercept fusion protein domain at the amino-terminal end of the CH2 domain fused to the amino-terminus of the CH2 domain; (3) a trebananib peptide (or other biological peptide): (i) inserted within each of the two CH3 domains **(FIG. 4A);** (ii) fused to the amino-terminus of the CH2 domain **(FIG. 4B);** (iii) fused to the carboxy-terminal end of the CHI domain **(FIG. 4C)**

[0382] FIGS. 5A-5G show seven different trispecific antitumor antagonist, TS-LPT-1, TS-LPT-2, TS-LPT-3, TS-LPT-4, TS-LPT-5, TS-M3, TS-M4, respectively; (1) anti-PD-1 or other checkpoint antibody variable domains (VH1, VL1) (i) fused to the amino-terminus of the CH1 and CL1, respectively **(FIG. 5F)**; (ii) fused to the amino-terminus of the VH1 and VL1, respectively **(FIG. 5G)**; (2) Lucentis, bevacizumab or other anti-VEGF antibody variable domains (VH1, VL1); (i) fused to the amino-terminus of the VH1 and VL1, respectively fused to the amino-terminus of the CH1 and CL1, respectively **(FIG. 5F)**; (ii) fused to the amino-terminus of the CH1 and CL1, respectively **(FIG. 5G)**; (iii) fused to the carboxy terminus of the CH3 domain and the amino-terminus of CH1 and CL1 domains, respectively (3) a trebananib peptide (or other biological peptide): (i) inserted within each of the two CH3 domains **(FIG. 5A)**; (ii) fused to the carboxy-terminus of the CL1 domain **(FIG. 5B)**; (iii) fused to one or two of the carboxy-terminal ends of the CHI domain **(FIG. 5C-5E)**; (iv) fused to the carboxy-terminus of the CH3 domain **(FIG. 5F-5G).**

[0383] The amino acid sequences demonstrated in **SEQ ID NOS:200 and 201** (LC) shows exemplary sequences of TS-ZPT-3S. The amino acid sequences demonstrated in **SEQ ID NOS:201 and 202** show exemplary sequences of TS-ZPT-3L. The amino acid sequences demonstrated in **SEQ ID NOS:187 and 188** show exemplary sequences of TS-LPT-1. The amino acid sequences demonstrated in **SEQ ID NOS:201 and 203** show exemplary sequences of TS-ZPT-2. The amino acid sequences demonstrated in **SEQ ID NOS:201 and 204** show exemplary sequences of TS-ZPT-5. The amino acid sequences demonstrated in **SEQ ID NOS:234 and 235** show exemplary sequences for TS-ZPT-6.

Example 3: Characterization of trispecific antitumor antagonists made with the anti-PD-1 antibody 2P16.

[0384] Versions of the trispecific antitumor antagonists depicted in FIGS. 3A-3H were made utilizing the 2P16 anti-PD-1 antibody, trebananib-long peptide, and the VEGF binding domain of aflibercept. TS-ZPT-1.1 (**SEQ ID NOS:236-237**), TS-ZPT-1.2(**SEQ ID NOS:238-239**), TS-ZPT-1.3(**SEQ ID NOS:240-241**), TS-ZPT-2(**SEQ ID NOS:242-243**), TS-ZPT-3(**SEQ ID NOS:201-202**), TS-ZPT-5(**SEQ ID NOS:244 and 298**), TS-ZPT-6 were constructed and compared to TS-LPT-1(**SEQ ID NOS:234-235**), a trispecific antitumor antagonist that utilized the 2P16 antibody, trebananib and the Fab portion of Lucentis.

[0385] FIGS. 6A and 6B show non-reducing SDS-PAGE analysis of the trispecific antitumor antagonists expressed by transiently transfected HEK293 cells. Samples were assessed before and after purification of the molecules. The results show all the molecules expressed and could be purified, and that TS-ZPT-2(2P16), TS-ZPT-3(2P16), TS-ZPT-5(2P16) expressed better than TS-ZPT-6(2P16), and TS-LPT-1(2P16).

[0386] A blocking assay was carried out to calculate the IC50 for selected trispecific molecules. Briefly, 2 or 3 fold serial dilutions of anti-human PD-1 mAb were prepared. Human PD-1 transfected CHO-K1 cells were washed with FACS buffer (0.5%BSA 2mM EDTA in PBS) and re-suspended at a concentration of 10^6 cells/ml. FITC labeled human PD-L1-Fc protein was added to the human PD-1 transfected CHO-K1 cells at a final concentration of 7 µg/ml and mixed well. Without incubation, 2,000 of these CHO-K1 cells (with PD-L1 Protein) in 20 µl FACS buffer was immediately added to a 96-well round bottom plate and 20 µl or 2 or 3 fold serial diluted trispecific molecules were immediately added to the cells and incubated at 4°C for 30 mins. The cells were then washed and re-suspended in 30 µl 7AAD solution; 35 µl 10% neutral buffered formalin solution was then added and incubated for 15 mins before analysis using the iQue intellicyt system.

[0387] FIGS. 7A-B show the IC50s for the exemplary trispecific antitumor antagonists, TS-ZPT-1.1(2P16), TS-ZPT-1.2(2P16), TS-ZPT-1.3(2P16), TS-ZPT-2(2P16), TS-ZPT-3(2P16), TS-ZPT-5(2P16), TS-ZPT-6(2P16) along with an anti-PD-1 mAb and a positive control or benchmark ("BM") antibody (nivolumab). The IC50s were comparable to the benchmark antibodies in blocking this interaction.

[0388] The ability of the trispecific antitumor antagonists TS-ZPT-2(2P16), TS-ZPT-3(2P16), TS-ZPT-5(2P16), TS-ZPT-6(2P16) to block the interaction between VEGF and its receptor, VEGFR2 was determined. In brief, 96-well assay plates were coated with 0.5ug/ml of recombinant human VEGF 165 (R&D) in carbonate-bicarbonate buffer (pH 9.6) at 4oC overnight followed by blocking with 1% BSA/PBS for 1 hour at room temperature. Serially

diluted antibodies were then added to the plate and incubated for 30 minutes at room temperature. Recombinant human VEGFR-2 was added and incubated for 1 hour at room temperature. The plate was washed with Wash Buffer (0.1% Tween-20 in PBS) and then incubated with anti-VEGFR-2 antibody for 1 hour at room temperature. Followed by washing with Wash Buffer, goat anti-mouse IgG-HRP were added to the plate and incubated for 1 hour at room temperature. The amount of VEGFR-2 binding was detected by measuring light absorbance at 650 nm after addition of TMB to the plate. IC50 values were determined as the mid-point in the activity curve.

[0389] FIGS. 8A and **8B** show the IC50 values calculated from the VEGF-VEGFR2 blocking assays using the trispecific antagonists TS-ZPT-1.2(2P16), TS-ZPT-2(2P16), TS-ZPT-5(2P16), TS-ZPT-6(2P16) and TS-LPT-1(2P16), which were compared to positive controls corresponding to the aflibercept peptide and the bevacizumab antibody. The results of these analyses showed that the trispecific antagonists exhibited comparable or lower (*i.e.*, better) IC50 values than the corresponding controls.

[0390] The ability of the trispecific antagonists TS-ZPT-1.2(2P16), TS-ZPT-2(2P16), TS-ZPT-5(2P16), TS-ZPT-6(2P16) and TS-LPT-1 to block the interaction between Ang-2 and its receptor, Tie2 was determined. Briefly, 96-well assay plates were coated with 1ug/ml of recombinant human Ang2 (R&D) in PBS at 37°C for 1 hour and then blocked with 1% BSA/PBS for 1 hour at room temperature. Serially diluted antibodies and recombinant human Tie2 mixture were then added to the plate and incubated for 2 hours at room temperature. The plate was washed with Wash Buffer (0.1% Tween-20 in PBS) and then incubated with anti-Tie2 antibody for 1 hour at room temperature. After washing with Wash Buffer, goat anti-mouse IgG-HRP were added to the plate and incubated for 1 hour at room temperature. Tie2 binding was detected by measuring light absorbance at 650 nm after addition of TMB to the plate. IC50 values were determined as the mid-point in the activity curve.

[0391] FIG. 9 shows the IC50 values calculated from the Tie2-Ang2 blocking assay testing the trispecific antagonists TS-ZPT-1.2(2P16), TS-ZPT-2(2P16), TS-ZPT-5(2P16), TS-ZPT-6(2P16) and TS-LPT-1(2P16) which were compared to positive controls corresponding to an antagonist, bevacizumab-trebananib. The results of this analysis showed that the trispecific antagonists exhibited comparable or lower (*i.e.*, better) IC50 values than the corresponding controls.

Example 4: Characterization of TS-ZPT-2, TS-ZPT-3L and TS-ZPT-5 molecules constructed with the anti-PD-1 antibody 2P17

[0392] TS-ZPT-2, TS-ZPT-3L and TS-ZPT-5 trispecific antitumor antagonists were made with the anti-PD-1 antibody 2P17 (**SEQ ID NOS:203 and 201, 233 and 201, 204 and**

201, respectively) and characterized for homogeneity and stability by size exclusion chromatography after purification by protein A chromatography. SEC chromatography was performed using a Tosoh TSKgel UP-G3000SWXL column. The mobile phase system was 100 mM sodium phosphate, 400 mM sodium chloride with 6% isopropyl alcohol, pH 6.8 at 0.2 mL/minute. Detection was done at 214 nm for relative area integration

[0393] FIG. 10 shows the results of size exclusion chromatography analysis of TS-ZPT-2(2P17), TS-ZPT-3L(2P17) and TS-ZPT-5(2P17), and parental 2P17 antibody produced by HEK293 transiently transfected cells. The percentages of high molecular weight (HWM) species and low molecular weight (LMW) species in comparison to dimerized molecule (Dimer) indicate that of the three trispecific antagonists, TS-ZPT-3L(2P17) has the lowest percentage of LMW species and the highest percentage of Dimers.

[0394] FIG. 11 shows the relative amounts of HWM and LMW species in comparison to Dimers of three trispecific antagonists: TS-ZPT-2(2P17), TS-ZPT-3L (2P17)(purified from two pools) and TS-ZPT-5(2P17) produced from stable CHO cell pools. The results show that of the three trispecific antagonists, TS-ZPT-3L(2P17) has the lowest percentage of LMW species and the highest percentage of Dimers.

[0395] Therefore, in both transient and stable cell lines, TS-ZPT-3L(2P17) exhibited lowest amount of clipping, indicating that inserting the trebananib peptide into the CH3 region protects the aflibercept fusion protein, and reduces clipping.

[0396] FIGS. 12A and 12B shows that the Dimer, HMW and LMW species of TS-ZPT-3L(2P17) exhibit good stability for at least 112 days at 4° C.

Example 5: Expression and purification of TS-ZPT-3S and TS-ZPT-3L trispecific antitumor antagonists.

[0397] TS-ZPT-3 versions were made using the anti-PD-1 antibodies 2P16 and 2P17 with both the full length trebananib peptide, TS-ZPT-3L, and a single copy, or trebananib-short, peptide for TS-ZPT-3S. These four molecules were compared to trispecific molecules utilizing the VH and VL of bevacizumab, TS-M3 and TS-M4 (depicted in **FIG. 5F**), utilizing anti-PD-1 antibodies nivolumab, 2P16 and 2P17 whose variable domain sequences are shown in **FIG. 1** with sequences **SEQ ID NOS:247-254**.

[0398] FIG. 13A shows the heavy- and light chain amino acid sequences for an exemplary trispecific antitumor antagonist with the trebananib peptide, *i.e.*, TS-ZPT-3L(2P17). **FIG. 13B** depicts an exemplary molecule derived from these sequences.

[0399] FIG. 14A shows the heavy and light chains amino acid sequences for an exemplary trispecific antitumor antagonist with the trebananib Short peptide, *i.e.*, TS-ZPT-3S(2P17). **FIG. 14B** depicts an exemplary molecule derived from these sequences.

[0400] FIG. 15 shows Coomassie stained non-reducing SDS-PAGE analysis of trispecific the TS-ZPT-3S(2P17), TS-ZPT-3L(2P17), TS-M3(2P17), TS-M4(2P17), TS-M3(2P16) and TS-M3(nivolumab) trispecific antitumor antagonist after production by transiently transfected HEK293 cells. The results show that trispecific antitumor antagonists TS-ZPT-3S(2P17), TS-ZPT-3L(2P17), which contains aflibercept fusion protein domains, exhibited higher levels of expression than trispecific antitumor antagonists TS-M3(nivolumab), TS-M4(nivolumab), TS-M3(2P17) and TS-M3(2P16), which contains anti-VEGF variable domains.

[0401] FIG. 16 Shows the results from a size exclusion chromatography analysis of TS-ZPT-3L(2P17), TS-ZPL-3S(2P17), TS-ZPT-3L(2P16), TS-ZPT-3S(2P16) after purification by protein A chromatography. The percentages of high molecular weight (HWM) species and low molecular weight (LMW) species in comparison to dimerized molecule (Dimer) indicate the molecules comprising the 2P17 antibody have higher levels of the preferred dimer species.

[0402] FIG. 17 shows the size exclusion chromatography analysis of TS-ZPT-3S(2P17) and TS-ZPT-3L(2P17) stored at 4 degrees C for 48 days, indicating both molecules are stable when stored.

Example 6: Functional characterization of trispecific antagonist TS-ZPT-3

[0403] The PD-1-PDL1 cell-based blocking assay in Example 5 was used to evaluate the ability of four variations of the trispecific antitumor antagonist, TS-ZPT-3S(2P16), TS-ZPT-3L(2P16), TS-ZPT-3S(2P17) and TS-ZPT-3L(2P17), to block the interaction between PD-1 and its ligand, PD-L1. The results were used to calculate the corresponding IC50s along with 2P17 and a benchmark antibody (nivolumab). **FIGS. 18A-18B** show that the IC50s were comparable to the benchmark antibodies in blocking this interaction.

[0404] The VEGF-VEGFR-2 bioassay was used to evaluate the ability of TS-ZPT-3S(2P16), TS-ZPT-3L(2P16), TS-ZPT-3S(2P17) and TS-ZPT-3L(2P17) to block the interaction between VEGF and its receptor, VEGFR-2 and to calculate the corresponding IC50s along with a benchmark antibody (bevacizumab). Briefly, VEGFR2 / NFAT Reporter – HEK293 Recombinant Cell Line (BPS Bioscience Catalog #: 79387) was plated at a density of 30k cells per well into a white clear-bottom 96-well microplate. After 24 hours serial dilutions of indicated molecules and human VEGF165 were added to the wells. After 4 hours of incubation, 100 µl of ONE-Step™ Luciferase reagent was added, the plates were rocked at room temperature for ~15minutes, and the plates read for the luminescence signal. **FIGS. 19A-19B** show that the IC50s were comparable to the benchmark antibodies in blocking this interaction.

[0405] The Ang2-Tie2 blocking assay in Example 5 was used to evaluate the ability of TS-ZPT-3S(2P16), TS-ZPT-3L(2P16), TS-ZPT-3S(2P17) and TS-ZPT-3L(2P17) to block the

interaction between Tie2 and its ligand, Ang-2 and to calculate the corresponding IC50s along with a benchmark antibody (trebananib). **FIGS. 20A-20B** shows that the IC50s of TS-ZPT-3L(2P16) and TS-ZPT-3L(2P17) with two Ang-2 blocking peptides were comparable to the benchmark, while TS-ZPT-3S(2P16) and TS-ZPT-3S(2P17) with only one Ang-2 blocking peptide have higher IC50s than the benchmark.

[0406] The bio-layer interferometry assay in Example 9 was used to evaluate the binding affinity of TS-ZPT-3S(2P16), TS-ZPT-3L(2P16), TS-ZPT-3S(2P17) and TS-ZPT-3L(2P17) against PD-1 protein. **FIGS. 21A-21F** show the binding and the resultant binding affinity constants. The results show that TS-ZPT-3S(2P16), TS-ZPT-3L(2P16), TS-ZPT-3S(2P17) and TS-ZPT-3L(2P17) exhibited higher affinity than the benchmark (nivolumab).

[0407] A similar bio-layer interferometry assay was used to evaluate the binding affinity of TS-ZPT-3S(2P16), TS-ZPT-3L(2P16), TS-ZPT-3S(2P17) and TS-ZPT-3L(2P17) against VEGF165. **FIGS. 22A-22F** show the binding and the resultant binding affinity constants. The results show that TS-ZPT-3S(2P16), TS-ZPT-3L(2P16), TS-ZPT-3S(2P17) and TS-ZPT-3L(2P17) exhibited higher affinity than the benchmark (bevacizumab).

[0408] A similar bio-layer interferometry assay was used to evaluate the binding affinity of TS-ZPT-3S(2P16), TS-ZPT-3L(2P16), TS-ZPT-3S(2P17) and TS-ZPT-3L(2P17) against Ang-2. **FIGS. 23A-23F** show the binding and the resultant binding affinity constants. The results show that TS-ZPT-3S(2P16), TS-ZPT-3L(2P16), TS-ZPT-3S(2P17) and TS-ZPT-3L(2P17) exhibited similar affinity than the benchmark (trebananib).

[0409] **FIG.24** shows the use of bio-layer interferometry to characterize the sequential binding of TS-ZPT-3S(2P16), TS-ZPT-3L(2P16), TS-ZPT-3S(2P17) and TS-ZPT-3L(2P17) to each of its three binding partners using the Octet RED96 System (ForteBio), largely as described in Example 11 above. Briefly, 20nM of TS-ZPT-3 trispecific antibodies were loaded onto the anti-human IgG capture biosensors. To monitor sequential binding of the three antigens to the four TS-ZPT-3 molecules, biosensors were placed into wells containing saturating concentrations (> 10 K_D) of each antigen (240 nM His tagged human PD-I, 120 nM human VEGF165, 40 nM His tagged human Angiopoietin-2) for 5 min followed by 1 min dissociation. Three different sequential binding combinations were observed. These results show that TS-ZPT-3S(2P16), TS-ZPT-3L(2P16), TS-ZPT-3S(2P17) and TS-ZPT-3L(2P17) are able to bind to VEGF, Ang-2 and PD-1 simultaneously.

[0410] To evaluate the pharmacokinetic properties of TS-ZPT-3L(2P17) in vivo, pharmacokinetic profiles were generated. Briefly, 10 mg/kg of the antagonist was intravenously injected into the tail vein of four 6-10 week old female CD1 mice (n=2 mice per molecule). Serum was harvested at 3 minutes, 3 hours, 1 day, 3 days, 7 days and 10 days post injection. To

detect the antibodies in the serum, 96 well ELISA plates were coated with 5 μg/ml goat anti-human IgG F(ab')2 fragment and then blocked with 5% milk in PBS. Serially diluted mouse serum in 5% milk and serially diluted purified protein molecule as standard were added to the plates. Following incubation with peroxidase conjugated mouse anti-human IgG and further washes, TS-ZP17T-3 were detected following incubation with TMB-ELISA substrate. The results of this analysis showed that the half-life (T1/2) of TS-ZP17T-3 is about 2 days (**FIG. 25**).

[0411] Thus, the insertion of trebananib, or a variant thereof, allows for the creation of a stable and potent trispecific therapeutic molecule.

[0412] FIGS. 26A-26B show two additional trispecific molecules utilizing two checkpoint antibodies and the trebananib peptide inserted into the CH3 domain. These molecules comprise sequences shown in **FIG. 1** with (1) the VH and VL from the first checkpoint antibody, for example anti-PD-1 or anti-PD-L1, (2) trebananib inserted into the CH3, (3) the VH and VL with a 3-6xG4S linker from a second checkpoint inhibitor, for example anti-TIGIT or anti-LAG3.

[0413] FIG. 27 shows an additional trispecific molecule, TS-A1BT-1 (**SEQ ID NOS:229-230**), with (1) the VH and VL domains encoding bevacizumab, or another anti-VEGF antibody, (2) trebananib peptide inserted into the CH3 domain, (3) the TGFBR-II ECD fused to the carboxy-terminus of the CH3 domain. This molecule was produced by transiently transfected HEK 293 cells. In **FIG. 28**, a non-reducing SDS-PAGE analysis of unpurified supernatants from the HEK293 cells indicate the molecule expresses well. **FIG. 29** show a size exclusion chromatography analysis of protein A chromatography purified TS-A1BT-1.

Example 7: Design of trispecific antitumor antagonists comprising a TGF-β RII extracellular domain (TGF-β-RII ECD)

[0414] FIGS. 30A-30F show six trispecific antitumor antagonists, TS-ZPB-1, TS-ZPB-2, TS-ZPB-3, TS-ZPB-4, TS-ZPB-5 and TS-ZPB-6, respectively. These antagonists comprise: (1) anti-PD-1 or another checkpoint antibody variable domains (VH1, VL1) (**FIGS. 30A-30F**); (2) an aflibercept fusion protein domain: (i) fused to the amino-terminal end of each heavy chain VH1 region (**FIGS. 30A, 30D, 30F**) or (ii) fused to the carboxy-terminal end of each heavy chain CH3 region (**FIGS. 30B, 30C, 30E**) each carboxy-terminal end; and (3) a TGF-β RII extracellular domain (ECD): (i) fused to the carboxy-terminal end of each heavy chain CH3 region (**FIG. 30A**), (ii) fused to the amino-terminal end of each heavy chain VH1 region (**FIG. 30B**), (iii) inserted within the Fc loop in each heavy chain CH3 region (**FIGS. 30C, 30F**), (iv) fused to the amino terminal end of each heavy chain, upstream of the aflibercept fusion protein domain (**FIG. 30D**), or (v) fused to the carboxyl-terminal end of each heavy chain, downstream of the aflibercept fusion protein domain (**FIG. 30E**).

[0415] The amino acid sequences demonstrated in **SEQ ID NOS:187 and 188** show exemplary sequences of TS-ZPLB-1. The amino acid sequences demonstrated in **SEQ ID NOS:270 and 271** show exemplary sequences of TS-ZPB-1. The amino acid sequences demonstrated in **SEQ ID NOS:272 and 273** show exemplary sequences for TS-ZPB-2. The amino acid sequences demonstrated in **SEQ ID NOS:188 and 189** show exemplary sequences of TS-ZPLB-3. The amino acid sequences demonstrated in **SEQ ID NOS:190 and 191** show exemplary sequences of TS-ZPB-3. The amino acid sequences demonstrated in **SEQ ID NOS:192 and 193** show exemplary sequences of TS-ZPB-5.

Example 8: Transient expression of TS-ZPB and TS-ZPLB molecules

[0416] TS-ZPB-1(2P17), TS-ZPB-2(2P17), TS-ZPB-3(2P17), TS-ZPB-5(2P17) and TS-ZPLB molecules were transiently expressed in human embryonic kidney HEK293 cells, which were cultured for 7 days. Titers were quantified using a POROS A 20 μm column, 2.1x30mm, 0.1 mL by Applied Biosystems. Standard curves were generated using purified recombinant TIGIT mAb at wavelength 280 nm. Samples were injected neat at 30μL of supernatant into the HPLC. Mobile phase A was PBS at pH 7.3; mobile phase B was PBS at pH 2.2. Multiple step gradients were done starting at 100% A for 0.2 minute, followed by 100% B from 0.22-1.22 minute, and 1.23-2.30 minute was back to 100% A, then 2.32-3.32 minute with 100% B, and finalized with 3.4-4.00 minute at 100% A. Flow rate used was 1 mL/min with a total of 4 minutes/run.

[0417] As shown in **FIG. 31A**, an SDS-PAGE analysis of TS-ZPB-1(2P17), TS-ZPB-2(2P17), TS-ZPB-3(2P17), TS-ZPB-5(2P17) and TS-ZPLB produced by transiently transfected HEK293 cells. The results of this analysis all of the molecules express well (**FIG. 31B**).

[0418] **FIG. 32A** shows exemplary size exclusion chromatograph (SEC) profiles for TS-ZPB-1(2P17), TS-ZPB-2(2P17), TS-ZPB-3(2P17), TS-ZPB-5(2P17) and TS-ZPLB using a Tosoh TSKgel UP-G3000SWXL column with detection at 214 nm. The results show that TS-ZPLB-1 and TS-ZPB-5(2P17) retain the most Dimers compared to other TS-ZPB molecules.

Example 9: Functional characterization of the TS-ZPB trispecific antagonists relative to controls

[0419] **FIG. 33A** shows the results of a cell-based assay measuring the ability of TS-ZPB-1(2P17), TS-ZPB-2(2P17), TS-ZPB-3(2P17), and TS-ZPB-5(2P17) to block the interaction between PD-1 and PD-L1. In this experiment, 3 fold serial dilutions of the TS-ZPB molecules (as indicated; highest Ab concentration: 300nM; triplicates for each mAb) were prepared; human PD-1 transfected CHOK1 cells were washed with FACS buffer (0.5% BSA 2mM EDTA in PBS) and re-suspended at a concentration of 10^6 cells/ml; FITC-labeled human PD-L1-Fc protein was added to human PD-1 transfected CHOK1 cells at a final concentration of 7μg/ml;

mixed well; without incubation, $20x10^3$ of the transfected CHOK1 cells (with PD-L1 Protein) were immediately added to 20µl FACS buffer in a 96-well round bottom plate; 20µl 3-fold serial dilutions of the TS-ZPB molecules were then immediately added to the cells, which were then incubated at 4°C for 30 mins; cells were then washed and re-suspended in 30µl 7AAD to which 35µl of 10% neutral buffered formalin solution was added. Incubate for 15mins before analysis with the iQue intellicyt system. **FIG. 33B** shows the IC50 values (nM) obtained from this analysis indicating all of the molecules are able to inhibit the interaction of PD-1 with PD-L1.

[0420] FIG. 34A shows the results of a cell-based assay using a TGFβ/SMAD Signaling Pathway SBE Reporter –HEK293 Cell Line (BPS Bioscience, Inc., San Diego, CA, Catalog #: 60653) to measure the ability of TS-ZPB-1(2P17), TS-ZPB-2(2P17), TS-ZPB-3(2P17), TS-ZPB-5(2P17) and TS-ZPLB and a benchmark, TGFβ1 RII-Fc to block TGFβ1 signaling. Briefly, SBE reporter-HEK293 cells were seeded at a density of ~35,000 cells per well in a white clear-bottom 96-well microplate in 100 µl of growth medium. 24 hours after seeding, the cells were treated with fivefold serial dilutions of TS-ZPB molecules in 90 µl assay medium. Then, 10 µl of diluted human TGFβ1 was added to stimulated wells (final TGFβ1 concentration = 10 ng/ml); 10 µl of assay medium was added to unstimulated control wells (for determining the basal activity), while 100 µl of assay medium was added to cell-free control wells (for determining background luminescence). The plates were then incubated at 37°C in a CO_2 incubator overnight (~18 hours). Following the overnight incubation, a luciferase assay was conducted using the ONE-Step™ Luciferase Assay System (BPS Bioscience, Inc.) according to the protocol provided (*i.e.*, 100 µl of ONE-Step™ Luciferase reagent was added per well and rocked at room temperature for ~15 to 30 minutes before reading luminescence signal. **FIG. 34B** shows the IC50 values (nm) obtained from this analysis and indicates all of the molecules are able to block the bioactivity of TGFβ1.

[0421] FIG. 35A shows the results of an ELISA assay measuring the ability of TS-ZPB-1(2P17), TS-ZPB-2(2P17), TS-ZPB-3(2P17), TS-ZPB-5(2P17) and TS-ZPLB to block the interaction between VEGF and VEGFR-2, as compared to the benchmark antibody, bevacizumab. Briefly, 0.5ug/ml of recombinant human VEGF-165 (R&D #293-VE-050/CF) in carbonate-bicarbonate buffer was coated to the wells in a 96-well assay plate and incubated overnight at 4°C. The wells were then washed 3 times with 0.05% Tween 20 in PBS (wash buffer), blocked with 1% BSA in PBS for 1 hour at room temperature, and washed 3 times with wash buffer. 50µl 5-fold serially diluted antibodies were then added to wells in the plate and incubated for 30 minutes at room temperature. Then, 50µl 0.6µg/ml recombinant human VEGFR-2-hu IgG1-His Tag (R&D #357-KD-050) was added to the antibodies in the wells and incubated for 1 hour at room temperature, followed by 3 washes with wash buffer. 100µl anti-

His tag HRP (1:10,000) was then added and the plates were incubated for 40mins at room temperature and washed 3 times with wash buffer. VEGFR-2 binding (His-Tag) was detected by measuring light absorbance at 650 nm after addition of 3,3',5,5'-Tetramethylbenzidine to each well in the plate. **FIG. 35B** shows the IC50 values (nM) obtained from this analysis and indicates all of the molecules are able to inhibit the binding of VEGF with VEGFR-2.

Example 10: Functional characterization of TS-ZPT-5 variant with a-glycosylated VEGFR (aflibercept)

[0422] Two variants of TS-ZPB-5 were produced to improve the pharmacokinetics of TS-ZPB-5. The heavy chain of TS-ZPB-5A (**SEQ ID NO:205**), was mutated at codon for the carboxy-terminal lysine of the CH3 domain to reduce potential proteolytic activity by proteases present in the blood. The heavy chain of TS-ZPB-5B (**SEQ ID NO:206**) additionally has mutations that eliminate the glycosylation sites in aflibercept, to reduce clearance by ASGRI and ASGRII.

[0423] FIGS. 36A-36C depict the three trispecific antitumor antagonists TS-ZPB-5, TS-ZPB-5A and TS-ZPB-5B.

[0424] FIG. 37 shows transient HEK293 cell expression levels of TS-TPB-5, TS-ZPB-5A and TS-ZPB-5B are similar to the parental 2P17 antibody.

[0425] The VEGF-VEGFR-2 blocking assay in Example 10 was used to evaluate the ability of TS-TPB-5, TS-ZPB-5A and TS-ZPB-5B to block the interaction between VEGF and VEGFR-2 (**FIG. 38**). The results show that binding and bioactivity of the VEGFR component are retained after mutation of aflibercept glycosylation sites from the Asn to Glu. The TGFB signaling assay described in Example 10 was used to evaluate the ability of TS-TPB-5, TS-ZPB-5A and TS-ZPB-5B to block the TGFβ signaling (**FIG. 39**). The results show that binding and bioactivity of TGFβR-II are retained after mutation of aflibercept glycosylation sites from the Asn to Glu.

[0426] The in vivo pharmacokinetic properties of TS-ZPT-5B variant with a-glycosylated VEGFR compared to the glycosylated version, TS-ZPT-5A was performed as described in Example 7.

[0427] FIG. 39 shows the TS-ZPB-5B variant with the mutation of aflibercept glycosylation sites from the Asn to Glu improved the pharmacokinetics by approximately five fold.

Example 11: Production and characterization of Bispecific inhibitors of PD-1/PD-L1 and VEGF

[0428] FIGS. 41A-41C show bispecific molecules TS-ZPL-1 (or TS-ZP-1), TS-ZPL-2 (or TS-ZP-2) and TS-ZPL-3 (or TS-ZP-3) comprising (1) a PD-1, PD-L1 or other checkpoint

antibody variable domains (VH and VL) (2) aflibercept fuse (i) to the amino-terminus of the VH domain (**FIGS. 40A and 40B**); (ii) to the carboxy-terminus of the CH3 domain (**FIG. 40C**). Exemplary sequences for these molecules are **SEQ ID NOS:218-225.**

[0429] FIG. 42A shows the results of a cell-based binding assay measuring the ability of Bi-ZPL-1 molecules to block the interaction between PD-L1 and PD-1. Briefly, 2 or 3 fold serial dilutions of Anti-human PD-L1 mAb or bispecific Ab (Highest Ab concentration: 128nM; Triplicates for each mAb) were prepared. Human PD-L1 transfected CHOK1 cells were washed with FACS buffer (0.5%BSA 2mM EDTA in PBS) and re-suspend to a concentration of 10^6 cells/ml. FITC labeled human PD-1-Fc protein at 7ug/ml was added to the cells and 20ul of the mixture was added to the 2 or 3 fold serial dilutions of Anti-human PD-L1 mAb or bispecific Ab. After incubation at 4 degree for 30mins, cells were washed and re-suspend cells in 30ul 7AAD and 35ul 10% neutral buffered formalin solution was added. 15 minutes later the wells were analyzed with an iQue Intellicyt system. The resulting IC50 values (nM) obtained from this analysis are shown in **FIG. 42B,** and indicate Bi-ZPL-1 retains the ability to inhibit PD-1-PD-L1 binding.

[0430] FIG. 43A the bioassay of example 10 testing the ability of Bi-ZP-2 and Bi-ZPL-3 to block the bioactivity of VEGF165 on VEGFR-2 expressing cells. The tabular results are shown in **FIG. 43B**, and indicate the molecules retain bioactivity relative the benchmark antibody, bevacizumab.

[0431] Both Bi-ZP-2 and Bi-ZPL-3 were assessed by size exclusion chromatography and had similar percentages of HMW, Dimer, and LMW species, as shown in **FIG. 44**.

[0432] FIG. 45 shows the pharmacokinetics of Bi-ZP-2 and Bi-ZPL-3 in two mice each. Bi-ZP-2 exhibits a longer half-life in vivo than Bi-ZPL-3.

[0433] The above description is for the purpose of teaching a person of ordinary skill in the art how to practice the present invention, and it is not intended to detail all those obvious modifications and variations of it which will become apparent to the skilled worker upon reading the description. It is intended, however, that all such obvious modifications and variations be included within the scope of the present invention, which is defined by the following claims. The claims are intended to cover the claimed components and steps in any sequence which is effective to meet the objectives there intended, unless the context specifically indicates the contrary.

WHAT IS CLAIMED IS:

1. A trispecific antagonist, comprising:

an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain;

a first targeting domain comprising one or more immunoglobulin variable domains selected from the group consisting of anti-PD-1 variable domains, anti-PD-L1 variable domains, anti-TIGIT variable domains and anti-LAG-3 variable domains;

a second targeting domain that binds specifically to VEGF and comprises one or more peptide domains derived from VEGFR; and

a third targeting domain comprising a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway.

2. The trispecific antagonist of Claim 1, wherein the second targeting domain is structurally linked to a carboxy-terminal of the CH3 domain and wherein the third targeting domain is inserted within the CH3 domain.

3. The trispecific antagonist of Claim 1 or 2, wherein the first targeting domain comprises one or more anti-PD-1 variable domains.

4. The trispecific antagonist of any one of Claims 1-3, wherein the first targeting domain comprises:

(1) an immunoglobulin heavy chain variable region comprising three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3,

wherein the HCDR1 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:48, 51, 54, 56 and 59,

wherein the HCDR2 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:49, 52, 57 and 60, and

wherein the HCDR3 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:50, 53, 55, 58 and 61;

(2) an immunoglobulin light chain variable region comprising three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3,

wherein the LCDR1 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:62, 65, 68, 69, 70 and 73,

wherein the LCDR2 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:63, 66, 71 and 74, and

wherein the LCDR3 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:64, 67, 72 and 75.

5. The trispecific antagonist of any one of Claims 1-5, wherein the first targeting

domain comprises:

(1) an immunoglobulin heavy chain variable region (HCVR) comprising an amino acid sequence that is 80% to 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NOS:127, 129, 131, 133, 135 and 137; and

(2) an immunoglobulin light chain variable region (LCVR) comprising an amino acid sequence that is 80% to 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NOS:128, 130, 132, 134, 136 and 138.

6. The trispecific antagonist of any one of Claims 1-5, wherein the first targeting domain comprises:

(1) an immunoglobulin HCVR comprising the amino acid sequence of SEQ ID NO:137; and

(2) an immunoglobulin LCVR comprising the amino acid sequence of SEQ ID NO: 138.

7. The trispecific antagonist of any one of Claims 1-3, wherein the first targeting domain comprises:

(1) an immunoglobulin HCVR comprising the amino acid sequence of SEQ ID NO:135; and

(2) an immunoglobulin LCVR comprising the amino acid sequence of SEQ ID NO:136.

8. The trispecific antagonist of any one of Claims 1-5, wherein the first targeting domain comprises:

(1) an HCVR comprising three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3,

wherein the HCDR1 comprises the amino acid sequence of SEQ ID NO: 56,

wherein the HCDR2 comprises the amino acid sequence SEQ ID NO:57, and

wherein the HCDR3 comprises the amino acid sequence of SEQ ID NO:58; and

(2) an LCVR comprising three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3,

wherein the LCDR1 comprises the amino acid sequence of SEQ ID NO:70,

wherein the LCDR2 comprises the amino acid sequence of SEQ ID NO:71, and

wherein the LCDR3 comprises the amino acid sequence of SEQ ID NO:72.

9. The trispecific antagonist of any one of Claims 1-8, wherein the second targeting domain comprises the amino acid sequence of SEQ ID NO:185.

10. The trispecific antagonist of any one of Claims 1-9, wherein the third targeting domain comprises only one copy of the amino acid sequence of SEQ ID NO:194.

11. The trispecific antagonist of any one of Claims 1-9, wherein the third targeting domain comprises the amino acid sequence of SEQ ID NO:182.

12. The trispecific antagonist of any one of Claims 1-8, comprising an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO: 201 and/or an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:200 or SEQ ID NO:202.

13. The trispecific antagonist of any one of Claims 1-8, comprising:

an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO: 232 and/or and an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO:231 or SEQ ID NO:233.

14. The trispecific antagonist of Claim 2, wherein the second targeting domain is structurally linked to the immunoglobulin scaffold via a peptide linker.

15. The trispecific antagonist of Claim 14, wherein the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 284-289.

16. A trispecific antagonist, comprising:

an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain;

a first targeting domain comprising one or more anti-PD-1 variable domains or one or more anti-PD-L1 variable domains;

a second targeting domain comprising a component of VEGFR; and

a third targeting domain comprising a TGF-β pathway inhibitor.

17. The trispecific antagonist of Claim 16, wherein the second targeting domain is structurally linked to a carboxy-terminal of the CH3 domain, and wherein the third targeting domain is structurally linked to a carboxy-terminal of the second targeting domain.

18. The trispecific antagonist of Claim 16 or 17, wherein the first targeting domain comprises one or more anti-PD-1 variable domains.

19. The trispecific antagonist of any one of Claims 16-18, wherein the first targeting domain comprises:

(1) an immunoglobulin HCVR comprising three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3,

wherein the HCDR1 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:48, 51, 54, 56 and 59,

wherein the HCDR2 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:49, 52, 57 and 60, and

wherein the HCDR3 comprises an amino acid sequence selected from the group

consisting of SEQ ID NOS:50, 53, 55, 58 and 61; and

(2) an immunoglobulin LCVR comprising three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3,

wherein the LCDR1 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:62, 65, 68, 69, 70 and 73,

wherein the LCDR2 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:63, 66, 71 and 74, and

wherein the LCDR3 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:64, 67, 72 and 75.

20. The trispecific antagonist of any one of Claims 16-19, wherein the first targeting domain comprises:

(1) an immunoglobulin HCVR comprising an amino acid sequence that is 80% to 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NOS:127, 129, 131, 133, 135 and 137; and/or

(2) an immunoglobulin LCVR comprising an amino acid sequence that is 80% to 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NOS:128, 130, 132, 134, 136 and 138.

21. The trispecific antagonist of any one of Claims 16-20, wherein the first targeting domain comprises:

(1) an immunoglobulin HCVR comprising the amino acid sequence of SEQ ID NO:137; and

(2) an immunoglobulin LCVR comprising the amino acid sequence of SEQ ID NO:138.

22. The trispecific antagonist of any one of Claims 16-21, wherein the first targeting domain comprises:

(1) an immunoglobulin HCVR comprising an amino acid sequence that is 80% to 100% identical to the amino acid sequence of SEQ ID NO:135; and

(2) an immunoglobulin LCVR comprising an amino acid sequence that is 80% to 100% identical to the amino acid sequence of SEQ ID NO:136.

23. The trispecific antagonist of any one of Claims 16-20, wherein the first targeting domain comprises:

(1) an immunoglobulin HCVR comprising three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3,

wherein the HCDR1 comprises the amino acid sequence of SEQ ID NO:59,

wherein the HCDR2 comprises the amino acid sequence of SEQ ID NO:60, and

wherein the HCDR3 comprises the amino acid sequence of SEQ ID NO:61; and/or

(2) an immunoglobulin LCVR, wherein the light chain variable region comprises three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3,

wherein the LCDR1 comprises the amino acid sequence of SEQ ID NO:73,

wherein the LCDR2 comprises the amino acid sequence of SEQ ID NO:74, and

wherein the LCDR3 comprises the amino acid sequence of SEQ ID NO:75.

24. The trispecific antagonist of any one of Claims 16-20, wherein the first targeting domain comprises:

(1) an immunoglobulin HCVR comprising three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3,

wherein the HCDR1 comprises the amino acid sequence of SEQ ID NO:56,

wherein the HCDR2 comprises the amino acid sequence of SEQ ID NO:57, and

wherein the HCDR3 comprises the amino acid sequence of SEQ ID NO:58; and/or

(2) an immunoglobulin LCVR comprising three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3,

wherein the LCDR1 comprises the amino acid sequence of SEQ ID NO:70,

wherein the LCDR2 comprises the amino acid sequence of SEQ ID NO:71, and

wherein the LCDR3 comprises the amino acid sequence of SEQ ID NO:72.

25. The trispecific antagonist of any one of Claims 16-24, wherein the second targeting domain comprises the amino acid sequence of SEQ ID NO:185.

26. The trispecific antagonist of any one of Claims 16-24, wherein the second targeting domain comprises the amino acid sequence of SEQ ID NO: 207.

27. The trispecific antagonist of any one of Claims 16-26, wherein the third targeting domain comprises a TGFβ1 RII extracellular domain (ECD).

28. The trispecific antagonist of Claim 17, comprising:

an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO: 193, and/or

an immunoglobulin heavy chain comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 192, 205 and 206.

29. The trispecific antagonist of Claim 17, wherein the first targeting domain is structurally linked to the amino-terminal of the immunoglobulin scaffold via a peptide linker, and wherein the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 284-289.

30. The trispecific antagonist of Claim 17, wherein the second targeting domain is structurally linked to the carboxyl-terminal of the immunoglobulin scaffold via a peptide

linker, and wherein the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 284-289.

31. The trispecific antagonist of Claim 17, wherein the third targeting domain is structurally linked to the carboxyl-terminal of the second targeting domain via a peptide linker, and wherein the peptide linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 284-289.

32. A method for treating a cell proliferative disorder, comprising:

administering to a subject in need thereof an effective amount of any one of the trispecific antagonists of Claims 1-31.

Exemplary Functional Checkpoint Sequences

VH/VL or Fusion Protein Domain	Amino Acid Sequences of Functional Domains
Anti-PD-L1 HCVR (PL-08)	QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWMHWVRQAPGQGLEWMGMIHPNSGGNNYNE KFKSRVTMTRDTSISTAYMELSRLRSDDTAVYYCARSWYGSSPYYFDYWGQGTLVTVSS (SEQ ID NO: 153)
Anti-PD-L1 LCVR (PL-08)	DIQMTQSPSSLSASVGDRVTISCRASQDIDNYLNWYQQKPGKAPKLLIKYTSRLHSGVPSRFSGSGSG TDFTLTISSLQPEDFATYFCQQGYTLPWTFGGGTKVEIK (SEQ ID NO: 154)
Anti-PD-1 HCVR (nivolumab)	QVQLVESGGGVVQPGRSLRLDCKASGITFSNSGMHWVRQAPGKGLEWVAVIWYDGSKRYYADSV KGRFTISRDNSKNTLFLQMNSLRAEDTAVYYCATNDDYWGQGTLVTVSS(SEQ ID: 245)
Anti-PD-2 LCVR (nivolumab)	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGSGSGT DFTLTISSLEPEDFAVYYCQQSSNWPRTFGQGTKVEIK(SEQ ID NO: 246)
Anti-PD-1 HCVR (2P17 or PD-06)	QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYYIHWVRQAPGQGLEWMGWIFPGSGNSKYNENF KGRVTLTADTSTSTVYMELSSLRSEDTAVYYCASETYDYGDYWGQGTLVTVSS (SEQ ID NO: 137)
Anti-PD-1 LCVR (2P17 or PD-06)	DIQMTQSPSFLSASVGDRVTITCKASQNVGTNVAWYQQKPGKAPKALIYSASYRYSGVPSRFSGSGS GTEFTLTISSLQPEDFATYYCQQYYSYPYTFGQGTKLEIK (SEQ ID NO: 138)
Anti-PD1 HCVR (2P16 or PD-05)	QVQLVQSGAEVKKPGASVKVSCKASGYTFTTYYIYWVRQAPGQGLEWMGGINPGNGGTNFNEKF KIRVTMTRDTSISTAYMELSSLRSEDTAVYYCARRYHGYDGGLDYWGQGTLVTVSS (SEQ ID NO: 135)
Anti-PD1 LCVR (2P16 or PD-05)	DIVLTQSPASLAVSPGQRATITCRASKSVSTSGFSYIHWYQQKPGQPPKLLIYLASNLESGVPARFSGS GSGTDFTLTINPVEANDTANYYCQHTWELPNTFGGGTKVEIK(SEQ ID NO: 136)
Anti-TIGIT HCVR (B21-35 or T-10)	QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYYMHWVRQAPGQGLEWMGIINPSGGRTSYAQMF QGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARDREEQWPVGGFDYWGQGTLVTVSS (SEQ ID NO: 125)
Anti-TIGIT LCVR (B21-35 or T-10)	DIQMTQSPSSLSASVGDRVTITCRASQSIRRYLNWYQQKPGKAPKLLIYSASNLQSGVPSRFSGSGSG TDFTLTISSLQPEDFATYYCQQSYIIPPTFGQGTKVEIK (SEQ ID NO: 126)
Anti-Lag-3 HCVR (2L2A.1)	QVQLVQSGAEVKKPGASVKVSCKASGYTLTDYYMNWMRQAPGQGLEWMGVINPYNGDTSYNQ KFKGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCVRDDGYYVHYFDYWGQGTLVTVSS(SEQ ID NO: 171)
Anti-Lag-3 LCVR (2L2A.1)	DIQMTQSPSSLSASVGDRVTITCRASQDISSRLTWLQQEPEKAPKRLIYATSSLDSGVPKRFSGSGSGT DFTLTISSLQPEDFATYYCLQYASSPLTFGGGTKVEIK(SEQ ID NO: 175)

FIG. 1

Functional Domain Sequences

VH/VL or Fusion Protein Domain	Amino Acid Sequences of Functional Domains
Trebananib long peptide	AQQEECEWDPWTCEHMGSGSATGGSGSTASSGSGSATHQEECEWDPWTCEHM (SEQ ID NO: 182)
Trebananib-short peptide	QEECEWDPWTCEHM(SEQ ID NO: 194)
Bevacizumab (Avastin) HCVR (wt)	EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTYAADF KRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSS (SEQ ID NO: 233)
Bevacizumab (Avastin) HCVR (mt)	EVQLVQSGGGVVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTYAAD FKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSS (SEQ ID NO: 235)
Bevacizumab (Avastin) LVCR	DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPSRFSGSGSG TDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIK (SEQ ID NO: 234)
Ranibizumab (Lucentis) HCVR	EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVRQAPGKGLEWVGWINTYTGEPTYAADF KRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPYYYGTSHWYFDVWGQGTLVTVSS (SEQ ID NO: 305)
Ranibizumab (Lucentis) LCVR	DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPSRFSGSGSGT DFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIK (SEQ ID NO: 306)
Aflibercept VEGF binding domain	GRELVIPCRVTSPNITVTLKKFPLDTLIPDGKRIIWDSRKGFIISNATYKEIGLLTCEATVNGHLYKTNYLT HRQTNTIIDVVLSPSHGIELSVGEKLVLNCTARTELNVGIDFNWEYPSSKHQHKKLVNRDLKTQSGSE MKKFLSTLTIDGVTRSDQGLYTCAASSGLMTKKNSTFVRVHEK (SEQ ID NO: 185)
TGF-β RII-ECD	IPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCVAVWR KNDENITLETVCHDPKLPYHDFILEDAASPKCIMKEKKKPGETFFMCSCSSDECNDNIIFSEEYNTSNP D (SEQ ID NO: 186)

FIG. 2

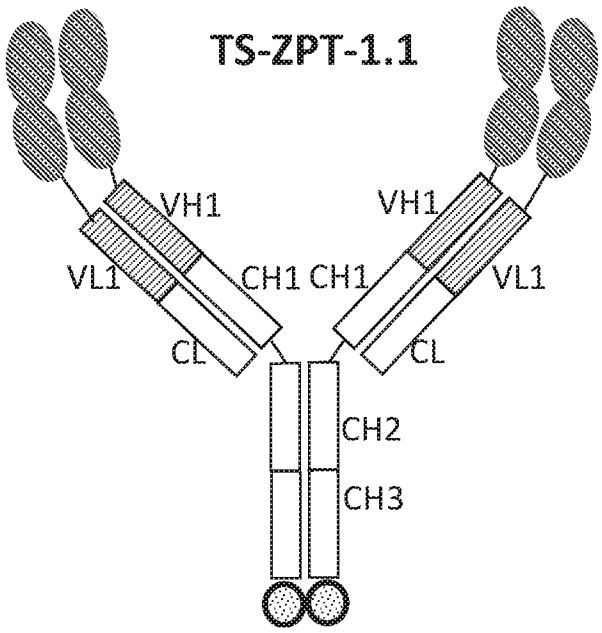


FIG. 3A

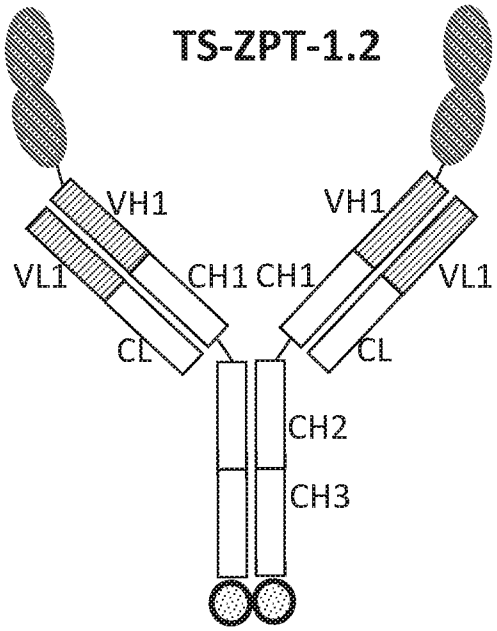


FIG. 3B

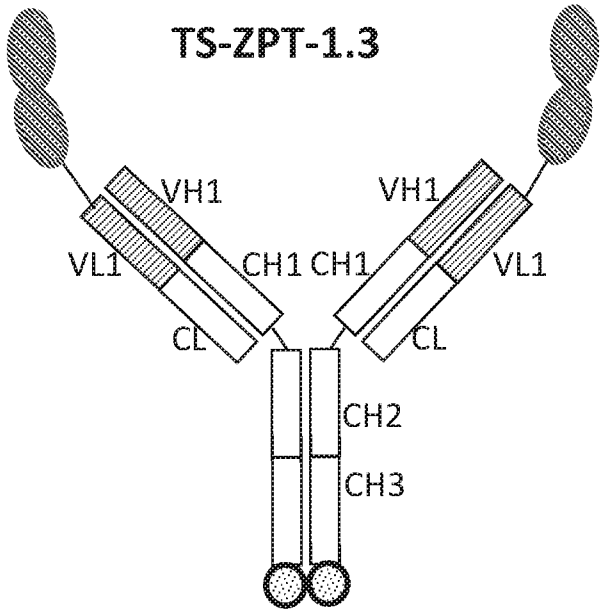


FIG. 3C

VH1-VL1 = Anti-PD1 or other Checkpoint, e. g., Anti-PDL1, Lag3, TIGIT, etc variable domain

Trebananib peptide

Aflibercept domain

TS-ZPT-2

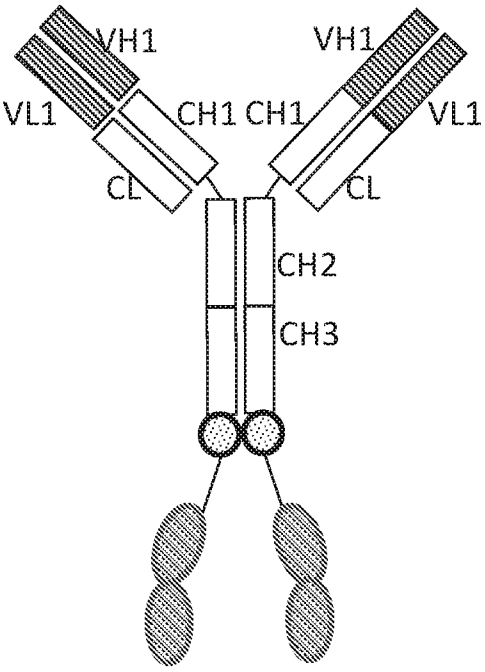


FIG. 3D

TS-ZPT-3L

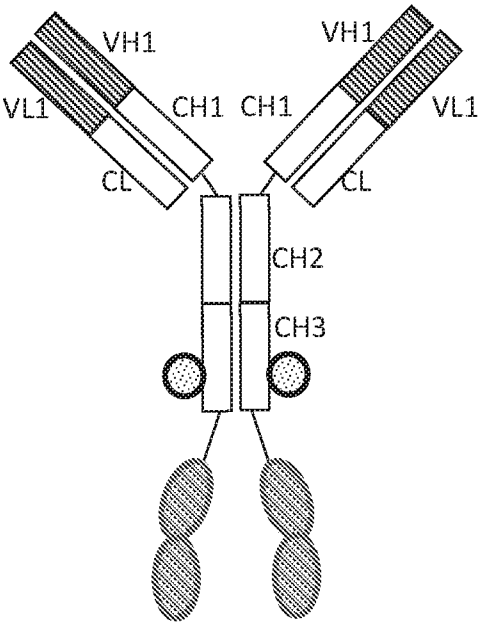


FIG. 3E

VH1-VL1 = Anti-PD1 or other Checkpoint Ab, e. g.,
Anti-PDL1, Lag3, TIGIT, etc variable domain

Trebananib peptide

Aflibercept domain

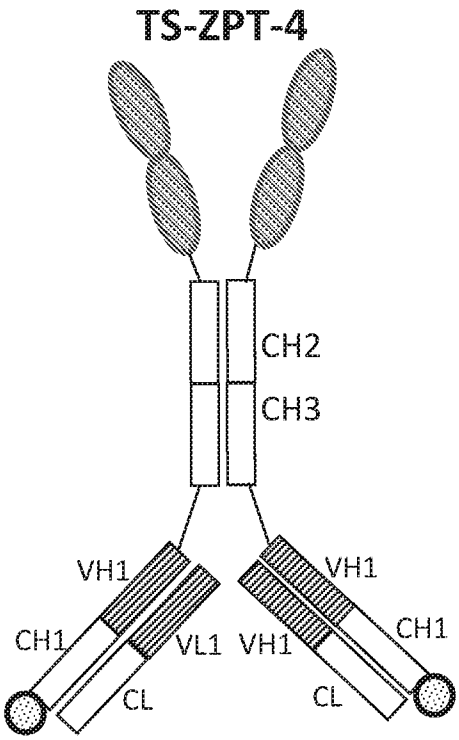


FIG. 3F

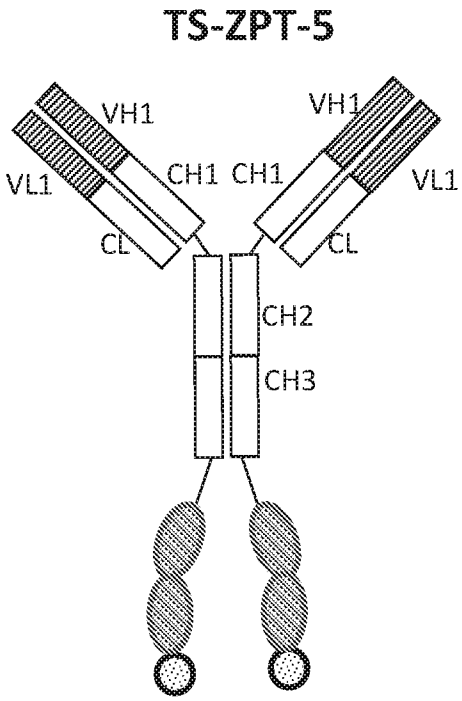


FIG. 3G

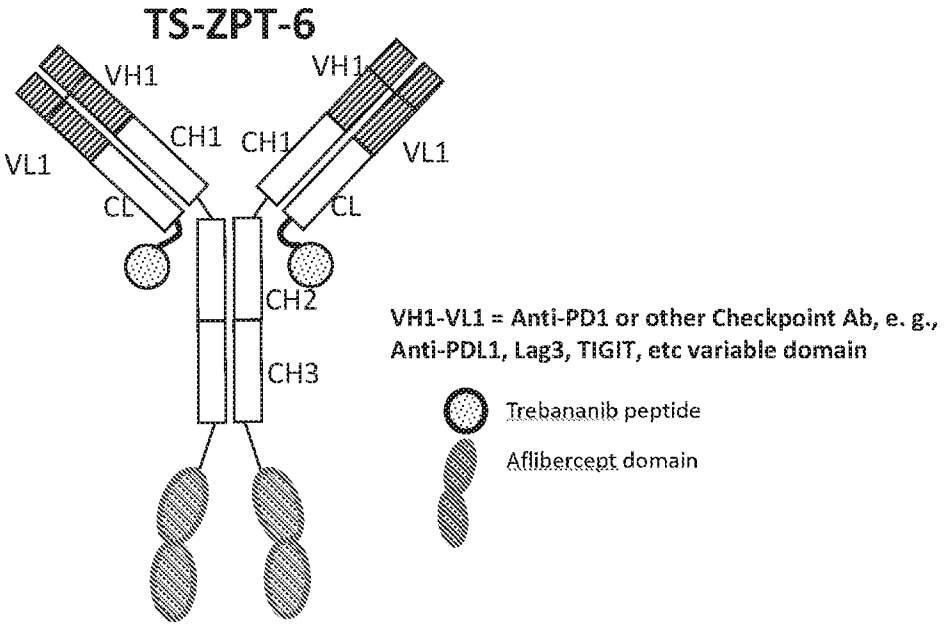


FIG. 3H

TS-ZPT-7

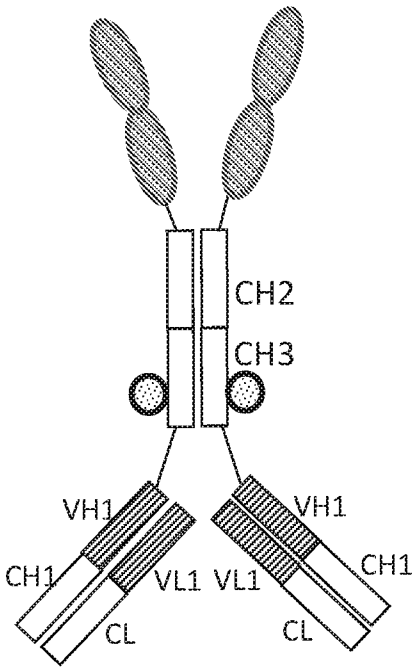


FIG. 4A

TS-ZPT-8

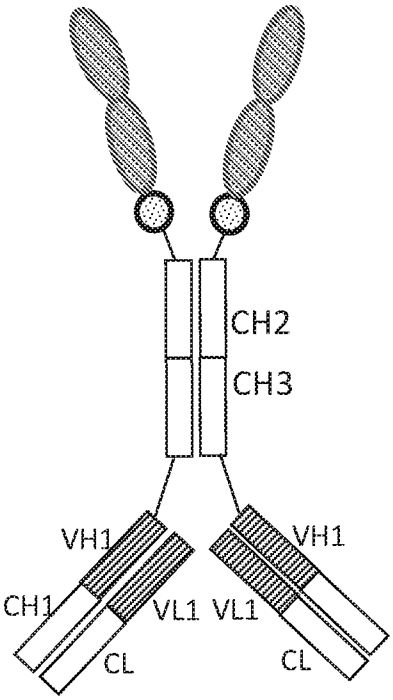


FIG. 4B

TS-ZPT-9

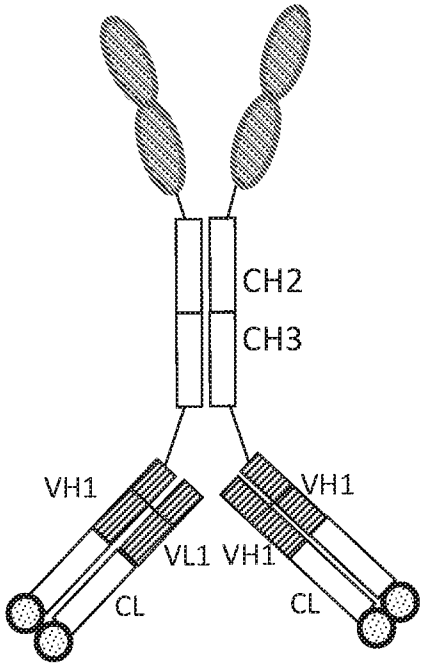


FIG. 4C

VH1-VL1 = Anti-PD1 or other Checkpoint Ab, e. g., Anti-PDL1, Lag3, TIGIT, etc variable domain

Trebananib peptide

Aflibercept domain

TS-LPT-1

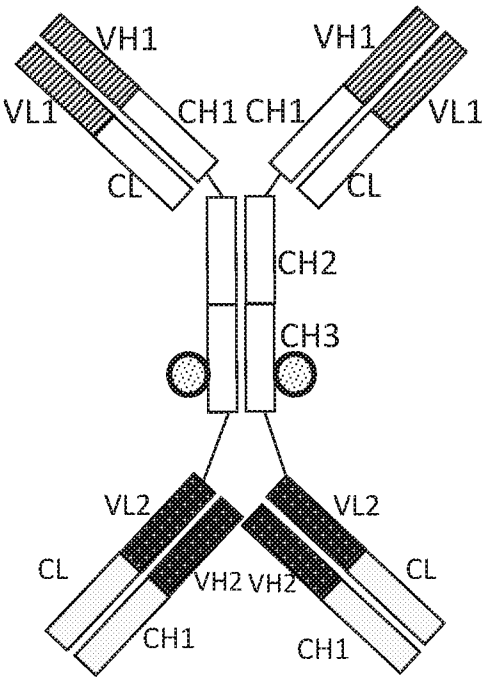


FIG. 5A

TS-LPT-2

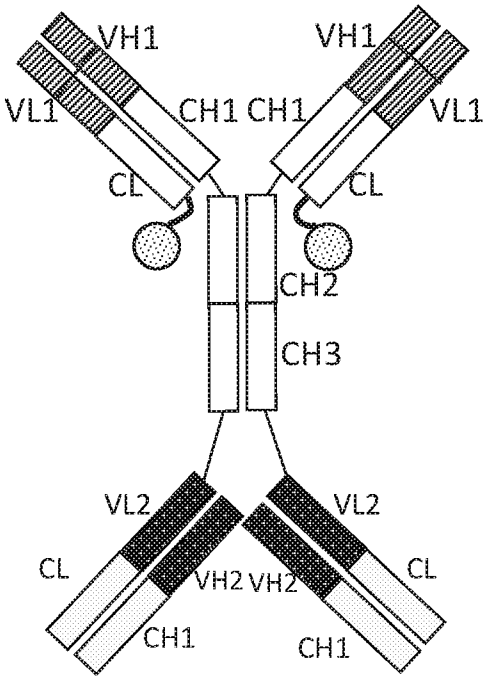


FIG. 5B

VH1-VL1 = Anti-PD1 or other Checkpoint Ab, e. g., Anti-PDL1, Lag3, TIGIT, etc variable domain

VH2-VL2 = Lucentis, Avastin, or other anti-VEGF

Trebananib peptide

TS-LPT-3

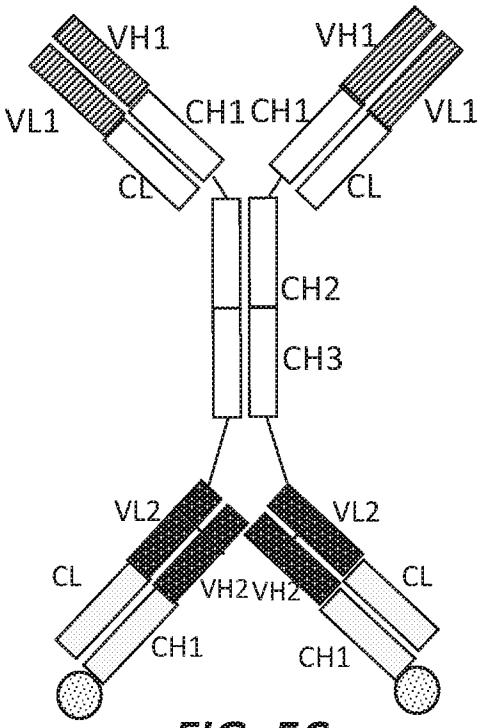


FIG. 5C

TS-LPT-4

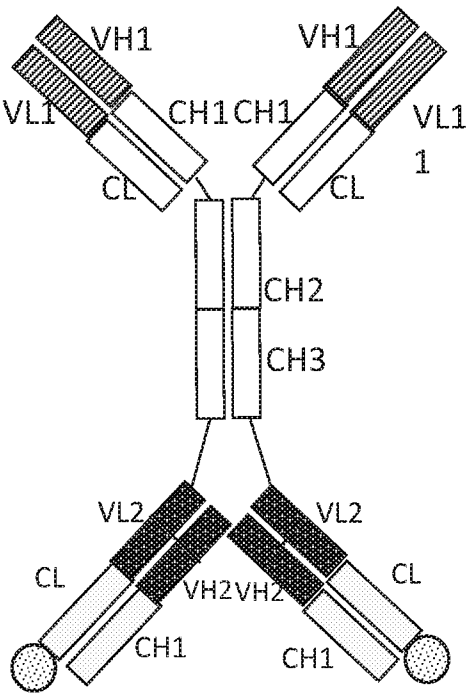


FIG. 5D

TS-LPT-5

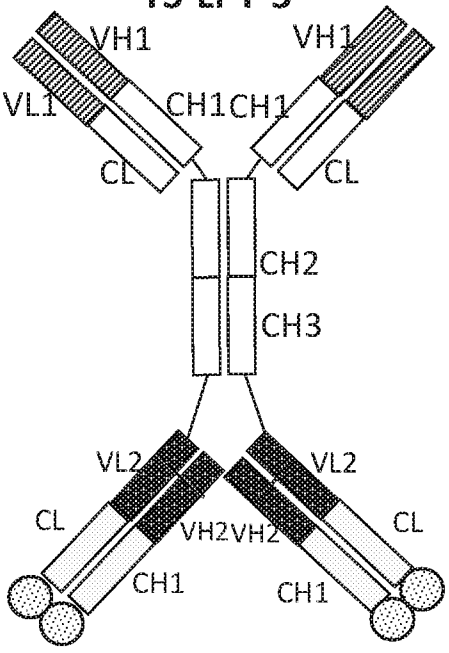


FIG. 5E

VH1-VL1 = Anti-PD1 or other Checkpoint Ab, e. g., Anti-PDL1, Lag3, TIGIT, etc variable domain

VH2-VL2 = Lucentis, Avastin, or other anti-VEGF

Trebananib peptide

TS-M3

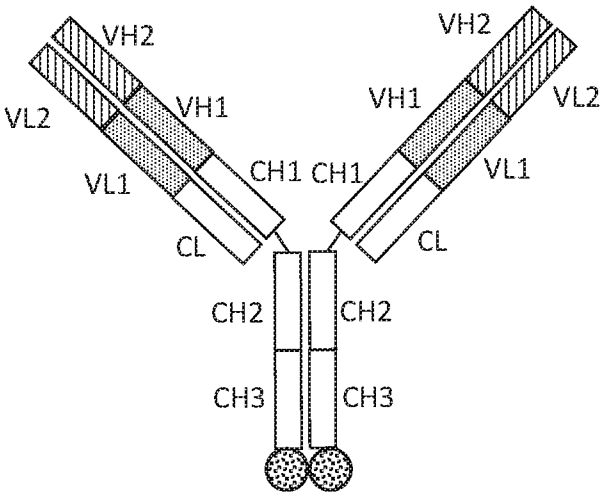


FIG. 5F

TS-M4

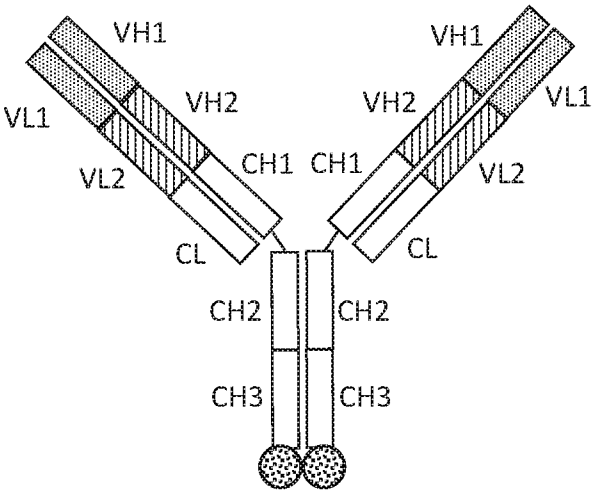


FIG. 5G

VH1-VL1 = Anti-PD1 or other Checkpoint Ab, e. g., Anti-PDL1, Lag3, TIGIT, etc variable domain

VH2-VL2 = Lucentis, Avastin, or other anti-VEGF

Trebananib peptide

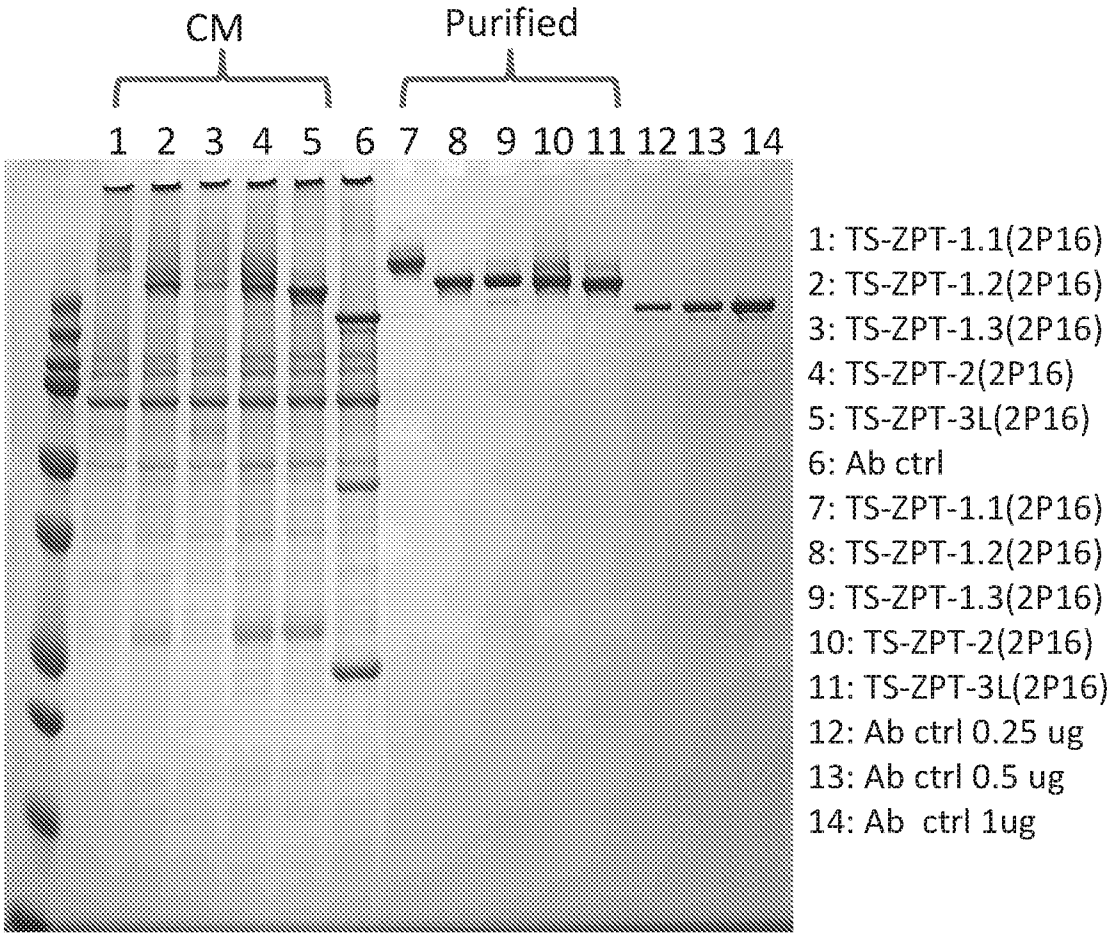

CM
Purified
1 2 3 4 5 6 7 8 9 10 11 12 13 14
1: TS-ZPT-1.1(2P16)
2: TS-ZPT-1.2(2P16)
3: TS-ZPT-1.3(2P16)
4: TS-ZPT-2(2P16)
5: TS-ZPT-3L(2P16)
6: Ab ctrl
7: TS-ZPT-1.1(2P16)
8: TS-ZPT-1.2(2P16)
9: TS-ZPT-1.3(2P16)
10: TS-ZPT-2(2P16)
11: TS-ZPT-3L(2P16)
12: Ab ctrl 0.25 ug
13: Ab ctrl 0.5 ug
14: Ab ctrl 1ug

FIG. 6A

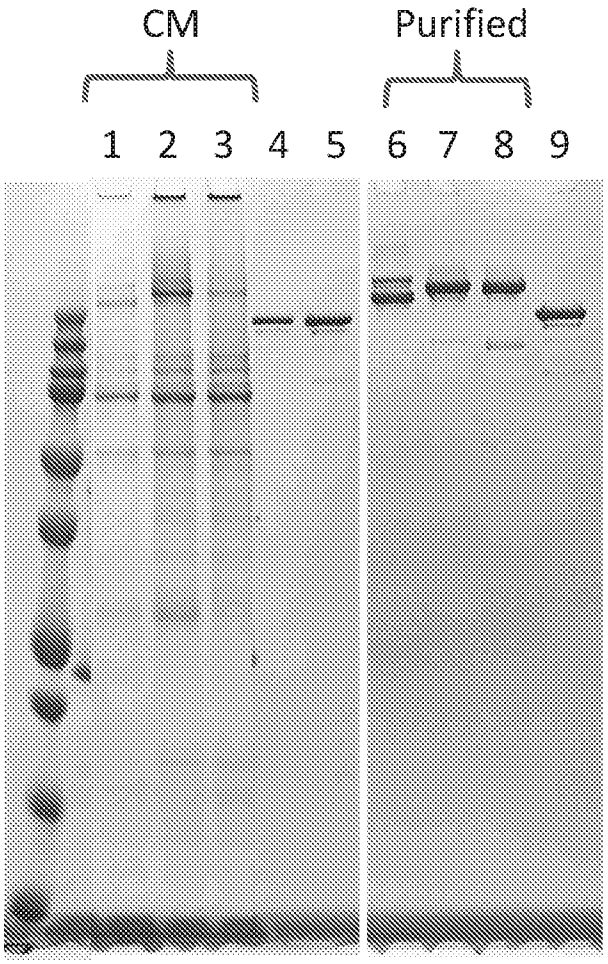

CM
Purified
1 2 3 4 5 6 7 8 9
1: TS-LPT-1(2P16)
2: TS-ZPT-5(2P16)
3: TS-ZPT-6(2P16)
4: Ab ctrl 0.5 ug
5: Ab ctrl 1 ug
6: TS-LPT-1(2P16)
7: TS-ZPT-5(2P16)
8: TS-ZPT-6(2P16)
9: Ab ctrl 1 ug

FIG. 6B

Trispecific Antitumor Antagonists Block Interaction of PD-1 and PD-L1

Antagonist	IC50 (nM)
Benchmark	2.89
TS-ZPT-1.1(2P16)	139.70
TS-ZPT-1.2(2P16)	14.77
TS-ZPT-1.3(2P16)	32.97
TS-ZPT-2(2P16)	4.62
TS-ZPT-3(2P16)	3.55
anti-PD-1 mAb	2.48

FIG. 7A

Antagonist	IC50 (nM)
Benchmark	0.64
TS-ZPT-5(2P16)	1.30
TS-ZPT-6(2P16)	1.16

FIG. 7B

Trispecific Antitumer Antagonists Block Interaction of VEGF and VEGFR-2

Antagonist	IC50 (nM)
TS-ZPT-2(2P16)	0.11
TS-ZPT-3(2P16)	0.50
TS-ZPT-5(2P16)	0.26
Aflibercept	0.20
Bevacizumab	1.57

FIG. 8A

Antagonist	IC50 (nM)
TS-ZPT-2(2P16)	0.17
TS-ZPT-5(2P16)	1.78
TS-ZPT-6(2P16)	0.66
Aflibercept	0.29
BVZ	1.36

FIG. 8B

Trispecific Antitumer Antagonists Block Interaction of Ang2 and Tie2

Antagonist	IC50 (nM)
TS-ZPT-1.2	0.95
TS-ZPT-2	0.65
TS-ZPT-5	0.40
TS-ZPT-6	0.55
TS-LPT-1	1.26
BVZ-Trebananib	0.57

FIG. 9

Size Exclusion Chromatography of HEK293 transiently produced molecules

Construct	HMW%	Dimer%	LMW%
2P17	1.0	97.3	1.7
TS-ZPT-2(2P17)	0.0	81.5	18.5
TS-ZPT-3L(2P17)	0.0	95.1	4.9
TS-ZPT-5(2P17)	0.6	65.3	34.1

FIG. 10

Size Exclusion Chromatography of stable CHO cell produced molecules

Construct	HMW%	Dimer%	LMW%
TS-ZPT-2(2P17)	0.6	89.9	9.7
TS-ZPT-5(2P17)	0.7	92.4	6.9
TS-ZPT-3L(2P17) Pool 1	0.5	97.7	1.8
TS-ZPT-3L(2P17) Pool 2	0.9	97.4	1.7

FIG. 11

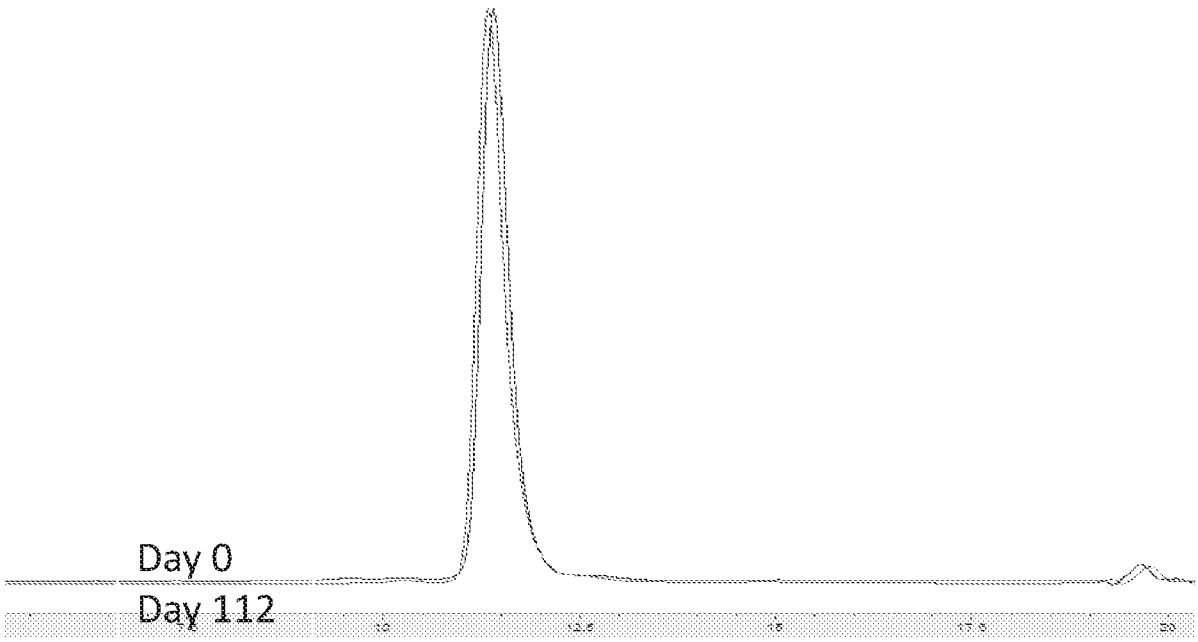


FIG. 12A

Size Exclusion Chromatography of 4 degree stability sample

Day	HMW%	Dimer%	LMW%
0	1.3	97.2	1.5
112	0.8	97.1	2.1

FIG. 12B

Exemplary Amino acid sequences of TS-ZPT-3L

Heavy Chain with peptide and fusion protein

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYYIHWVRQAPGQGLEWMGWIFPGSGNS KYNENFKGRVTLTADTSTSTVYMELSSLRSEDTAVYYCASETYDYGDYWGQGTLVTVSSAST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLS SVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMGGAQQE ECEWDPWTCEHMGSGSATGGSGSTASSGSGSATHQEECEWDPWTCEHMGGTKNQVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS VMHEALHNHYTQKSLSLSPGKGGGGSGGGGSGGGGSSDTGRPFVEMYSEIPEIIHMTEG RELVIPCRVTSPNITVTLKKFPLDTLIPDGKRIIWDSRKGFIISNATYKEIGLLTCEATVNGHLYK TNYLTHRQTNTIIDVVLSPSHGIELSVGEKLVLNCTARTELNVGIDFNWEYPSSKHQHKKLV NRDLKTQSGSEMKKFLSTLTIDGVTRSDQGLYTCAASSGLMTKKNSTFVRVHEK (**SEQ ID NO: 202**)

Anti-PD-1 2P17 HCVR

Trebananib peptide

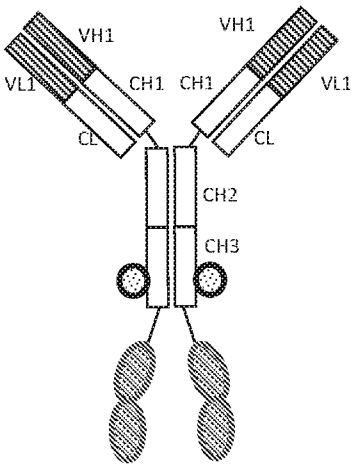
Aflibercept domain

Light Chain

DIQMTQSPSFLSASVGDRVTITCKASQNVGTNVAWYQQKPGKAPKALIYSASYRYSGVPS RFSGSGSGTEFTLTISSLQPEDFATYYCQQYYSYPYTFGQGTKLEIKRTVAAPSVFIFPPSDEQ LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKAD YEKHKVYACEVTHQGLSSPVTKSFNRGEC (**SEQ ID NO: 201**)

Anti-PD-1 2P17 LCVR

FIG. 13A



TS-ZPT-3L(2P17)

VH1-VL1 = Anti-PD1 (2P17)

Trebananib peptide

Aflibercept domain

FIG. 13B

Exemplary Amino acid sequences of TS-ZPT-3S

Heavy Chain with peptide and fusion protein

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYYIHWVRQAPGQGLEWMGWIFPGSGNS KYNENFKGRVTLTADTSTSTVYMELSSLRSEDTAVYYCASETYDYGDYWGQGTLVTVSSAST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLS SVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMGGQEECE WDPWTCEHMGGTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGGGSGGGGSGGGGSSD TGRPFVEMYSEIPEIIHMTEGRELVIPCRVTSPNITVTLKKFPLDTLIPDGKRIIWDSRKGFIIS NATYKEIGLLTCEATVNGHLYKTNYLTHRQTNTIIDVVLSPSHGIELSVGEKLVLNCTARTELN VGIDFNWEYPSSKHQHKKLVNRDLKTQSGSEMKKFLSTLTIDGVTRSDQGLYTCAASSGL MTKKNSTFVRVHEK (**SEQ ID NO: 200**)

Anti-PD-1 2P17 HCVR

Trebananib short peptide

Aflibercept domain

Light Chain

DIQMTQSPSFLSASVGDRVTITCKASQNVGTNVAWYQQKPGKAPKALIYSASYRYSGVPS RFSGSGSGTEFTLTISSLQPEDFATYYCQQYYSYPYTFGQGTKLEIKRTVAAPSVFIFPPSDEQ LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKAD YEKHKVYACEVTHQGLSSPVTKSFNRGEC (**SEQ ID NO: 201**)

Anti-PD-1 2P17 LCVR

FIG. 14A

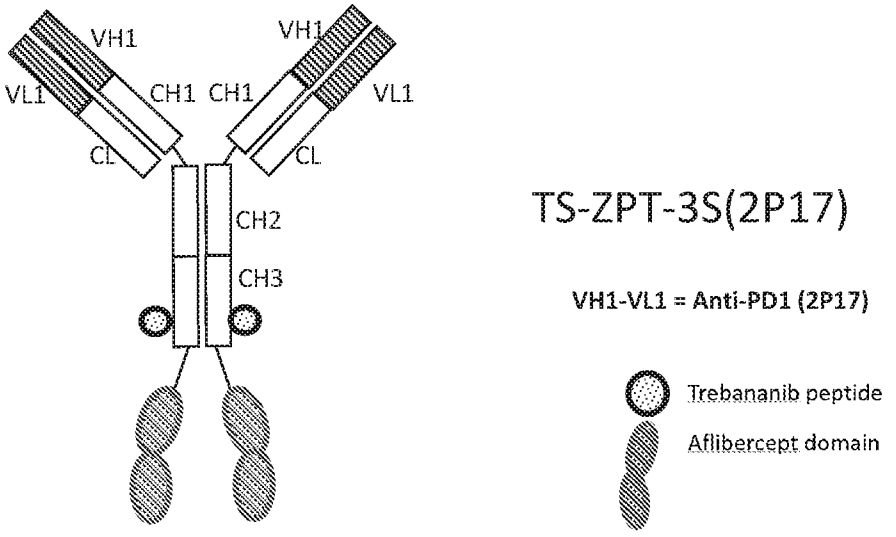
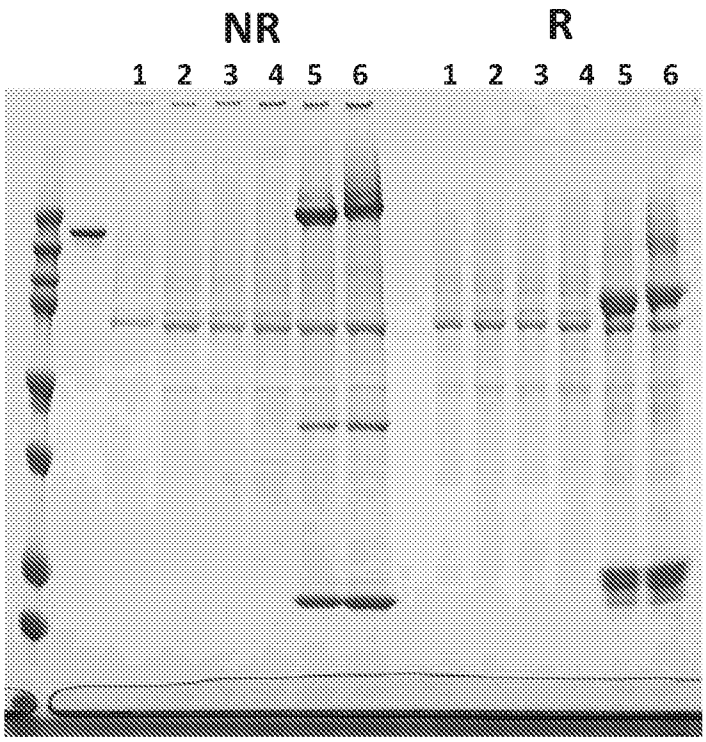


FIG. 14B



1: TS-M3 (Nivolumab)
2: TS-M4 (Nivolumab)
3: TS-M3(2P16)
4: TS-M3(2P17)
5: TS-ZPT-3S(2P17)
6: TS-ZPT-3L(2P17)

FIG. 15

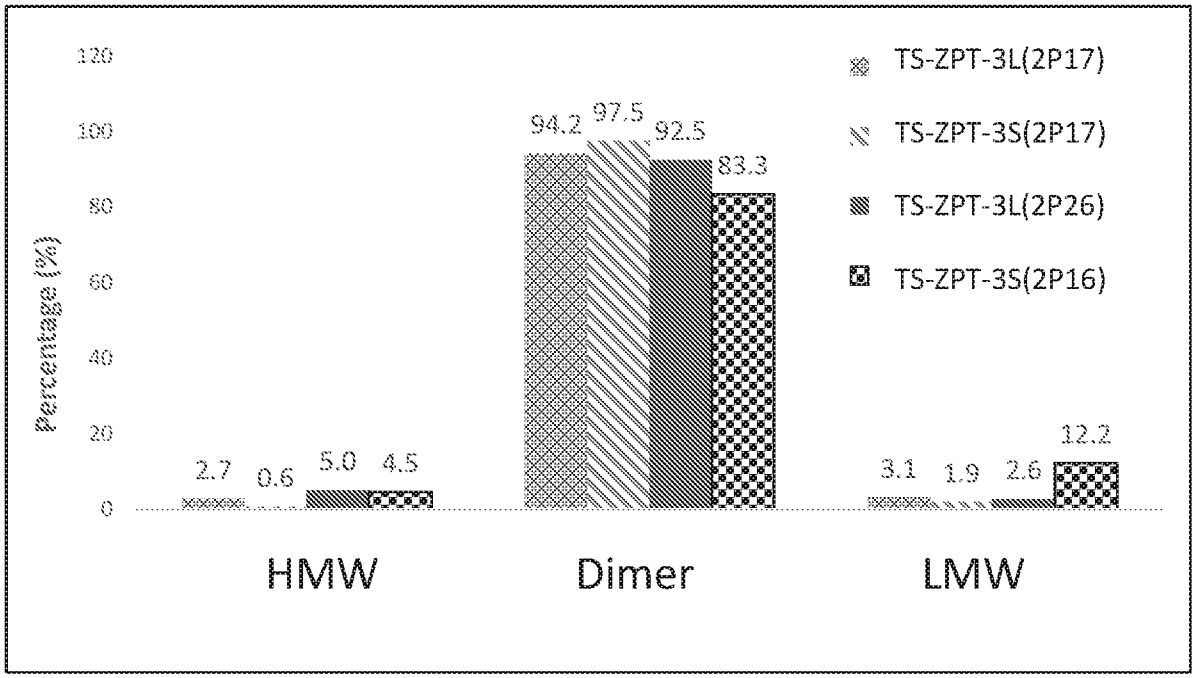

120
100
80
60
40
20
0
Percentage (%)
2.7
0.6
5.0
4.5
94.2
97.5
92.5
83.3
3.1
1.9
2.6
12.2
HMW
Dimer
LMW
TS-ZPT-3L(2P17)
TS-ZPT-3S(2P17)
TS-ZPT-3L(2P26)
TS-ZPT-3S(2P16)

FIG. 16

Day	TS-ZPT-3L(2P17)			TS-ZPT-3S(2P17)		
	HMW %	Dimer %	LMW %	HMW %	Dimer %	LMW %
0	3.0	93.7	3.4	0.5	97.9	1.6
7	3.2	93.8	3.0	0.8	97.9	1.4
14	3.2	93.7	3.0	0.8	97.9	1.3
20	3.3	93.6	3.1	0.6	07.7	1.7
28	3.3	93.6	3.1	0.7	07.6	1.6
35	3.5	93.2	3.3	0.6	97.6	1.8
42	3.4	93.2	3.4	0.6	97.7	1.7
48	3.2	93.2	3.6	0.8	97.5	1.7

FIG. 17

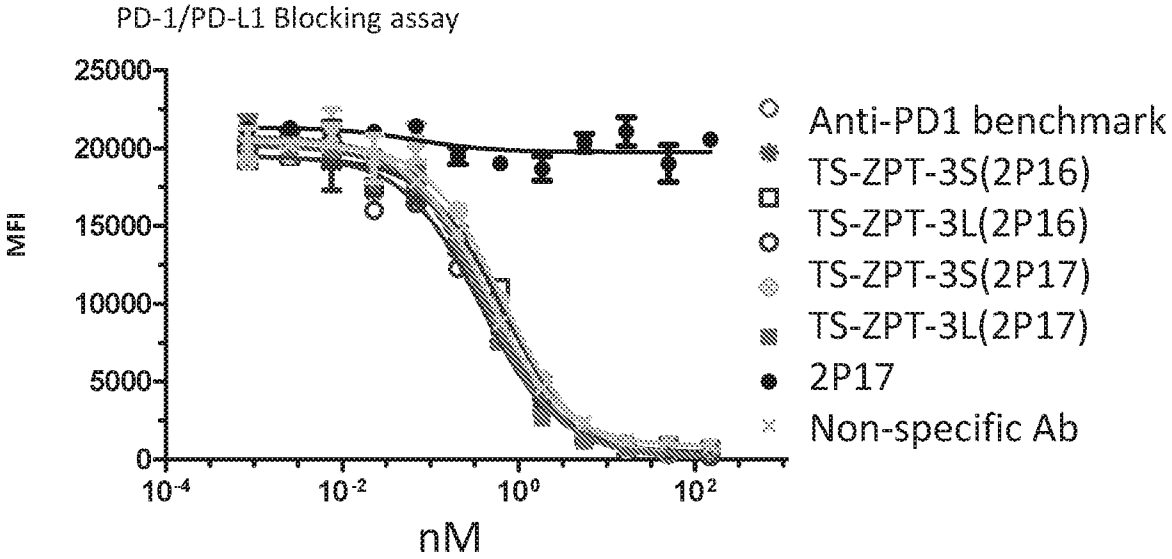


FIG. 18A

PD-1/PDL-1	IC50 (nM)
Anti-PD1 Benchmark Ab	0.44
TS-ZPT-3S(2P16)	0.43
TS-ZPT-3L(2P16)	0.64
TS-ZPT-3S(2P17)	0.37
TS-ZPT-3(2P17)	0.65
2P17	0.37
Control Ab	N/A

FIG. 18B

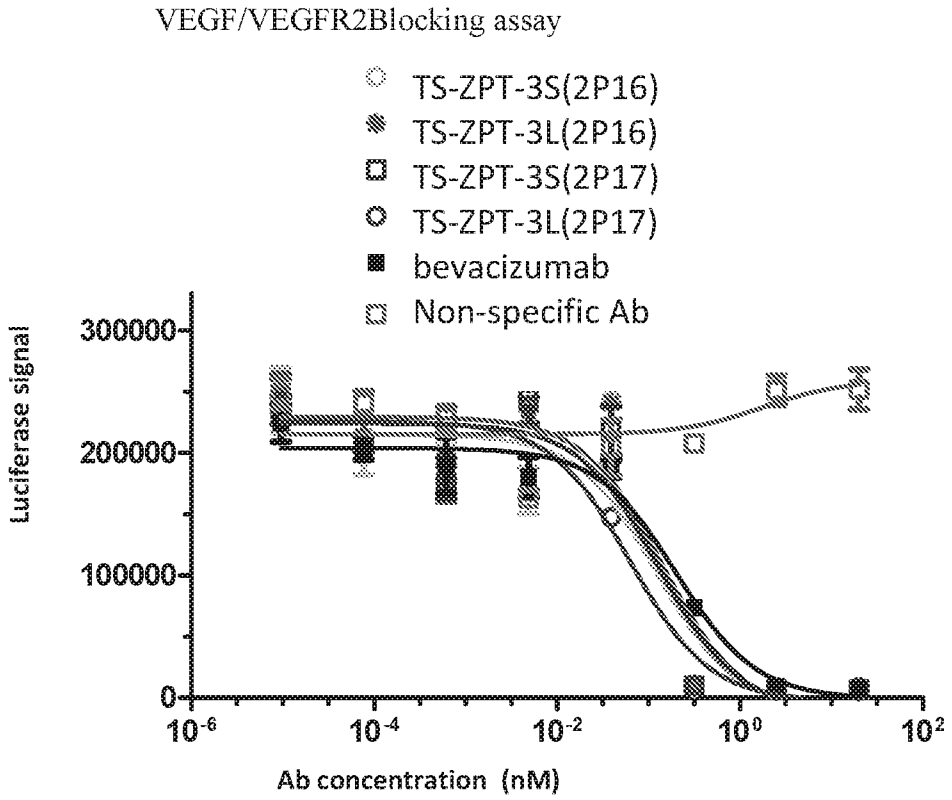


FIG. 19A

VEGF/VEGFR2	IC50 (pM)
TS-ZPT-3S(2P16)	123
TS-ZPT-3L(2P16)	142
TS-ZPT-3S(2P17)	129
TS-ZPT-3L(2P17)	63
Bevacizumab	202
Control	No blocking

FIG. 19B

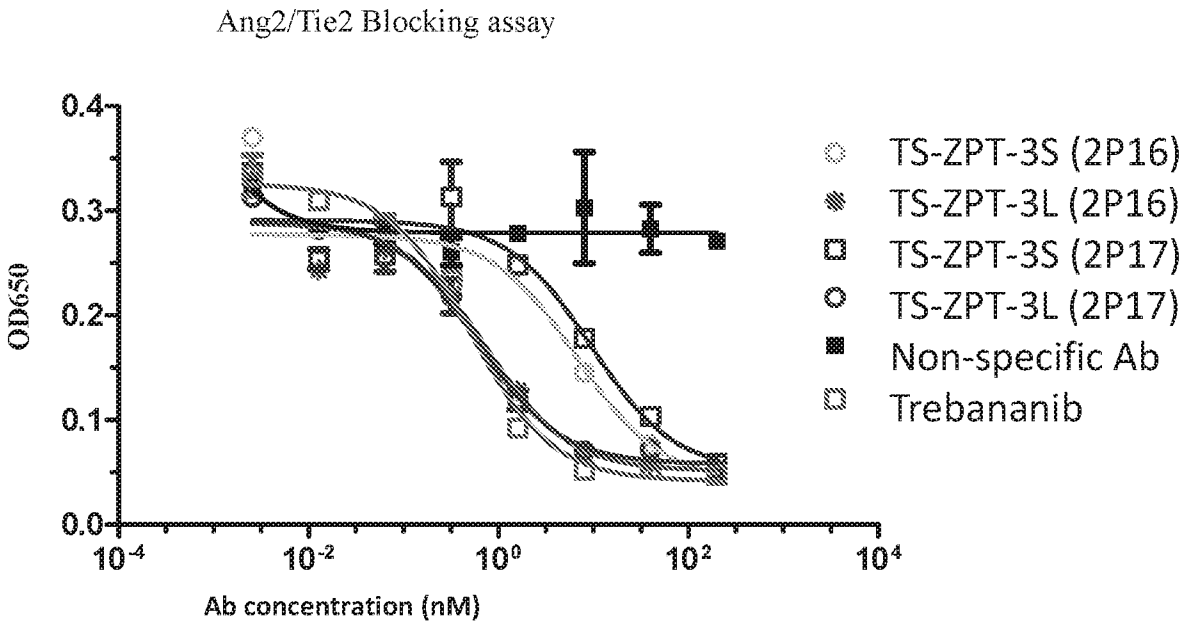


FIG. 20A

Ang2/Tie2	IC50 (nM)
TS-ZPT-3S(2P16)	6.71
TS-ZPT-3L (2P16)	0.73
TS-ZPT-3S(2P17)	9.34
TS-ZPT-3L(2P17)	0.60
Control Ab	N/A
Trebananib	0.50

FIG. 20B

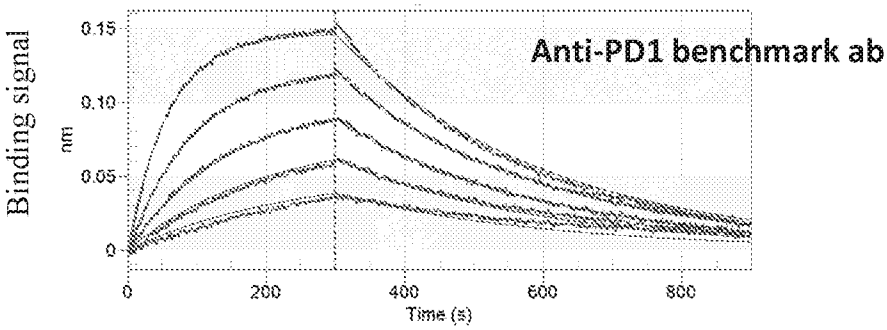


FIG. 21A

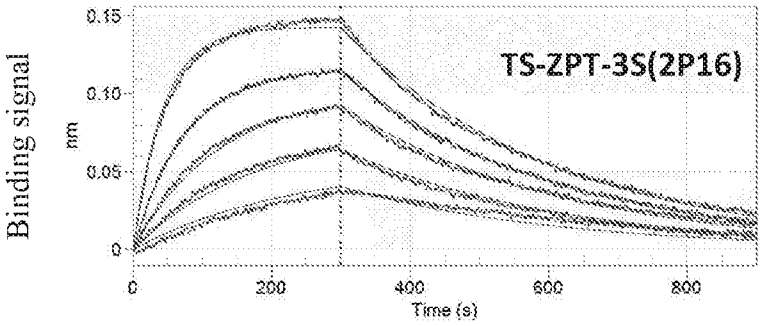


FIG. 21B

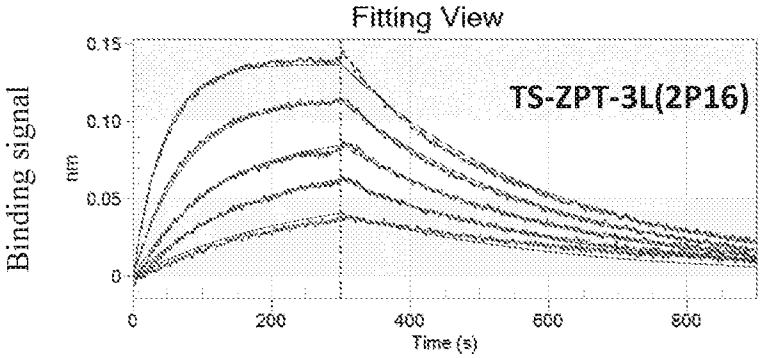


FIG. 21C

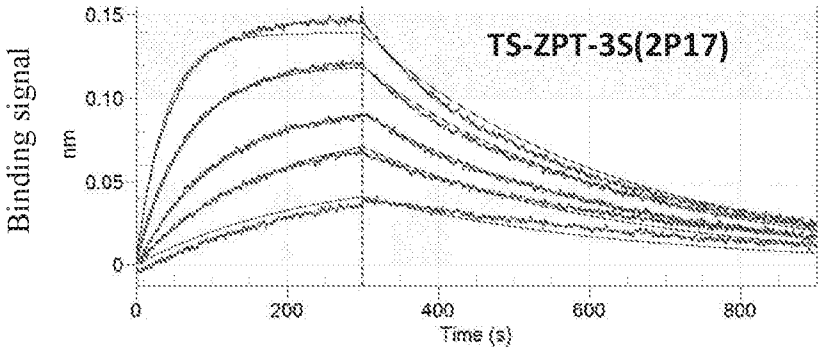


FIG. 21D

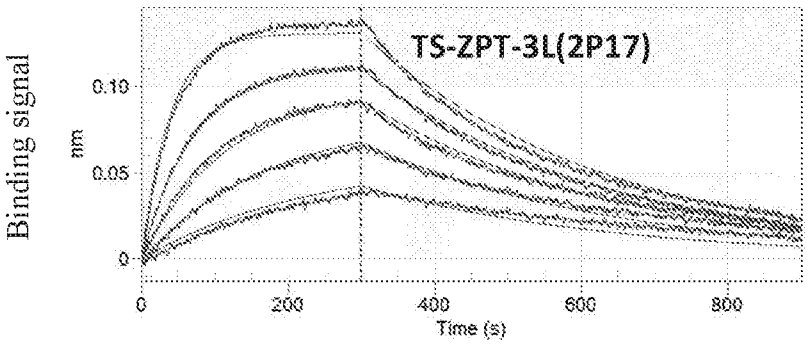


FIG. 21E

	PD-1-His (R&D)		
Ab name	K_D (nM)	$K_a(M^{-1}s^{-1})$	$K_d(s^{-1})$
Anti-PD1 Benchmark	7.32E-09	1.98E+05	1.45E-03
TS-ZPT-3S(2P16)	4.76E-09	3.35E+05	1.59E-03
TS-ZPT-3L(2P16)	4.67E-09	3.33E+05	1.55E-03
TS-ZPT-3S(2P17)	3.71E-09	3.94E+05	1.46E-03
TS-ZPT-3L(2P17)	3.84E-09	3.82E+05	1.47E-03

FIG. 21F

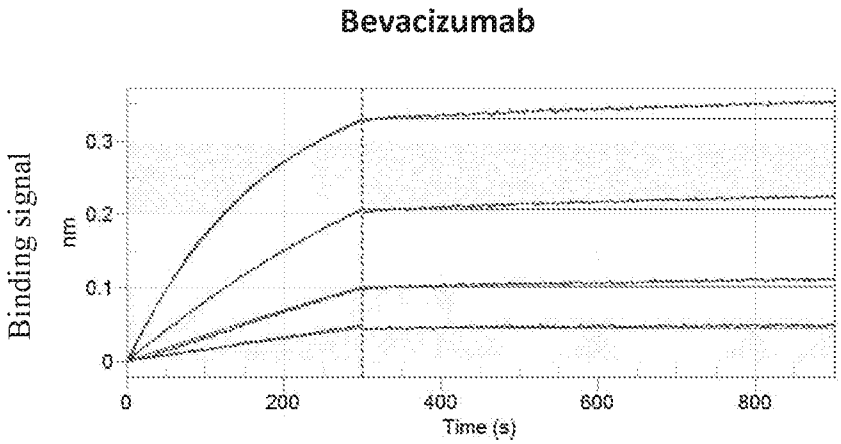


FIG. 22A

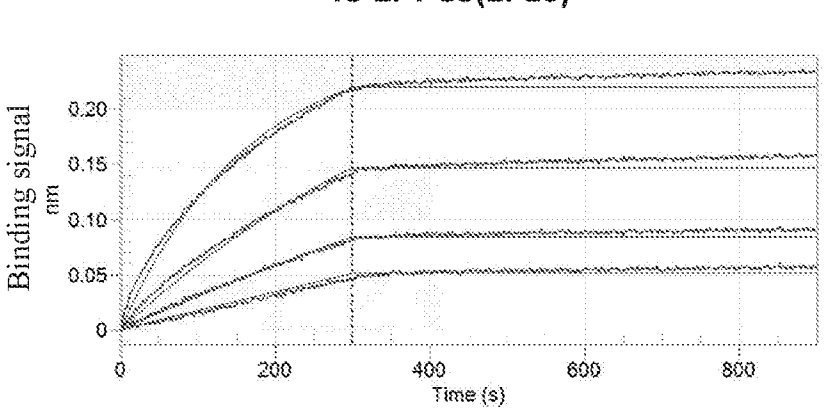


FIG. 22B

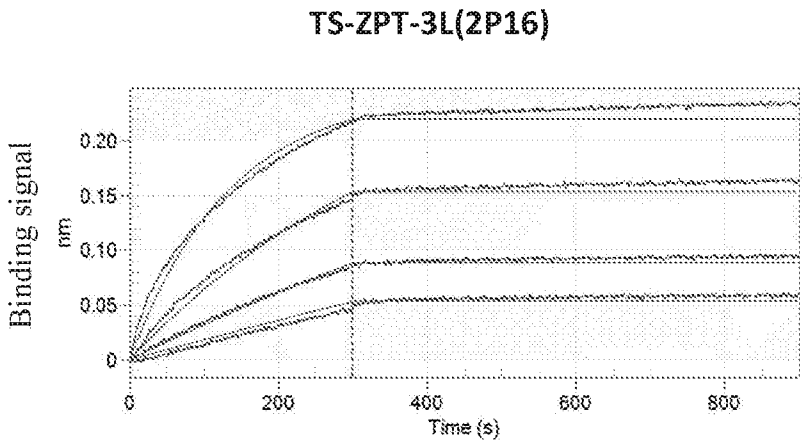


FIG. 22C

TS-ZPT-3S(2P17)

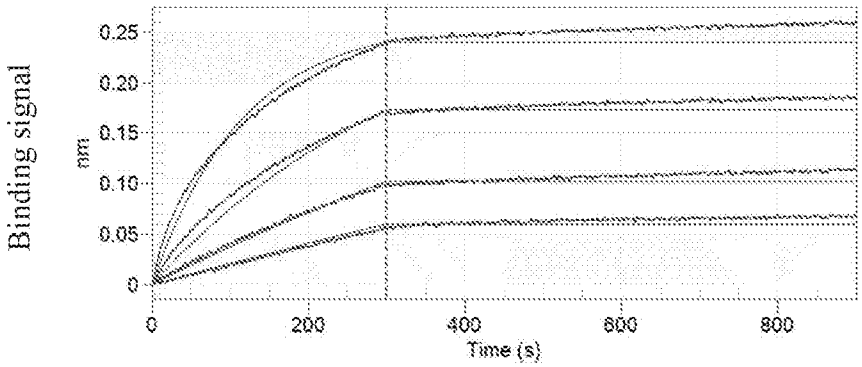


FIG. 22D

TS-ZPT-3L(2P17)

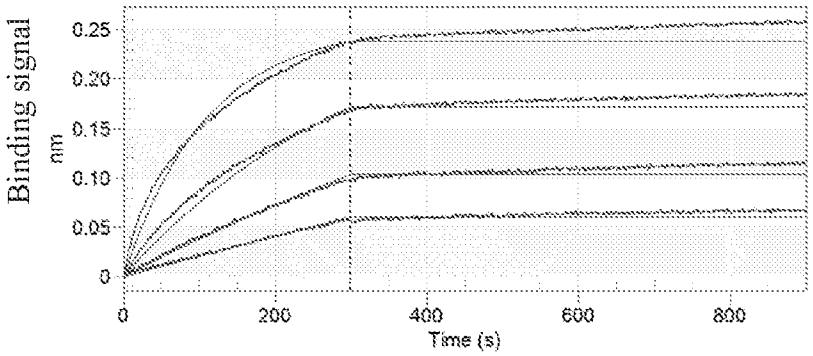


FIG. 22E

	rhVEGF165		
Ab name	K_D (pM)	$K_a(M^{-1}s^{-1})$	$K_d(s^{-1})$
Bevacizumab	20.4	1.97E+05	4.00E-06
TS-ZPT-3S(2P16)	6.3	6.83E+05	4.31E-06
TS-ZPT-3L(2P16)	5.3	8.45E+05	4.47E-06
TS-ZPT-3S(2P17)	5.2	9.74E+05	5.03E-06
TS-ZPT-3L(2P17)	4.9	1.02E+06	5.00E-06

FIG. 22F

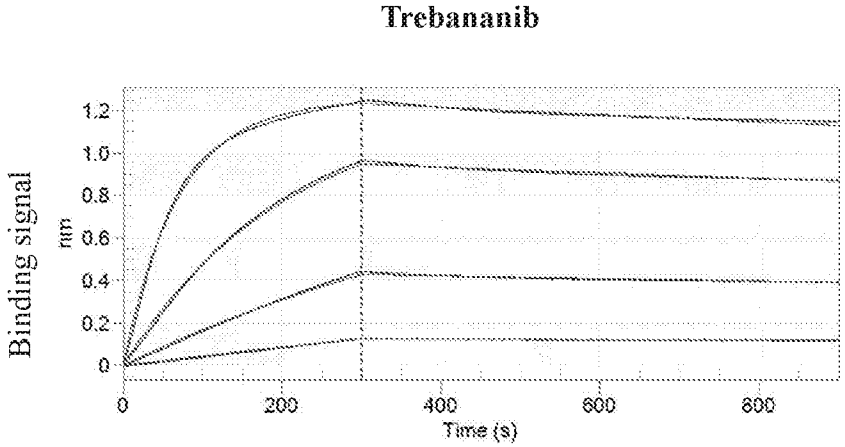


FIG. 23A

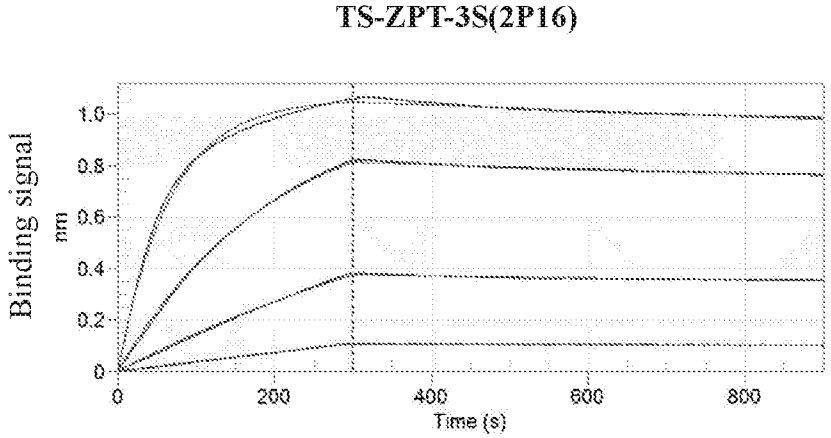


FIG. 23B

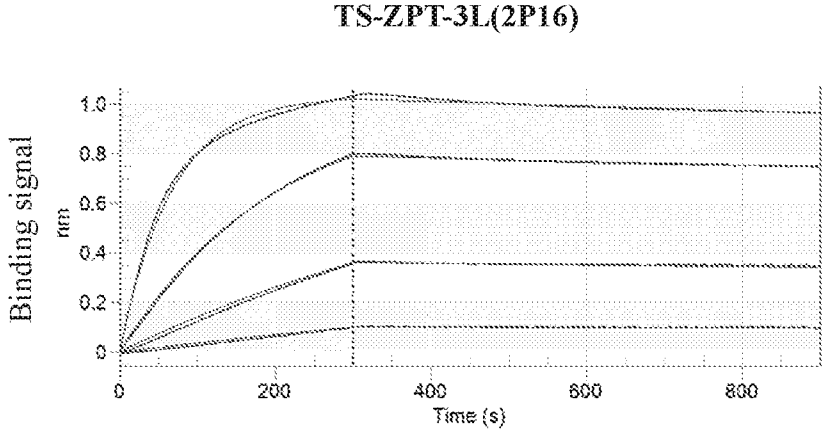


FIG. 23C

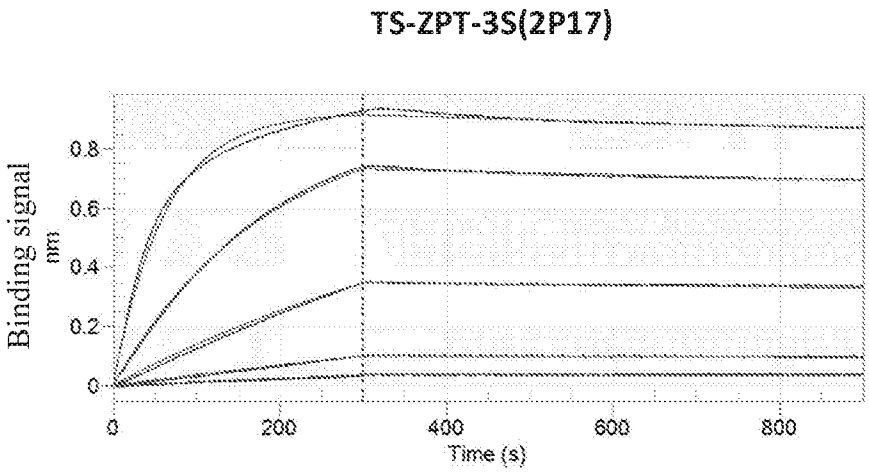


FIG. 23D

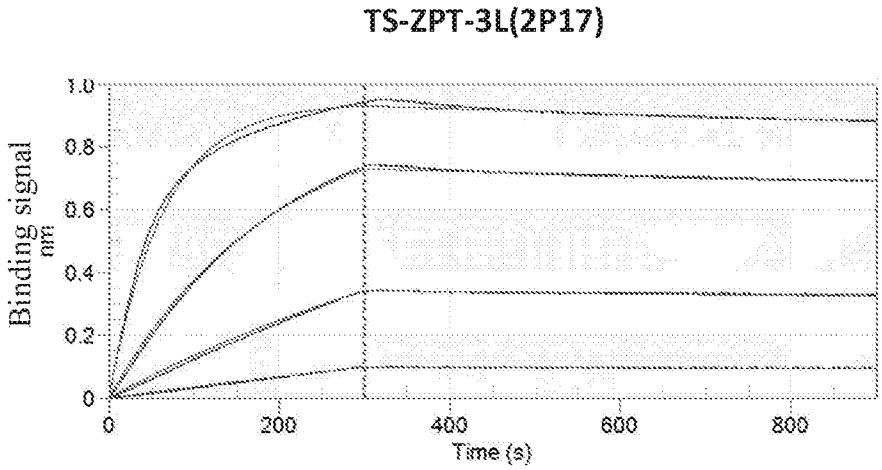


FIG. 23E

	rhAng2-His		
Ab name	K_D (nM)	$K_a(M^{-1}s^{-1})$	$K_d(s^{-1})$
Trebananib	1.23	1.19E+05	1.46E-04
TS-ZPT-3S(2P16)	0.87	1.26E+05	1.10E-04
TS-ZPT-3L(2P16)	0.76	1.24E+05	9.39E-05
TS-ZPT-3S(2P17)	0.65	1.34E+05	8.66E-05
TS-ZPT-3L(2P17)	0.70	1.30E+05	9.05E-05

FIG. 23F

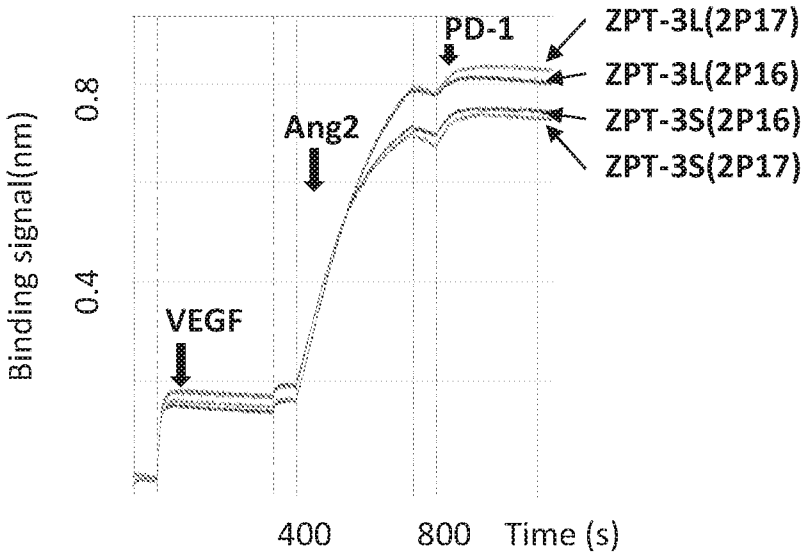

Binding signal(nm)
0.8
0.4
VEGF
Ang2
PD-1
ZPT-3L(2P17)
ZPT-3L(2P16)
ZPT-3S(2P16)
ZPT-3S(2P17)
400
800
Time (s)

FIG. 24

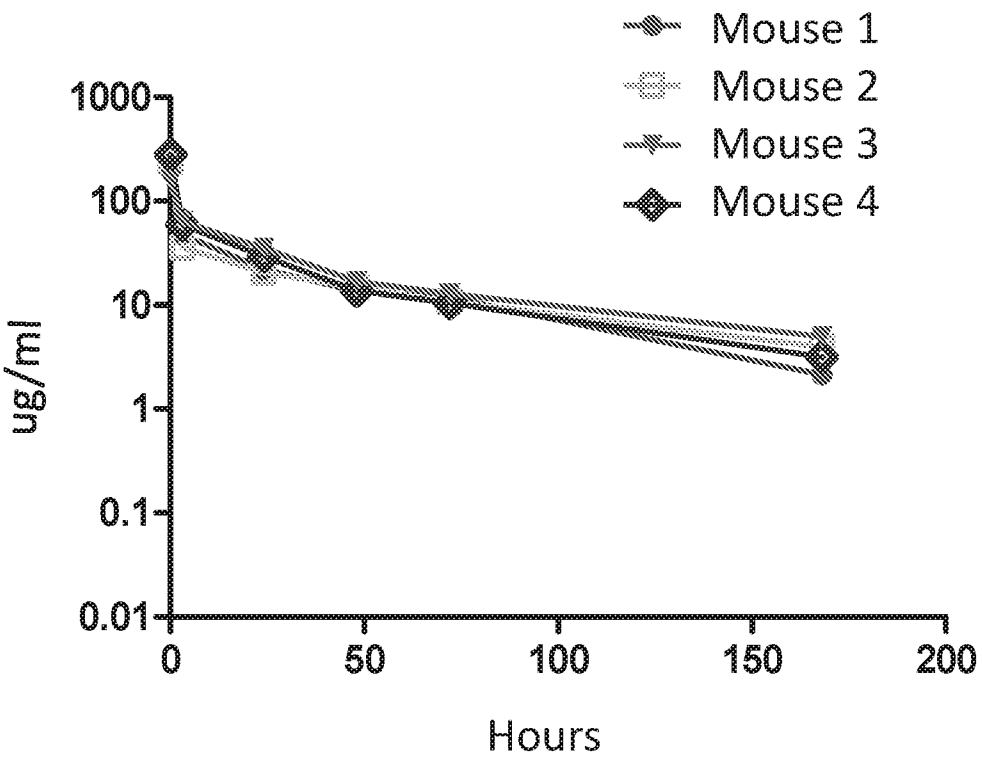

Mouse 1
Mouse 2
Mouse 3
Mouse 4
1000
100
10
1
0.1
0.01
ug/ml
0
50
100
150
200
Hours

FIG. 25

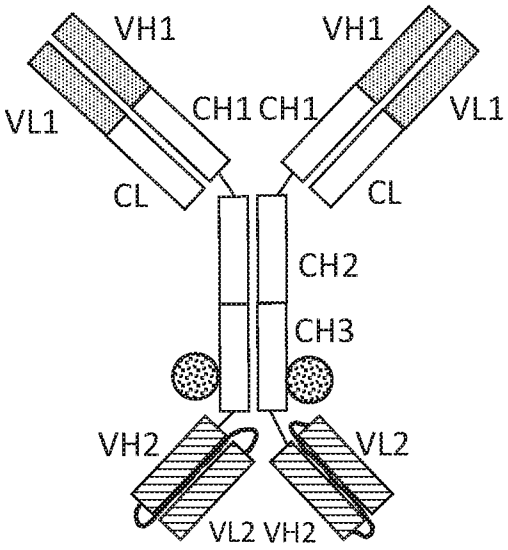


FIG. 26A

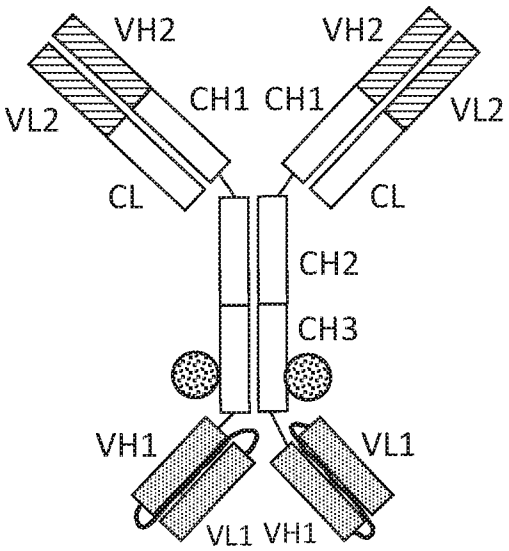
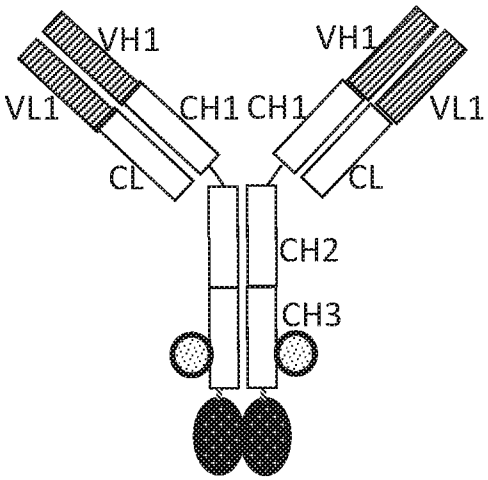


FIG. 26B

VH1 VL1 = Checkpoint #1 e.g. Anti-PD-1, anti-PD-L1 or any other mAb variable domain
VH2 VL2 = Checkpoint #2 Anti-LAG3, anti-TIGIT or any other mAb variable domain
= trebananib or other biological peptide

TS-A1BT-1



VH1-VL1 = Avastin (m), Avastin (WT), Lucentis, or other anti-VEGF Ab

TGFRβR II ECD

Trebananib peptide

FIG. 27

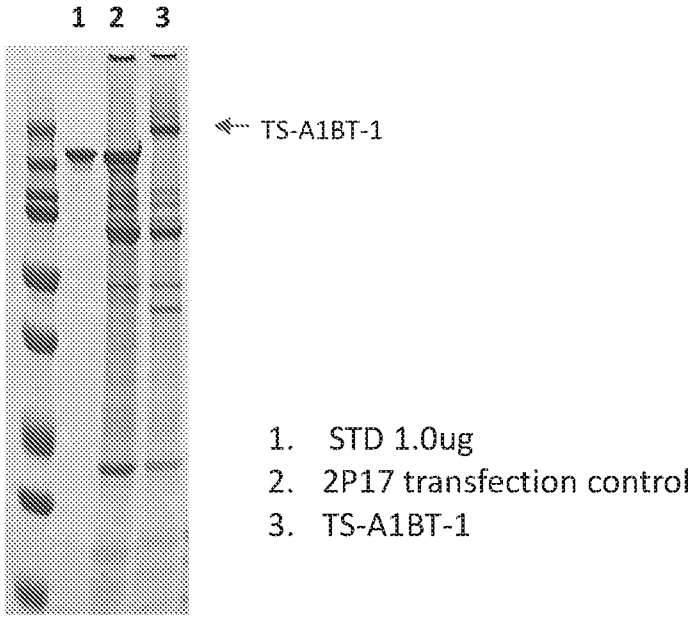

1 2 3
TS-A1BT-1
1. STD 1.0ug
2. 2P17 transfection control
3. TS-A1BT-1

FIG. 28

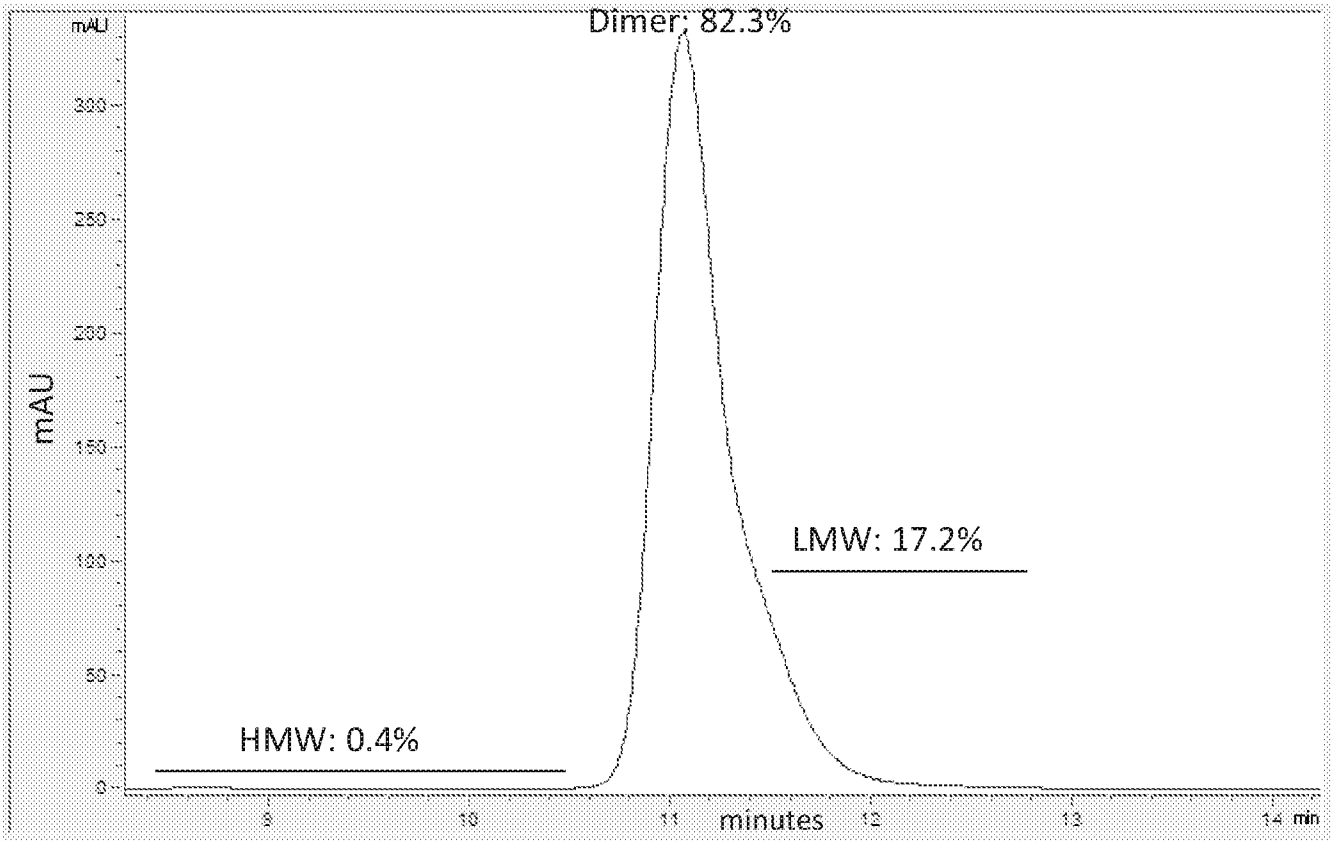

mAU
300
250
200
150
100
50
0
mAU
Dimer; 82.3%
LMW: 17.2%
HMW: 0.4%
9
10
11
minutes
12
13
14 min

FIG. 29

TS-ZPB-1

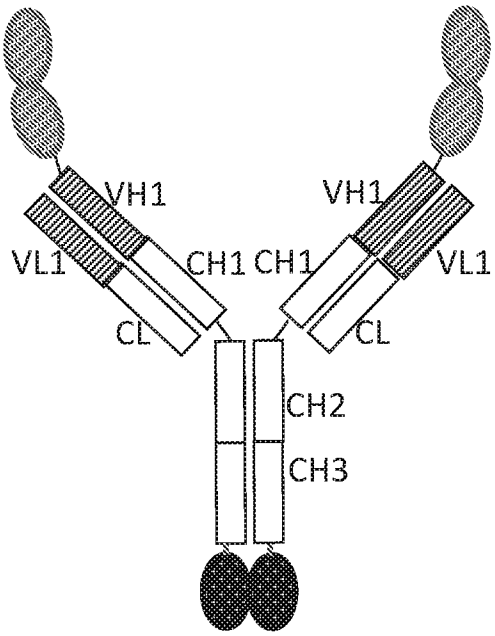


FIG. 30A

TS-ZPB-2

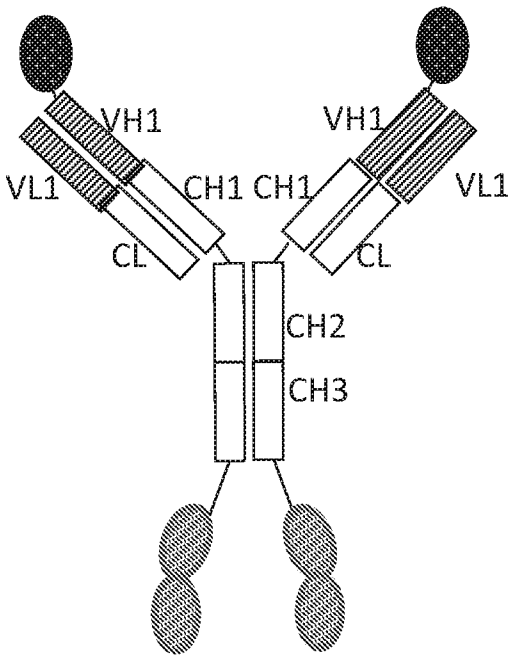
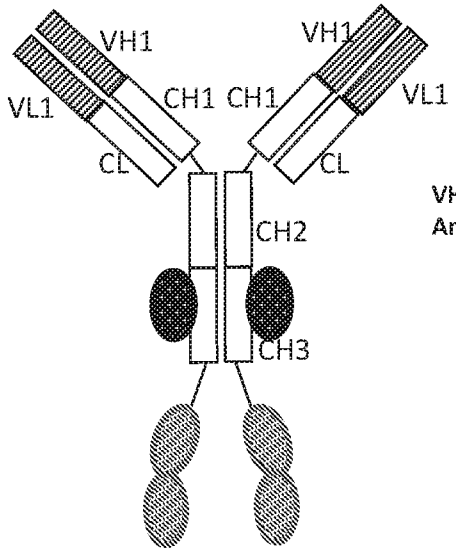


FIG. 30B

TS-ZPB-3



VH1-VL1 = Anti-PD1 or other Checkpoint, e. g., Anti-PDL1, Lag3, TIGIT, etc variable domain

TGFRβR-II ECD

Aflibercept VEFG binding domain

FIG. 30C

TS-ZPB-4

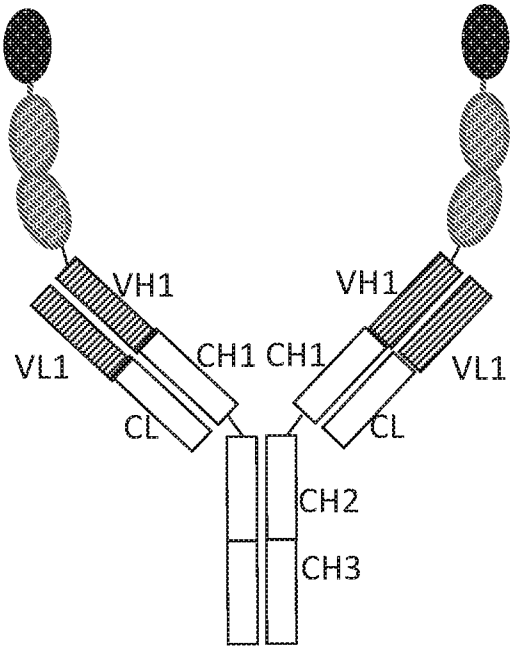


FIG. 30D

TS-ZPB-5

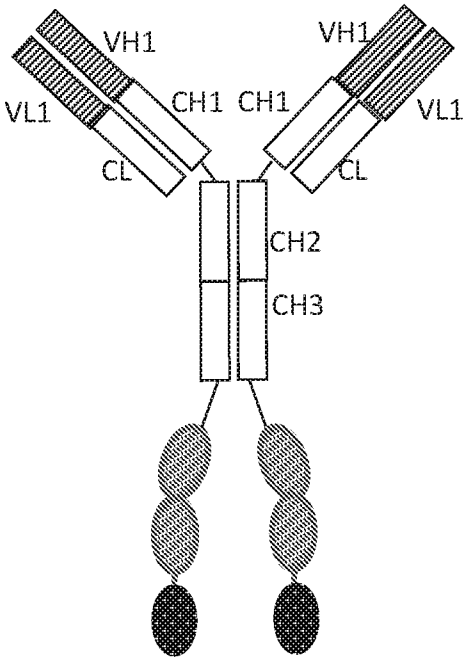
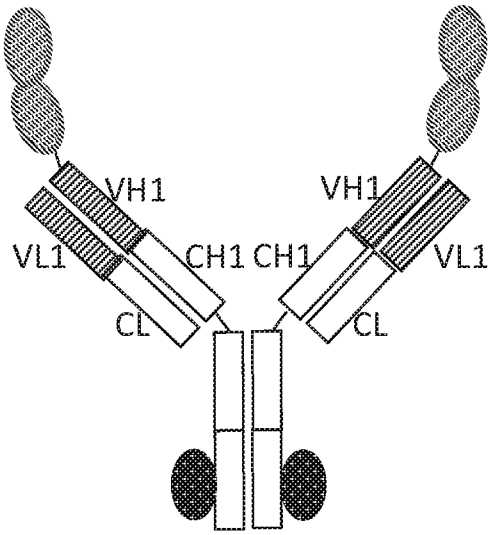


FIG. 30E

TS-ZPB-6



VH1-VL1 = Anti-PD1 or other Checkpoint, e. g., Anti-PDL1, Lag3, TIGIT, etc variable domain

TGFRβR-II ECD

Aflibercept VEFG binding domain

FIG. 30F

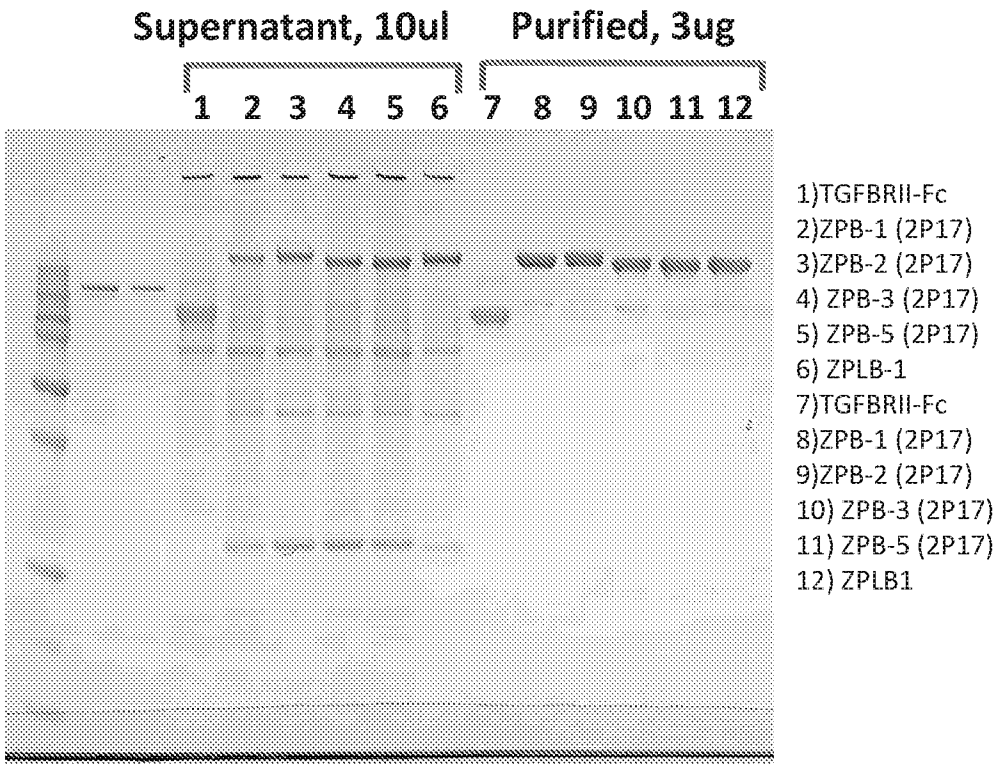

Supernatant, 10ul
Purified, 3ug
1 2 3 4 5 6 7 8 9 10 11 12
1)TGFBRII-Fc
2)ZPB-1 (2P17)
3)ZPB-2 (2P17)
4) ZPB-3 (2P17)
5) ZPB-5 (2P17)
6) ZPLB-1
7)TGFBRII-Fc
8)ZPB-1 (2P17)
9)ZPB-2 (2P17)
10) ZPB-3 (2P17)
11) ZPB-5 (2P17)
12) ZPLB1

FIG. 31A

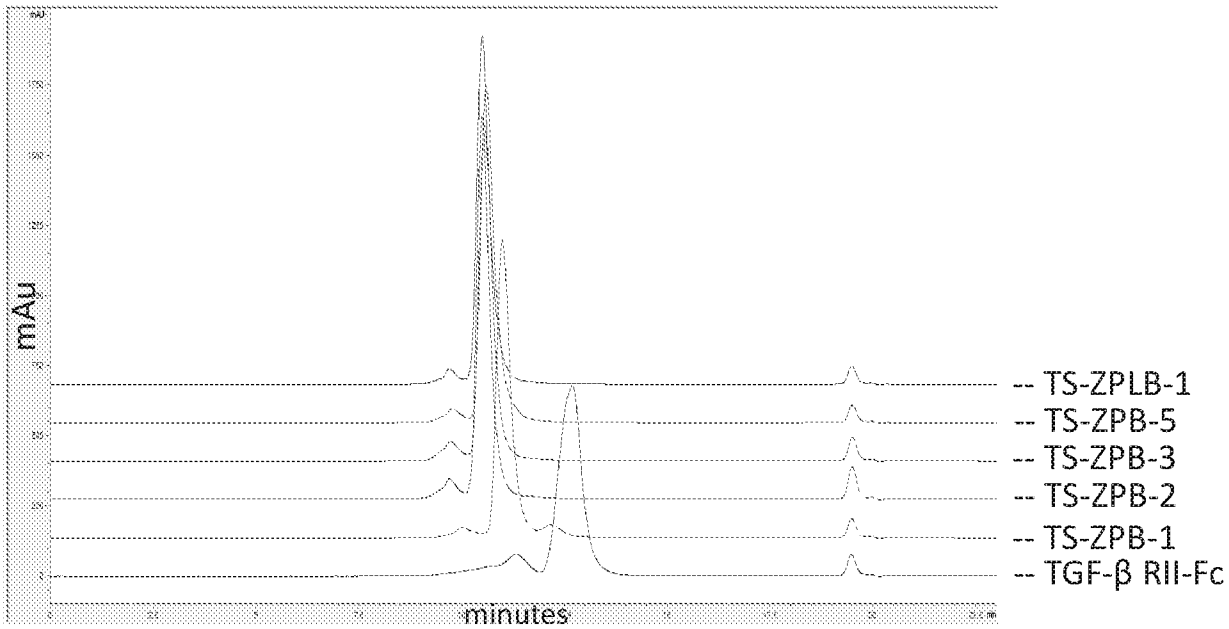


FIG. 32A

Construct	HMW%	Dimer%	LMW%
TS-ZPB-1(2P17)	4.5	89.5	6.0
TS-ZPB-2(2P17)	6.5	92.3	1.2
TS-ZPB-3(2P17)	7.1	91.3	1.5
TS-ZPB-5(2P17)	4.7	95.0	0.3
TS-ZPLB-1	4.8	94.0	1.3
TGFβR II-Fc	15.5	84.3	0.2

FIG. 32B

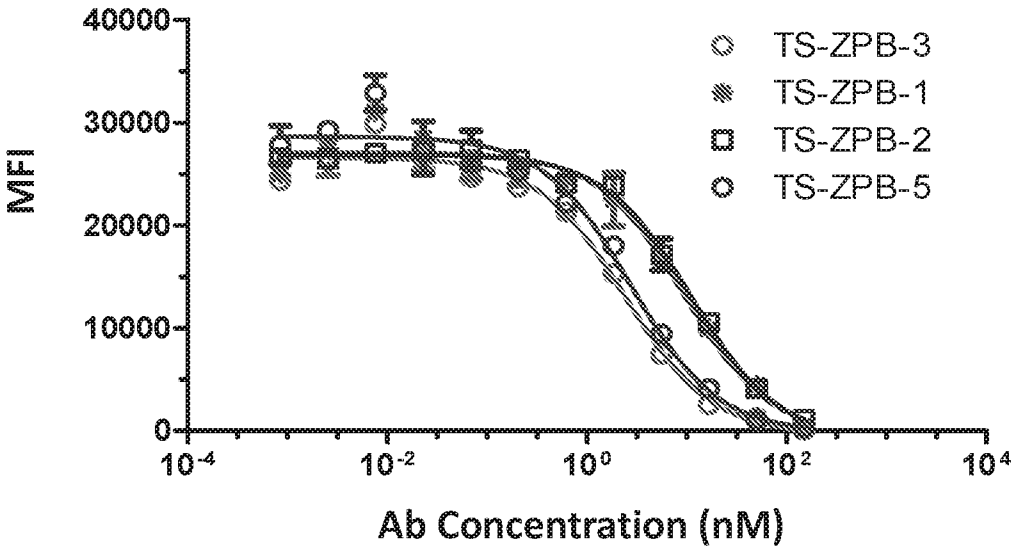


FIG. 33A

Antagonist	IC50 (nM)
TS-ZPB-1(2P17)	9.29
TS-ZPB-2(2P17)	11.05
TS-ZPB-3(2P17)	2.39
TS-ZPB-5(2P17)	2.72

FIG. 33B

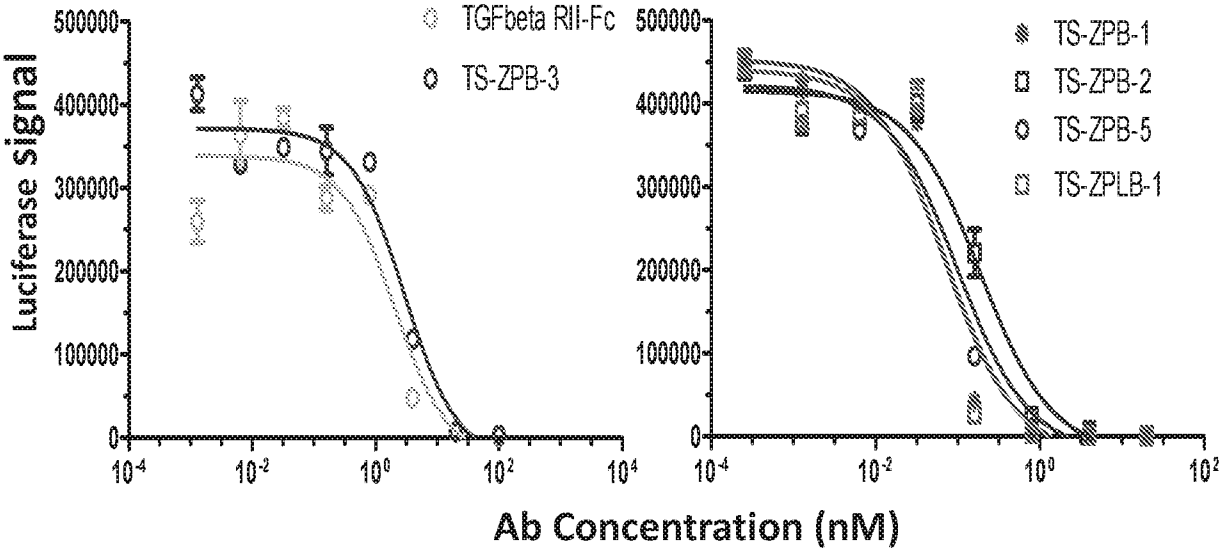


FIG. 34A

Antagonist	IC50 (nM)
TGFβ RII-Fc	2.02
TS-ZPB-1(2P17)	0.07
TS-ZPB-2(2P17)	0.24
TS-ZPB-3(2P17)	2.95
TS-ZPB-5(2P17)	0.17
TS-ZPLB-1	0.11

FIG. 34B

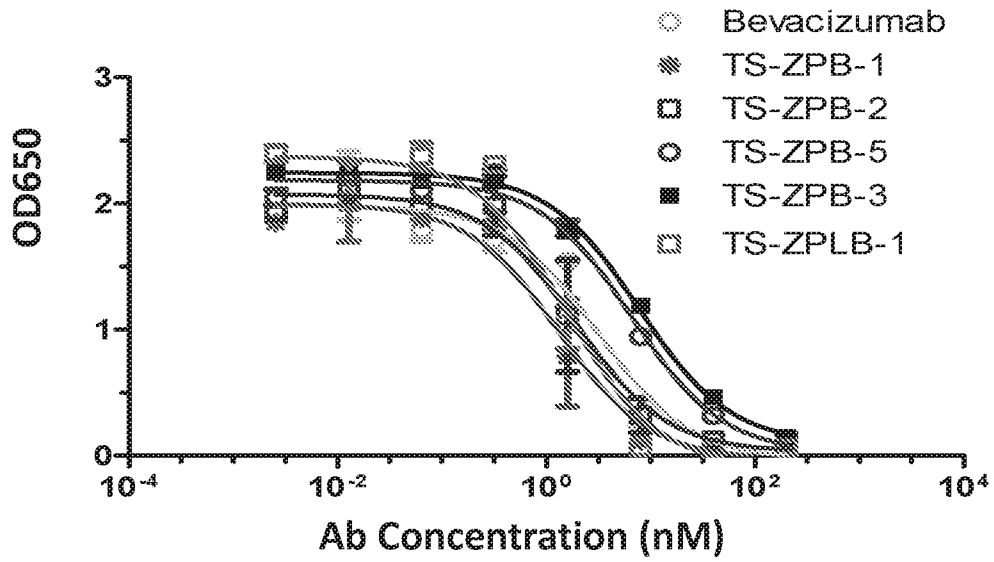


FIG. 35A

Antagonist	IC50 (nM)
Bevacizumab	3.11
TS-ZPB-1	1.32
TS-ZPB-2	1.92
TS-ZPB-3	7.71
TS-ZPB-5	6.58
TS-ZPLB-1	1.57

FIG. 35B

TS-ZPB-5

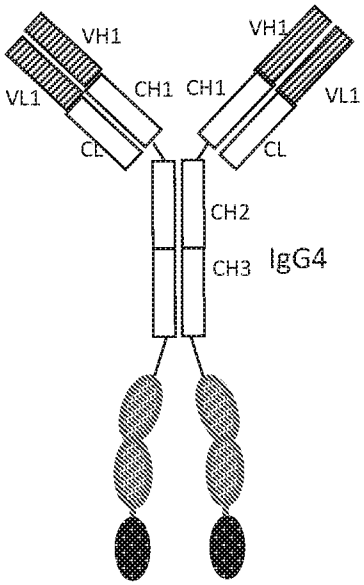


FIG. 36A

TS-ZPB-5A

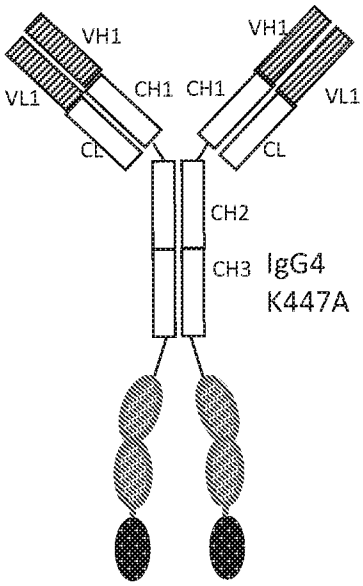


FIG. 36B

TS-ZPB-5B

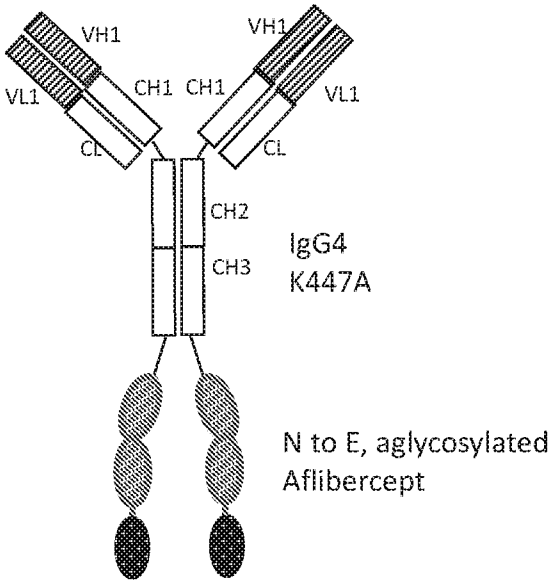


FIG. 36C

VH1-VL1 = Anti-PD1 or other Checkpoint, e. g., Anti-PDL1, Lag3, TIGIT, etc variable domain

TGFRβR II ECD

Aflibercept VEFG binding domain

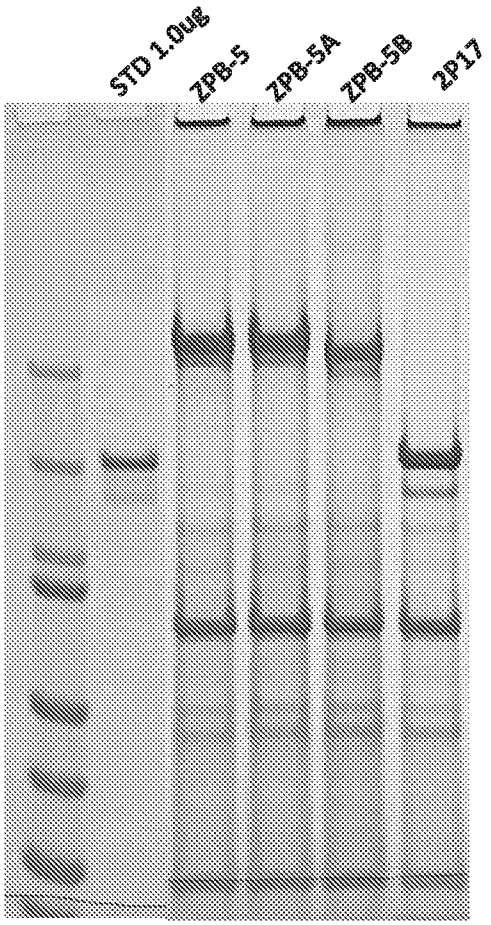


FIG. 37

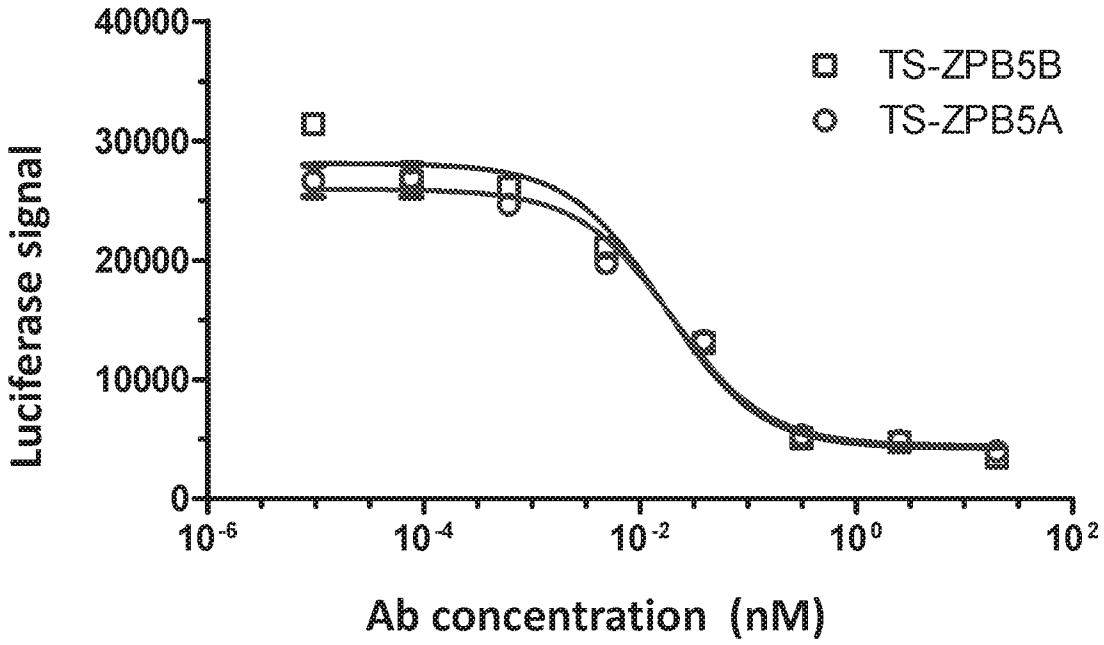

40000
30000
20000
10000
0
Luciferase signal
TS-ZPB5B
TS-ZPB5A
10-6
10-4
10-2
100
102
Ab concentration (nM)

FIG. 38

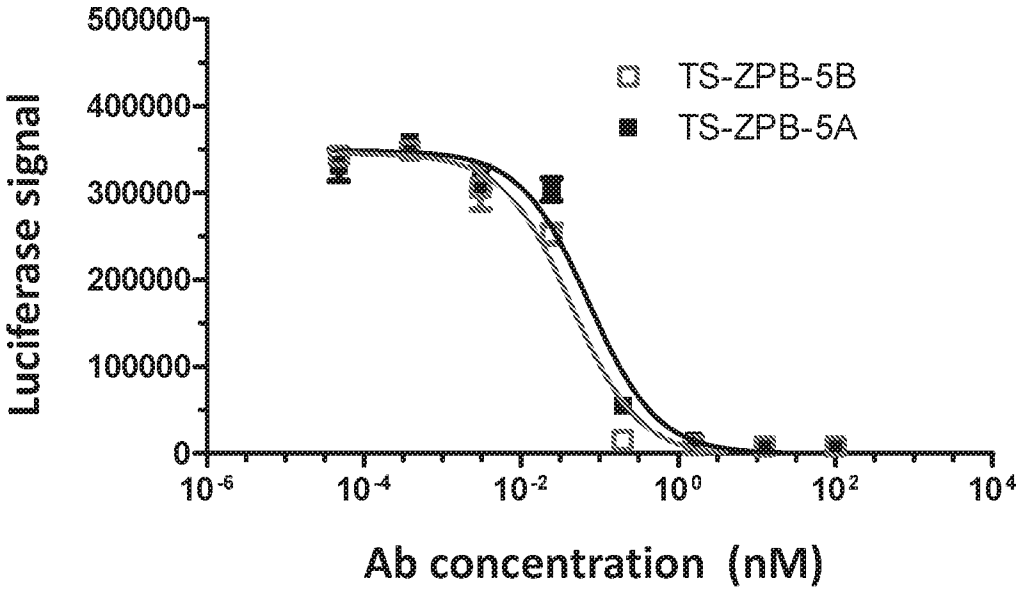

500000
400000
300000
200000
100000
0
Luciferase signal
TS-ZPB-5B
TS-ZPB-5A
10-6
10-4
10-2
100
102
104
Ab concentration (nM)

FIG. 39

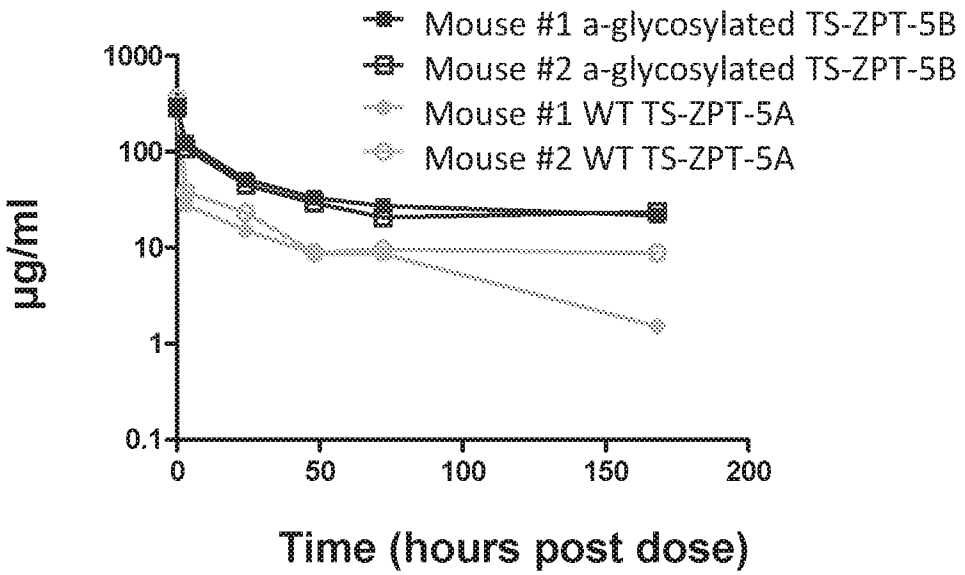

Mouse #1 a-glycosylated TS-ZPT-5B
Mouse #2 a-glycosylated TS-ZPT-5B
Mouse #1 WT TS-ZPT-5A
Mouse #2 WT TS-ZPT-5A
µg/ml
1000
100
10
1
0.1
0
50
100
150
200
Time (hours post dose)

FIG. 40

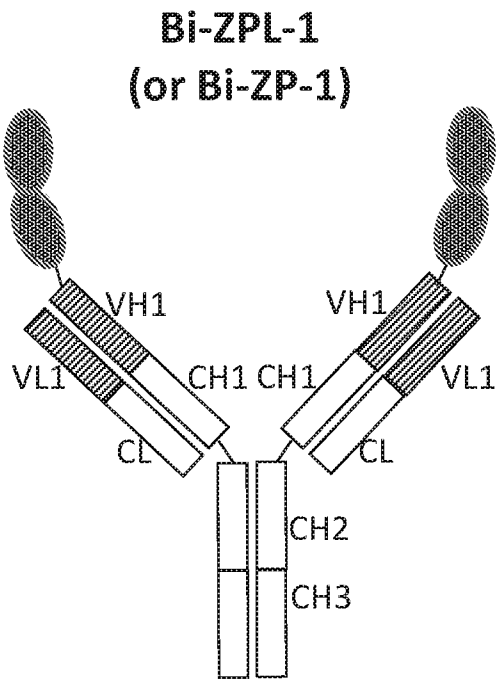


FIG. 41A

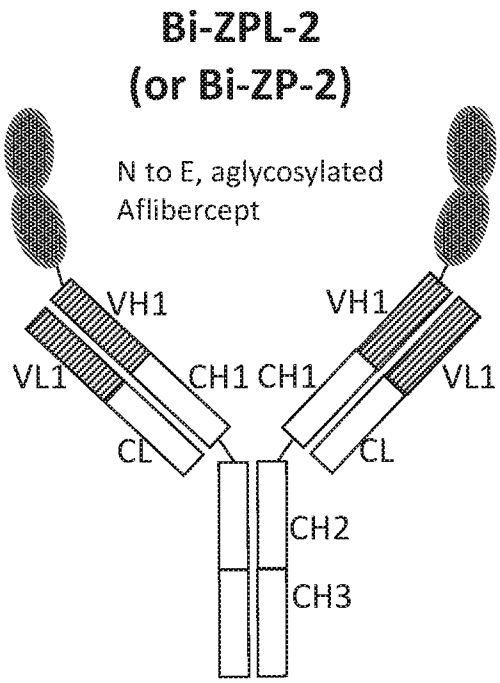


FIG. 41B

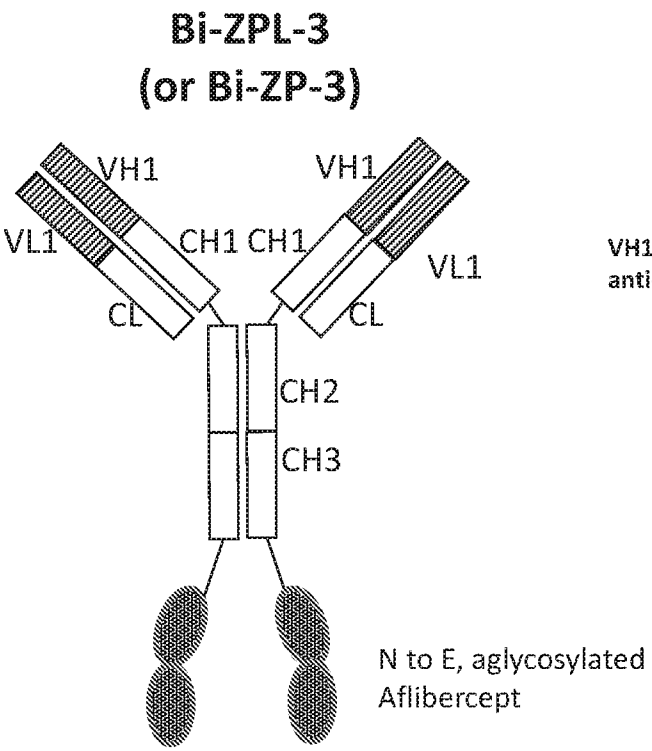


FIG. 41C

VH1-VL1 = Anti-PDL1, anti-PD1 or other Checkpoint, e. g., anti-Lag3, anti-TIGIT, etc variable domain

aflibercept

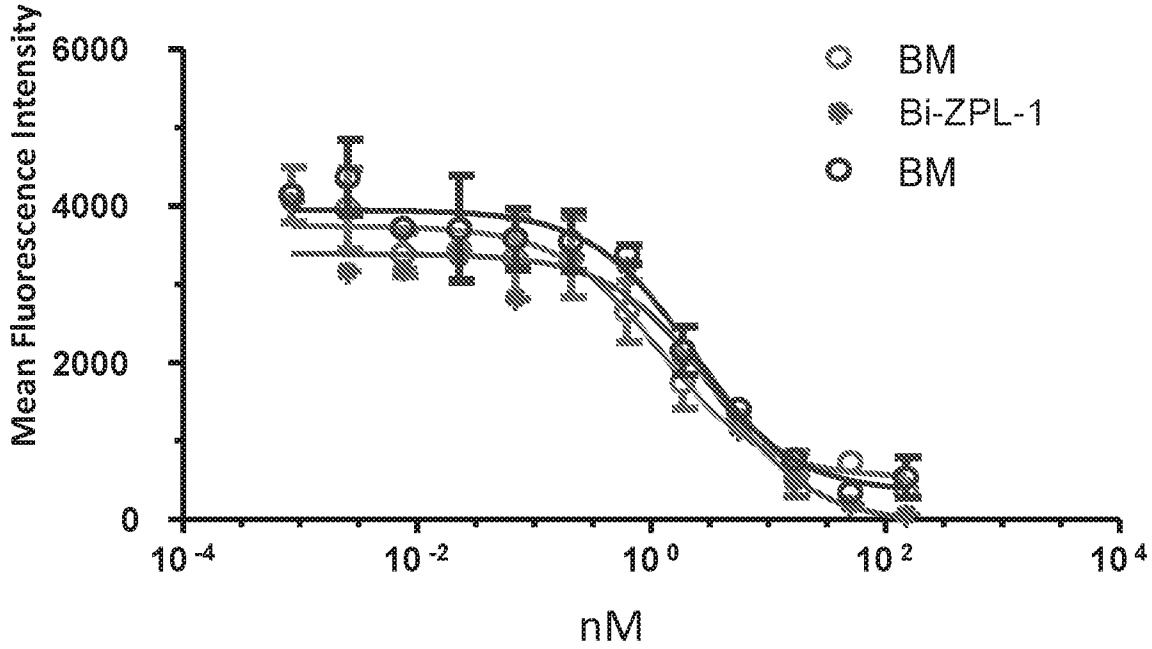


FIG. 42A

Antagonist	IC50 (nM)
BM	2.14
Bi-ZPL-1	3.30

FIG. 42B

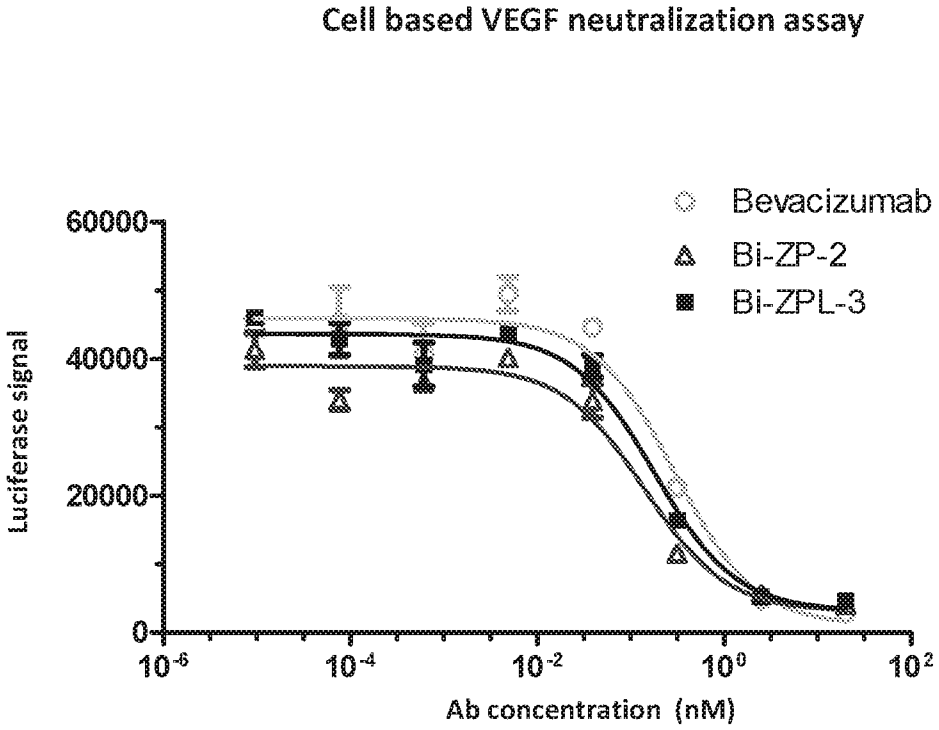


FIG. 43A

	IC50 (nM)
Bevacizumab	0.29
Bi-ZP-2	0.14
Bi-ZPL-3	0.18

FIG. 43B

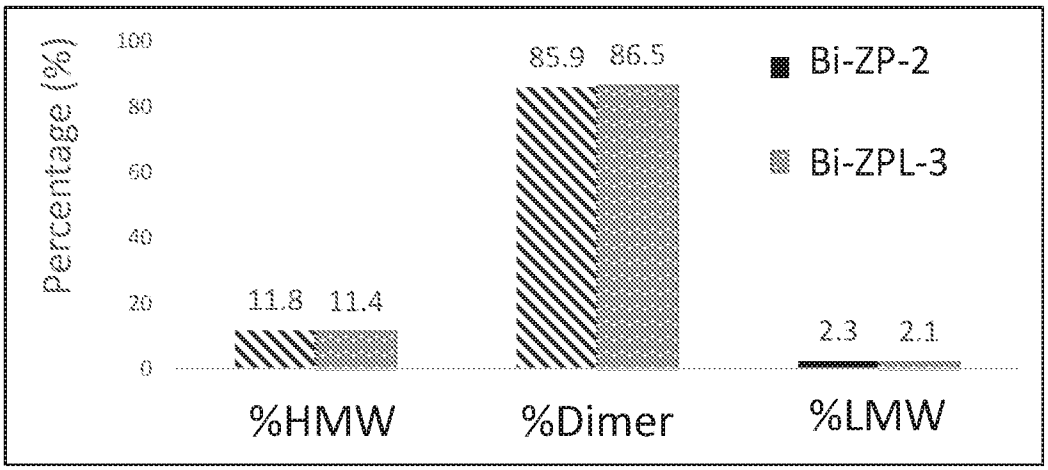

Percentage (%)
100
80
60
40
20
0
11.8
11.4
85.9
86.5
2.3
2.1
%HMW
%Dimer
%LMW
Bi-ZP-2
Bi-ZPL-3

FIG. 44

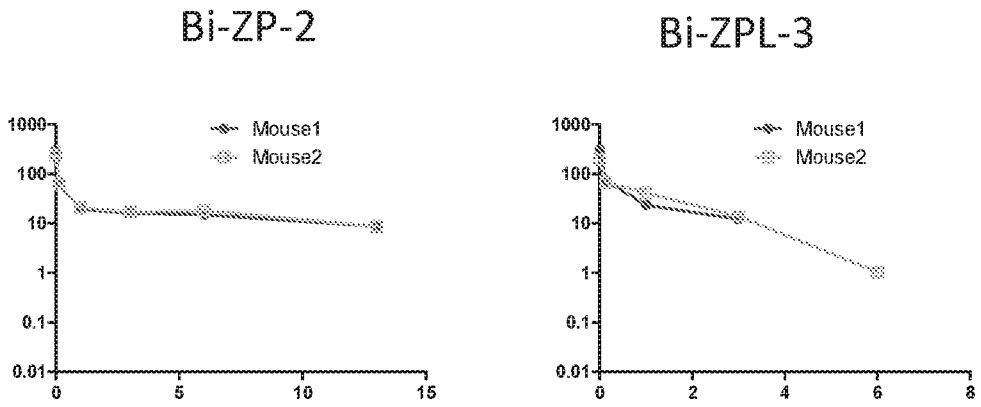

Bi-ZP-2
Mouse1
Mouse2
1000
100
10
1
0.1
0.01
0
5
10
15
Bi-ZPL-3
Mouse1
Mouse2
1000
100
10
1
0.1
0.01
0
2
4
6
8

FIG. 45

SEQ ID No.	Description	Sequence
299	T-01 HFR1	QVQLQESGPGLVKPSQTLSLTCTVSGYSIT
300	T-01, 02, 06, 07 HFR2	WIRQPPGKGLEWIG
301	T-01, 06 HFR3	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR
302	T-01 HFR4	WGQGTSVTVSS
303	T-01, 03, 04, 05, 06, 07, 10 LFR1	DIQMTQSPSSLSASVGDRVTITC
304	T-01 LFR2	WHQQKPGKAPKLLIY
305	T-01, 02 LFR3	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTP
306	T-01, 06 LFR4	FGGGTKLEIKR
307	T-02 HFR1	QVKLQESGPGLVKPSQTLSLTCTVTGYSIT
308	T-02 HFR3	RVTISVDTSKNQFSLKLSSVTAADTAVYSCAR
309	T-02, 06, 07 HFR4	WGQGTLVTVSA
310	T-02 LFR1	DIQMTQSPSSLSASVGDRVTIPC
311	T-02, 03, 04, 05, 06, 07, 10 LFR2	WYQQKPGKAPKLLIY
312	T-02 LFR4	FGEGTKLEIK
313	T-03, 04 HFR1	EVQLVQSGAEVKKPGATVKISCKVSGYTFT
314	T-03, 04, 05 HFR2	WVQQAPGKGLEWMG
315	T-03, 04 HFR3	RVTITADTSTDTAYMELSSLRSEDTAVYYCAT
316	T-03 HFR4	WGQGTLITVSVA
317	T-03, 05, 06, 10 LFR3	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC
318	T-03, 05 LFR4	FGAGTKLELK
319	T-04, 05, 08, 09, 10 HFR4	WGQGTLVTVSS
320	T-04 LFR3	GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC
321	T-04 LFR4	FGAGTKLEIK
322	T-05 HFR1	EVQLKQSGAEVKKPGATVKISCKVSGYTFT
323	T-05 HFR3	RVTITADTSTDTAYMELSSLRSEDTAVYFCAR
324	T-06 HFR1	QVQLQESGPGLVKPSQTLSLTCTVSGGSVS
325	T-07 HFR1	EVQLQESGPGLVKPSDTLSLTCAVSGYSIT
326	T-07 HFR3	RVTMSVDTSKNQFSLKLSSVTAVDTAVYYCTR
327	T-07 LFR3	GAPSRFSGSGSGTDFTLTISSLQPEDFGIYYC
328	T-07 LFR4	FGGGTKLEFK
329	T-08 HFR1	QVQLVQSGSELKKPGASVKVSCKASGYTFT
330	T-08, 10 HFR2	WVRQAPGQGLEWMG
331	T-08 HFR3	RFVFSLDTSVSTAYLQISSLKAEDTAVYYCAR
332	T-08 LFR1	DVVMTQSPLSLPVTLGQPASISC
333	T-08 LFR2	WFQQRPGQSPRVLIY
334	T-08 LFR3	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC
335	T-08 LFR4	FGRGTKLEIK
336	T-09 HFR1	QVTLKESGPTLVKPTQTLTLTCTFSGFSLS
337	T-09 HFR2	WIRQPPGKALEWLA
338	T-09 HFR3	RLTITKDTSKNQVVLTMTNMDPVDTATYYCAR
339	T-09 LFR1	QAVVTQEPSLTVSPGGTVTLTC
340	T-09 LFR2	WVQQKPGQLFRGLIG
341	T-09 LFR3	WVPARFSGSLIGDKAALTLSGVQPEDEAEYFC
342	T-09 LFR4	FGGGTKLTVL
343	T-10 HFR1	QVQLVQSGAEVKKPGASVKVSCKASGYTFT
344	T-10 HFR3	RVTMTRDTSTSTVYMELSSLRSEDTAVYYCAR
345	T-10 LFR4	FGQGTKVEIK

FIG. 46A

SEQ ID NO.	Description	Sequence
346	PD-01 HFR1	EVQLVESGGGLVQPGGSLRLSCAASGFTFS
347	PD-01 HFR2	WVRQAPGKGLEWVS
348	PD-01 HFR3	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK
349	PD-01 HFR4	WGQGTSVTVSS
350	PD-01 LFR1	DIQMTQSPSSVSASVGDRVTITC
351	PD-01, 02, 03, 04; PL-02, 03 LFR2	WYQQKPGKAPKLLIY
352	PD-01, 02, 03, 04 LFR3	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC
353	PD-01; PL-05 LFR4	FGGGTKLEIK
354	PD-02, 03, 04 HFR1	QVQLVQSGAEVKKPGASVKVSCKASDYTFT
355	PD-02 HFR2	WLRQAPGQGLEWMG
356	PD-02 HFR3	RTTSTRDTSISTAYMELSRLRSDDTVVYYCTR
357	PD-02, 04, 05, 06; PL-01, 02, 03, 04, 06, 07, 08 HFR4	WGQGTLVTVSS
358	PD-02, 04 LFR1	DIQMTQSPSSLSASVGDRVTFTC
359	PD-02, 04, 05; PL-01, 04, 06, 07, 08 LFR4	FGGGTKVEIK
360	PD-03, 04, 05, 06; PL-02, 03, 04, 08 HFR2	WVRQAPGQGLEWMG
361	PD-03, 04; PL-01 HFR3	RVTSTRDTSISTAYMELSRLRSDDTVVYYCA
362	PD-03; PL-05 HFR4	WGQGTTLTVSS
363	PD-03 LFR1	DIQMTQSPSSLSASVGDRVTITC
364	PD-03 LFR4	FGAGTKLDLK
365	PD-04, 05; PL-01, 04, 06, 07, 08 LFR4	FGGGTKVEIK
366	PD-05, 06; PL-01, 02, 03, 04, 06, 07, 08 HFR1	QVQLVQSGAEVKKPGASVKVSCKASGYTFT
367	PD-05 HFR3	RVTMTRDTSISTAYMELSSLRSEDTAVYYCAR
368	PD-05 LFR1	DIVLTQSPASLAVSPGQRATITC
369	PD-05 LFR2	WYQQKPGQPPKLLIY
370	PD-05 LFR3	GVPARFSGSGSGTDFTLTINPVEANDTANYYC
371	PD-06 HFR3	RVTLTADTSTSTVYMELSSLRSEDTAVYYCA
372	PD-06 LFR1	DIQMTQSPSFLSASVGDRVTITC
373	PD-06 LFR2	WYQQKPGKAPKALIY
374	PD-06 LFR3	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC
375	PD-06 LFR4	FGQGTKLEIK
376	PL-01 HFR2	WMKQAPGQGLEWMG
377	PL-01, 02, 03, 04, 08 LFR1	DIQMTQSPSSLSASVGDRVTISC
378	PL-01, 04, 08 LFR2	WYQQKPGKAPKLLIK
379	PL-01, 04, 08 LFR3	GVPSRFSGSGSGTDFTLTISSLQPEDFATYFC
380	PL-02 HFR3	KATMTRDKSSSTVYMELSSLRSEDTAVYYCAR
381	PL-02, 03 LFR3	GVPSRFSGSGSGTDFTFTISSLQPEDIATYFC
382	PL-02, 03 LFR4	FGQGTKVEIK
383	PL-03, 08 HFR3	RVTMTRDTSISTAYMELSRLRSDDTAVYYCAR
384	PL-04 HFR3	RVTMTRDTSTSTVYMELSSLRSEDTAVYYCAR
385	PL-05 HFR1	DVQLQESGPGLVKPSQSLSLTCTVTGYSIT
386	PL-05 HFR2	WIRQFPGNKLEWMG
387	PL-05 HFR3	RISITRDTSKNQFFLQLNSVTTEDTATYYCAN
388	PL-05 LFR1	DIVMTQSHKFMSTSVGDRVSITC
389	PL-05 LFR2	WYQQKPGQSPKLLIF
390	PL-05 LFR3	GVPDRFTGSGSGTDYTLTISSVQAEDLALYYC
391	PL-06, 07 HFR2	WVRQAPGQRLEWMGW
392	PL-06, 07 HFR3	RVTITRDTSASTAYMELSSLRSEDTAVYYCAR
393	PL-06 LFR1	DIQMTQSPSSLSAFVGDRVTITC
394	PL-06 LFR2	WYQQKPGKAPKLLIH
395	PL-06 LFR3	GVPSRFSGSGSGRDFTFTISSLQPEDIATYYC
396	PL-07 LFR1	EIVLTQSPVTLSLSPGERATLSC
397	PL-07 LFR2	WYLQKPGQAPRLLIK
398	PL-07 LFR3	IPARFSGSGSGSDFTLTISSLEPEDFAVYYC

FIG. 46B

SEQ ID NO.	Description	Sequence
399	2L2A.1 HFR1	QVQLVQSGAEVKKPGASVKVSCKASGYTLT
400	2L2A.1 HFR2	WMRQAPGQGLEWMG
401	2L2A.1 HFR3	RVTMTRDTSTSTVYMELSSLRSEDTAVYYCVR
402	2L2A.1, 2L2A.6, 3L1A HFR4	WGQGTLVTVSS
403	2L2A.1, 2L2A.6, 2L27B LFR1	DIQMTQSPSSLSASVGDRVTITC
404	2L2A.1 LFR2	WLQQKPEKAPKRLIY
405	2L2A.1, 2L2A.6, 2L27B LFR3	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC
406	2L2A.1, 2L2A.6, 2L27B LFR4	FGGGTKVEIK
407	2L2A.6 HFR1	QVQLVQSGAEVKKPGASVKVSCKASGYTFT
408	2L2A.6 HFR2	WVRQAPGQGLEWMG
409	2L2A.6 3L1A HFR3	RVTMTRDTSTSTVYMELSSLRSEDTAVYYCAR
410	2L2A.6, 3L1A LFR2	WLQQKPGKAPKRLIY
411	2L27B HFR1	QVQLVQSGAEVKKPGASVKVSCKASGFTFS
412	2L27B HFR2	WVRQAPGQGLEWMGL
413	2L27B HFR3	RVTMTRDTSTSTVYMELSSLRSEDTAVYFC
414	2L27B HFR4	FDYWDDGYYVEHFDYWGQGTLVTVSS
415	2L27B LFR2	WYQQKPGKAPKRLIY
416	3L1A HFR1	QVQLVQSGAEVKKPGASVKVSCKASGYTF
417	3L1A LFR1	DIQMTQSPSTLSASVGDRVTITC
418	3L1A LFR2	WLAWYQQKPGKAPKLLIY
419	3L1A LFR3	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC
420	3L1A LFR4	FGQGTKLEIK

FIG. 46C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/39982

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C07K 16/22, 16/28, 16/46 (2019.01)

CPC - C07K 16/22, 16/2818, 16/2863, 16/468, 16/2803

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2017/0275353 A1 (GENSUN BIOPHARMA INC.) 28 September 2017; abstract; paragraphs [0011], [0012], [0015], [0047], [0049], [0063], [0096], [0106], [0109], [0115], [0117], [0130]	1-2, 3/1-2, 14 --- 15
Y	US 2006/0099150 A1 (HOUSTON et al.) 11 May 2006; paragraphs [0511], [0767]	15
A	WO 2016/187594 A1 (HARPOON THERAPEUTICS, INC.) 24 November 2016; entire document	1-2, 3/1-2, 14-15

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 November 2019 (18.11.2019)	03 DEC 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/39982

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

3. ☒ Claims Nos.: 4-13, 19-27, 32
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:

-***-Please See Supplemental Page-***-

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.:

1-2, 3/1-2, 14-15; PD-1 variable domain (first targeting domain); SEQ ID NO: 284 (peptide linker)

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
Information on patent family members

International application No.
PCT/US19/39982

-***-Continued from Box No. III Observations where unity of invention is lacking: -***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+, Claims 1-3, 14-15, anti-PD-1 variable domain (first targeting domain), and SEQ ID NO: 284 (peptide linker) are directed toward trispecific antitumor antagonists that bind specifically to an immune checkpoint regulator and components of the angiogenesis pathways.

The antitumor antagonists will be searched to the extent that they encompass an anti-PD-1 variable domain (first exemplary first targeting domain) and SEQ ID NO: 284 (first exemplary peptide linker). Applicant is invited to elect additional targeting domain(s) and/or peptide linker(s), with specified SEQ ID NO: for each linker or with specified substitution(s) at specified site(s) of a SEQ ID NO:, such that the sequence of each elected species is fully specified (i.e. no optional or variable residues or substituents), and available as an option within at least one searchable claim, to be searched. Additional targeting domain(s) and/or linker sequence(s) will be searched upon the payment of additional fees. It is believed that claims 1-3 (each in-part) and 14-15 (each in-part), encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass an anti-PD-1 variable domain (first targeting domain) and SEQ ID NO: 284 (peptide linker). Applicants must specify the searchable claims that encompass any additionally elected targeting domain(s) and sequence(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be an anti-PD-L1 variable domain (first targeting domain).

Groups II+, Claims 16-18, 28-31, anti-PD-1 variable domain (first targeting domain); SEQ ID NO: 192 (heavy chain) and SEQ ID NO: 284 (peptide linker) are directed toward antitumor antagonists that bind specifically to an immune checkpoint regulator and components of the angiogenesis pathways and components of the TGF pathway.

The antitumor antagonists can be searched to the extent that they encompass an anti-PD-1 variable domain (first exemplary first targeting domain), SEQ ID NO: 192 (first exemplary heavy chain) and SEQ ID NO: 284 (first exemplary peptide linker). Applicant is invited to elect additional targeting domain(s) and/or heavy chain sequence(s) and peptide linker(s), with specified SEQ ID NO: for each heavy chain and/or linker or with specified substitution(s) at specified site(s) of a SEQ ID NO:, such that the sequence of each elected species is fully specified (i.e. no optional or variable residues or substituents), and available as an option within at least one searchable claim, to be searched. Additional targeting domain(s), and/or heavy chain sequence(s) and/or linker sequence(s) can be searched upon the payment of additional fees. It is believed that claims 16-18 and 28-31 (each in-part), encompass this first named invention and thus these claims can be searched without fee to the extent that they encompass an anti-PD-1 variable domain (first targeting domain), SEQ ID NO: 192 (heavy chain) and SEQ ID NO: 284 (peptide linker). Applicants must specify the searchable claims that encompass any additionally elected targeting domain(s) and/or heavy chain sequence(s) and/or linker chain sequence(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be an anti-PD-L1 variable domain (first targeting domain).

The inventions listed as Groups I+ and II+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Groups I+ include a targeting domain comprising a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway, not present in any of Groups II+; the special technical features of Groups II+ include a targeting domain comprising a TGF-B pathway inhibitor, not present in any of Groups I+.

Groups I+ and II+ share the technical features including: a trispecific antagonist, comprising: an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain; a first targeting domain comprising one or more anti-PD-1 variable domains or one or more anti-PD-L1 variable domains; a second targeting domain comprising a component of VEGFR; and a third targeting domain; and a linker comprising at least one of SEQ ID NOs: 284-289.

However, these shared technical features are previously disclosed by US 2017/0275353 A1 (GENSUN BIOPHARMA INC) (hereinafter 'Gensun').

Gensun discloses a trispecific antagonist (abstract), comprising: an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain (comprising: an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain; paragraph [0012]); a first targeting domain comprising one or more immunoglobulin variable domains selected from the group consisting of anti-PD-1 variable domains (abstract; paragraphs [0115], [0117]); a second targeting domain that binds specifically to VEGF (abstract) and comprises one or more peptide domains derived from VEGFR (binds to VEGFR (comprises one or more peptide domains derived from VEGFR); paragraphs [0049]; [0106]); and a third targeting domain (a third targeting domain comprising a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway; abstract; paragraph [0109]); and a linker linking the targeting domains (a linker linking the targeting domains; paragraph [0007]), wherein the linker is rich in glycine for flexibility, as well as serine for solubility (wherein the linker is rich in glycine for flexibility, as well as serine for solubility; paragraph [0058]); wherein the linker comprises SEQ ID NO: 285 (wherein the linker comprises the sequence Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser (wherein the linker comprises SEQ ID NO: 285); paragraph [0031], SEQ ID NO: 28, residues 212-221, and 345-354; wherein the sequence Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser is 100% identical to Applicants' SEQ ID NO: 285).

No technical features are shared between the sequences of Groups I+ and, accordingly, these groups lack unity a priori.

-***-Continued Within the Next Supplemental Box-***-

-***-Continued from Previous Supplemental Page-***-:

Additionally, even if Groups I+ were considered to share the technical features including: a trispecific antagonist, comprising: an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain; a first targeting domain comprising one or more immunoglobulin variable domains selected from the group consisting of anti-PD-1 variable domains, anti-PD-L1 variable domains, anti-TIGIT variable domains and anti-LAG-3 variable domains; a second targeting domain that binds specifically to VEGF and comprises one or more peptide domains derived from VEGFR; and a third targeting domain comprising a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway; these shared technical features are previously disclosed by Gensun, as above.

Gensun discloses a trispecific antagonist (abstract), comprising: an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain (comprising: an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain; paragraph [0012]); a first targeting domain comprising one or more immunoglobulin variable domains selected from the group consisting of anti-PD-1 variable domains (abstract; paragraphs [0115], [0117]); a second targeting domain that binds specifically to VEGF (abstract) and comprises one or more peptide domains derived from VEGFR (binds to VEGFR (comprises one or more peptide domains derived from VEGFR); paragraphs [0049]; [0106]); and a third targeting domain comprising a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway (a third targeting domain comprising a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway; abstract; paragraph [0109]).

No technical features are shared between the heavy chain sequences and linker sequences of Groups II+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups II+ were considered to share the technical features including: a trispecific antagonist, comprising: an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain; a first targeting domain comprising one or more anti-PD-1 variable domains or one or more anti-PD-L1 variable domains; a second targeting domain comprising a component of VEGFR; and a third targeting domain comprising a TGF-B pathway inhibitor; these shared technical features are previously disclosed Gensun, as above, in view of the publication entitled 'M7824, a Novel Bifunctional Anti-PD-L1/TGFb Trap Fusion Protein, Promotes Anti-tumor Efficacy as Monotherapy and in Combination with Vaccine' by Knudson, et al. (hereinafter 'Knudson').

Gensun discloses a trispecific antagonist (abstract), comprising: an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain (comprising: an immunoglobulin scaffold comprising a CH1 domain, a CH2 domain and a CH3 domain; paragraph [0012]); a first targeting domain comprising one or more immunoglobulin variable domains selected from the group consisting of anti-PD-1 variable domains (abstract; paragraphs [0115], [0117]); a second targeting domain that binds specifically to VEGF (abstract) and comprises one or more peptide domains derived from VEGFR (binds to VEGFR (comprises one or more peptide domains derived from VEGFR); paragraphs [0049]; [0106]); and a third targeting domain (a third targeting domain comprising a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway; abstract; paragraph [0109]). Gensun further discloses an angiogenesis pathway inhibitor (a third targeting domain comprising a peptide inhibitor of the angiopoietin/Tie-2 signaling pathway; abstract; paragraph [0109]).

Gensun does not disclose a TGF-B pathway inhibitor.

Knudson discloses an antitumor antagonist (abstract; page e1426519-8, column 2, first paragraph) comprising: a first targeting domain comprising a TGF-B1 RII extracellular domain (ECD) (abstract; page e1426519-8, column 2, first paragraph). Knudson further discloses that TGFB promotes tumor progression through its effects on angiogenesis (e1426519-1, second column).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the disclosure of Gensun, to provide include a TGF-B pathway inhibitor, as taught by Knudson, in order to provide a superior method of treating proliferative disorders with the antitumor antagonists.

Since none of the special technical features of the Groups I+ and II+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Gensun and Knudson reference, unity of invention is lacking.