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(54) Title: VEGFA/ANG2 COMPOUNDS

(57) Abstract: The present invention relates to compounds that bind to human vascular endothelial growth factor A (VEGFA) and human angiopoietin-2 (Ang2), and may be useful for treating angiogenic eye diseases, such as diabetic and other proliferative retinopathies, and for cancer, especially solid tumors driven by VEGFA and Ang2, including gastric, lung, hepatocellular carcinoma, ovarian, colorectal, and breast cancers.

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VEGFA/Ang2 Compounds

The present invention relates to the field of medicine. More particularly, the present invention relates to compounds that bind to human vascular endothelial growth factor A (VEGFA) and human angiopoietin-2 (Ang2), and may be useful for treating angiogenic eye diseases, such as diabetic and other proliferative retinopathies, and for cancer, especially solid tumors driven by VEGFA and Ang2, including gastric, lung, hepatocellular carcinoma, ovarian, colorectal, and breast cancers.

A hallmark of cancer is persistent new blood vessel formation, called angiogenesis. The vascular endothelial growth factor (VEGF) pathway is an important signaling cascade in the regulation of angiogenesis; human VEGFA is a key ligand in the VEGF pathway.

Angiopoietin-1 (Ang1) and Ang2 are members of another key pathway that regulate angiogenesis; Ang1 and Ang2 are secreted factors that bind to the endothelial cell–specific receptor tyrosine kinase Tie2. Ang1 is constitutively secreted by pericytes and stabilizes blood vessel integrity via the Tie2 receptor. Ang2 is released from endothelial cells only in response to stimulus (e.g. wound healing, tumor growth) and facilitates blood vessel sprouting and inhibits pericyte-endothelial cell interaction via Tie2 signaling. An antibody against human Ang2, when dosed in combination with the VEGF blocker aflibercept, has been shown to inhibit tumor growth and to decrease tumor vascularity in mouse xenograft tumor models (Daly et al., Cancer Res (2013) 73(1):108). Multiple investigational Ang2 antibodies are currently in clinical trials.

Inhibition of both the VEGF and Ang/Tie2 pathways of angiogenesis has been proposed for the potential to improve the outcome against cancer (see, for example, Daly et al., Cancer Res (2013) 73:108). Currently, co-administration of a VEGFA antibody and Ang-2 antibody would require either injections or infusions of two separate products or administration of a co-formulation of an antibody mixture. Separate administration would permit flexibility of dose amount and timing, but would be a potential issue for patient compliance and convenience due to increased infusion time. A co-formulation might also provide some flexibility of dosage amounts, but can be challenging to find formulation conditions that permit chemical and physical stability of both antibodies due

to different molecular characteristics of the two different antibodies. Furthermore, co-administration or co-formulation involves the additive costs of two drug therapies.

WO2012/009705 disclosed complexes containing one or more modular recognition domains (MRDs) attached to scaffolds that include antibodies. Ang2 was listed as contemplated for the MRD portion of the complex, and a VEGFA antibody was specified as an antibody which MRDs could be attached. A MRD against Ang2 attached to a VEGFA antibody was not exemplified. WO2010/040508, WO2011/117329, and WO2012/131078 claim bispecific antibodies to VEGFA and Ang-2.

There remains a need to provide compounds that inhibit two angiogenesis pathways by binding and neutralizing both human VEGFA and human Ang2. In particular, there remains a need to provide compounds that inhibit two angiogenesis pathways by binding and neutralizing both human VEGFA and human Ang2, and without compromising significant Ang2 *in vitro* binding activity due to the use of an Ang2 scFv, and without compromising significant *in vitro* cell-based assay activity due to the combination of the VEGFA antibody and Ang2 scFv into one compound. There remains a need to provide compounds that neutralize Ang2 mediated phosphorylation of Tie2, but not Ang1 mediated phosphorylation.

Accordingly, an embodiment of the present invention provides a compound, comprising an antibody fused by two linkers to two single chain fragment variable (scFv) polypeptides, wherein:

a) the antibody comprises two identical heavy chains (HCs) and two identical light chains (LCs), wherein each HC comprises a heavy chain variable region (HCVR) whose amino acid sequence is given in SEQ ID NO: 1, and wherein each LC comprises a light chain variable region (LCVR) whose amino acid sequence is given in SEQ ID NO: 4,

b) the two scFv polypeptides are identical and each comprise an HCVR operably linked to an LCVR, wherein each HCVR has the amino acid sequence given in SEQ ID NO: 7, and wherein each LCVR has the amino acid sequence given in SEQ ID NO: 8, and

c) the two linkers are identical glycine-rich linkers that each operably link the carboxy-terminus of one HC of the antibody to the amino-terminus of one of the scFv polypeptides.

In a further embodiment, the present invention provides a compound comprising an antibody fused by two linkers to two scFv polypeptides, wherein the two scFv polypeptides each comprise the carboxy-terminus of the HCVR of one scFv polypeptide operably linked to the amino-terminus of the LCVR of one scFv polypeptide.

In an embodiment, the present invention provides a compound comprising an antibody fused by two linkers to two scFv polypeptides, wherein the antibody comprises two heavy chains (HCs) and two light chains (LCs), wherein each HC has the amino acid sequence given in SEQ ID NO: 2, and each LC has the amino acid sequence given in SEQ ID NO: 5.

In an embodiment, the present invention provides a compound comprising an antibody fused by two linkers to two scFv polypeptides, wherein each scFv polypeptide has the identical amino acid sequence, which is that given in SEQ ID NO: 6.

In an embodiment, the present invention provides a compound comprising two first polypeptides and two second polypeptides wherein each of the first polypeptides has the amino acid sequence of SEQ ID NO: 3, and each of the second polypeptides has the amino acid sequence of SEQ ID NO: 5. As shown in Table 1, the two first polypeptides comprise the HC of the antibody, the linker, and the scFv polypeptide; the two second polypeptides comprise the LC of the antibody.

In an embodiment, the present invention further provides a compound comprising two first polypeptides and two second polypeptides wherein each of the first polypeptides forms an inter-chain disulfide bond with each of the second polypeptides, and the first polypeptide forms two inter-chain disulfide bonds with the other first polypeptide, and each of the first polypeptides forms seven intra-chain disulfide bonds.

In an embodiment, the present invention provides a compound that binds human VEGFA and human Ang2 comprising an antibody that binds human VEGFA (SEQ ID NO: 11) fused by two linkers to two scFv polypeptides that bind human Ang2 (SEQ ID NO: 12), wherein:

a) the antibody comprises two identical heavy chains (HCs) and two identical light chains (LCs), wherein each HC comprises a heavy chain variable region (HCVR) whose amino acid sequence is given in SEQ ID NO: 1, and wherein each LC comprises a light chain variable region (LVCR) whose amino acid sequence is given in SEQ ID NO: 4,

b) the two scFv polypeptides are identical and each comprise an HCVR operably linked to an LCVR, wherein each HCVR has the amino acid sequence given in SEQ ID NO: 7, and wherein each LCVR has the amino acid sequence given in SEQ ID NO: 8, and

c) the two linkers are identical glycine-rich linkers that each operably link the carboxy-terminus of one HC of the antibody to the amino-terminus of one of the scFv polypeptides.

In a further embodiment, the present invention provides a compound that binds human VEGFA and human Ang2 comprising an antibody that binds human VEGFA (SEQ ID NO: 11) fused by two linkers to two scFv polypeptides that bind human Ang2 (SEQ ID NO: 12), wherein the carboxy-terminus of the HCVR of each scFv polypeptide is operably linked to the amino-terminus of the LCVR.

In an embodiment, the present invention provides a compound that binds human VEGFA and human Ang2 comprising an antibody that binds human VEGFA (SEQ ID NO: 11) fused by two linkers to two scFv polypeptides that bind human Ang2 (SEQ ID NO: 12), wherein the antibody comprises two heavy chains (HCs) and two light chains (LCs), wherein each HC has the amino acid sequence given in SEQ ID NO: 2, and each LC has the amino acid sequence given in SEQ ID NO: 5.

In an embodiment, the present invention provides a compound that binds human VEGFA and human Ang2 comprising an antibody that binds human VEGFA (SEQ ID NO: 11) fused by two linkers to two scFv polypeptides that bind human Ang2 (SEQ ID NO: 12), wherein each scFv polypeptide has the identical amino acid sequence, which is that given in SEQ ID NO: 6.

In an embodiment, the present invention provides a compound that binds human VEGFA (SEQ ID NO: 11) and human Ang2 (SEQ ID NO: 12) comprising two first polypeptides and two second polypeptides wherein each of the first polypeptides has the

amino acid sequence of SEQ ID NO: 3, and each of the second polypeptides has the amino acid sequence of SEQ ID NO: 5.

In an embodiment, the present invention further provides a compound that binds human VEGFA (SEQ ID NO: 11) and human Ang2 (SEQ ID NO: 12) comprising two first polypeptides and two second polypeptides wherein each of the first polypeptides forms an inter-chain disulfide bond with each of the second polypeptides, and the first polypeptide forms two inter-chain disulfide bonds with the other first polypeptide, and each of the first polypeptides forms seven intra-chain disulfide bonds.

In an embodiment, the present invention provides an antibody that binds human VEGFA (SEQ ID NO: 11), comprising a light chain (LC) and a heavy chain (HC), wherein the light chain comprises a light chain variable region (LCVR) and the heavy chain comprises a heavy chain variable region (HCVR), wherein the LCVR has the amino acid sequence given in SEQ ID NO: 4, and the HCVR has the amino acid sequence given in SEQ ID NO: 1.

In an embodiment, the present invention provides an antibody that binds human VEGFA (SEQ ID NO: 11), comprising a light chain (LC) and a heavy chain (HC), wherein the LC has the amino acid sequence given in SEQ ID NO: 5, and the HC has the amino acid sequence given in SEQ ID NO: 2.

In a further embodiment, the present invention provides an antibody that binds VEGFA (SEQ ID NO: 11), comprising two light chains and two heavy chains, wherein each light chain has the amino acid sequence given in SEQ ID NO: 5, and each heavy chain has the amino acid sequence given in SEQ ID NO: 2.

In an embodiment, the present invention provides a mammalian cell comprising a DNA molecule comprising a polynucleotide sequence encoding a first polypeptide given by SEQ ID NO: 3 and a polynucleotide sequence encoding a second polypeptide given by SEQ ID NO: 5, wherein the cell is capable of expressing a compound comprising the first polypeptide and the second polypeptide.

In an embodiment, the present invention provides a process for producing a compound comprising two first polypeptides given by SEQ ID NO: 3, and two second polypeptides given by SEQ ID NO: 5, comprising cultivating the mammalian cell of the

present invention under conditions such that the compound is expressed, and recovering the expressed compound.

In an embodiment of the above-described processes, the two polynucleotide sequences in the mammalian cell of the present invention are part of the same nucleic acid molecule (SEQ ID NO: 9 and SEQ ID NO:10).

In an embodiment, the present invention provides a compound obtainable by one of the aforementioned processes.

In an embodiment, the present invention provides a pharmaceutical composition, comprising a compound of the present invention, and an acceptable carrier, diluent, or excipient.

In an embodiment, the present invention provides a method of treating cancer, comprising administering to a patient in need thereof, an effective amount of a compound of the present invention. In a further embodiment, the present invention provides a method of treating cancer, comprising administering to a patient in need thereof, an effective amount of a compound of the present invention, wherein the cancer is breast cancer, lung cancer, ovarian cancer, gastric cancer, colorectal cancer, hepatocellular carcinoma, or Von Hippel-Lindau syndrome.

In a further embodiment, these methods comprise the administration of an effective amount of the compound of the present invention in simultaneous, separate, or sequential combination with one or more anti-tumor agents selected from the group consisting of cisplatin, carboplatin, liposomal doxorubicin, docetaxel, cyclophosphamide and doxorubicin, navelbine, eribulin, paclitaxel protein-bound particles for injectable suspension, ixabepilone, capecitabine, ramucirumab, FOLFOX (leucovorin, fluorouracil, and oxaliplatin), FOLFIRI (leucovorin, fluorouracil, and irinotecan), and cetuximab.

In a further embodiment, these methods comprise the administration of an effective amount of the compound of the present invention in simultaneous, separate, or sequential combination with one or more immuno-oncology agents selected from the group consisting of nivolumab, ipilimumab, pidilizumab, pembrolizumab, and durvalumab.

In an embodiment, the present invention provides a method of treating proliferative retinopathy, comprising administering to a patient in need thereof, an

effective amount of a compound of the present invention. In a further embodiment, the present invention provides a method of treating proliferative retinopathy, comprising administering to a patient in need thereof, an effective amount of a compound of the present invention, wherein the proliferative retinopathy is diabetic retinopathy, or retinopathy of prematurity.

In an embodiment, the present invention provides a method of treating angiogenic eye disease, comprising administering to a patient in need thereof, an effective amount of a compound of the present invention. In a further embodiment, the present invention provides a method of treating angiogenic eye disease, comprising administering to a patient in need thereof, an effective amount of a compound of the present invention, wherein the angiogenic eye disease is neovascular glaucoma, age-related macular degeneration, diabetic macular edema, corneal neovascularization, corneal graft neovascularization, corneal graft rejection, retinal/choroidal neovascularization, neovascularization of the angle (rubeosis), ocular neovascular disease, vascular restenosis, or arteriovenous malformations (AVM).

In an embodiment, the present invention provides a compound of the present invention, for use in therapy. In an embodiment, the present invention provides a compound of the present invention, for use in the treatment of cancer. In a further embodiment, the present invention provides a compound of the present invention, for use in the treatment of cancer, wherein the cancer is breast cancer, lung cancer, ovarian cancer, gastric cancer, colorectal cancer, or hepatocellular carcinoma. In a further embodiment, for use in the treatment of cancer, the compound of the present invention in simultaneous, separate, or sequential combination with one or more anti-tumor agents selected from the group consisting of cisplatin, carboplatin, liposomal doxorubicin, docetaxel, cyclophosphamide and doxorubicin, navelbine, eribulin, paclitaxel protein-bound particles for injectable suspension, ixabepilone, capecitabine, ramucirumab, FOLFOX (leucovorin, fluorouracil, and oxaliplatin), FOLFIRI (leucovorin, fluorouracil, and irinotecan), and cetuximab.

In a further embodiment, for use in the treatment of cancer, the compound of the present invention in simultaneous, separate, or sequential combination with one or more

immuno-oncology agents selected from the group consisting of nivolumab, ipilimumab, pidilizumab, pembrolizumab, and durvalumab.

In an embodiment, the present invention provides a compound of the present invention, for use in the treatment of proliferative retinopathy. In a further embodiment, the present invention provides a compound of the present invention, for use in the treatment of proliferative retinopathy, wherein the proliferative retinopathy is diabetic retinopathy, retinopathy of prematurity, sickle cell retinopathy, post traumatic retinopathy, a hyperviscosity syndrome, an aortic arch syndrome, an ocular ischemic syndrome, carotid–cavernous fistula, multiple sclerosis, retinal vasculitis, systemic lupus erythematosus, arteriolitis with SS-A autoantibody, acute multifocal hemorrhagic vasculitis, vasculitis resulting from infection, vasculitis resulting from Behçet's disease, sarcoidosis, coagulopathies, a sickling hemoglobinopathy, AC and C-ß thalassemia, small vessel hyalinosis, incontinentia pigmenti, Eales' disease, branch retinal artery or vein occlusion, frosted branch angiitis, idiopathic retinal vasculitis, an aneurysm, neuroretinitis, retinal embolization, retinopathy of prematurity, uveitis, pars planitis, acute retinal necrosis, birdshot retinochoroidopathy, long-standing retinal detachment, choroidal melanoma, radiation retinopathy, familial exudative vitreoretinopathy, inherited retinal venous beading, retinoschisis, retinitis pigmentosa, or autosomal dominant vitreoretinochoroidopathy.

In an embodiment, the present invention provides a compound of the present invention, for use in the treatment of angiogenic eye disease. In a further embodiment, the present invention provides a compound of the present invention, for use in the treatment of angiogenic eye disease, wherein the angiogenic eye disease is neovascular glaucoma, age-related macular degeneration, diabetic macular edema, corneal neovascularization, corneal graft neovascularization, corneal graft rejection, retinal/choroidal neovascularization, neovascularization of the angle (rubeosis), ocular neovascular disease, vascular restenosis, or arteriovenous malformations (AVM). Examples of age-related macular degeneration are non-neovascular (also known as "dry") and neovascular (also known as "wet" or "exudative") macular degeneration.

In an embodiment, the present invention provides the use of a compound of the present invention for the manufacture of a medicament for the treatment of cancer. In a

further embodiment, the present invention provides the use of a compound of the present invention for the manufacture of a medicament for the treatment of cancer, wherein the cancer is breast cancer, lung cancer, ovarian cancer, gastric cancer, colorectal cancer, hepatocellular carcinoma, or Von Hippel-Lindau syndrome.

In a further embodiment , the present invention provides the use of a compound of the present invention in simultaneous, separate, or sequential combination with one or more anti-tumor agents selected from the group consisting of cisplatin, carboplatin, liposomal doxorubicin, docetaxel, cyclophosphamide and doxorubicin, navelbine, eribulin, paclitaxel protein-bound particles for injectable suspension, ixabepilone, capecitabine, ramucirumab, FOLFOX (leucovorin, fluorouracil, and oxaliplatin), FOLFIRI (leucovorin, fluorouracil, and irinotecan), and cetuximab for the manufacture of a medicament for the treatment of cancer.

In a further embodiment, the present invention provides the use of a compound of the present invention in simultaneous, separate, or sequential combination with one or more immuno-oncology agents selected from the group consisting of nivolumab, ipilimumab, pidilizumab, pembrolizumab, and durvalumab for the manufacture of a medicament for the treatment of cancer.

In an embodiment, the present invention provides the use of a compound of the present invention for the manufacture of a medicament for the treatment of proliferative retinopathy. In a further embodiment, the present invention provides the use of a compound of the present invention for the manufacture of a medicament for the treatment of proliferative retinopathy, wherein the proliferative retinopathy is diabetic retinopathy, retinopathy of prematurity, sickle cell retinopathy, post traumatic retinopathy, a hyperviscosity syndrome, an aortic arch syndrome, an ocular ischemic syndrome, carotid–cavernous fistula, multiple sclerosis, retinal vasculitis, systemic lupus erythematosus, arteriolitis with SS-A autoantibody, acute multifocal hemorrhagic vasculitis, vasculitis resulting from infection, vasculitis resulting from Behçet's disease, sarcoidosis, coagulopathies, a sickling hemoglobinopathy, AC and C-ß thalassemia, small vessel hyalinosis, incontinentia pigmenti, Eales' disease, branch retinal artery or vein occlusion, frosted branch angiitis, idiopathic retinal vasculitis, an aneurysm, neuroretinitis, retinal embolization, retinopathy of prematurity, uveitis, pars planitis, acute

retinal necrosis, birdshot retinochoroidopathy, long-standing retinal detachment, choroidal melanoma, radiation retinopathy, familial exudative vitreoretinopathy, inherited retinal venous beading, retinoschisis, retinitis pigmentosa, or autosomal dominant vitreoretinochoroidopathy.

In an embodiment, the present invention provides the use of a compound of the present invention for the manufacture of a medicament for the treatment of angiogenic eye disease. In a further embodiment, the present invention provides the use of a compound of the present invention for the manufacture of a medicament for the treatment of angiogenic eye disease, wherein the angiogenic eye disease is neovascular glaucoma, age-related macular degeneration, diabetic macular edema, corneal neovascularization, corneal graft neovascularization, corneal graft rejection, retinal/choroidal neovascularization, neovascularization of the angle (rubeosis), ocular neovascular disease, vascular restenosis, or arteriovenous malformations (AVM). Examples of age-related macular degeneration are non-neovascular (also known as "dry") and neovascular (also known as "wet" or "exudative") macular degeneration.

A compound of the present invention is an engineered, non-naturally occurring polypeptide complex. A DNA molecule of the present invention is a non-naturally occurring DNA molecule that comprises a polynucleotide sequence encoding a polypeptide having the amino acid sequence of one of the polypeptides in a compound of the present invention.

The antibody portion of the compound of the present invention is designed to have engineered CDRs and have some portions of the antibody (all or parts of the frameworks, hinge regions, and constant regions) to be of human origin that are identical with or substantially identical (substantially human) with frameworks and constant regions derived from human genomic sequences. Fully human frameworks, hinge regions, and constant regions are those human germline sequences as well as sequences with naturally-occurring somatic mutations and those with engineered mutations. The antibody portion of the compound of the present invention may comprise framework, hinge, or constant regions derived from a fully human framework, hinge, or constant region containing one or more amino acid substitutions, deletions, or additions therein. Further, the antibody

portion of the compound of the present invention is preferably substantially non-immunogenic in humans.

The antibody portion of the compound of the present invention is an IgG type antibody and has four amino acid chains (two "heavy" chains and two "light" chains) that are covalently stabilized via intra- and inter-chain disulfide bonds. Each heavy chain is comprised of an N-terminal HCVR and a heavy chain constant region ("HCCR"). Each light chain is comprised of a LCVR and a light chain constant region ("LCCR"). When expressed in certain biological systems, antibodies having native human Fc sequences are glycosylated in the Fc region. Typically, glycosylation occurs in the Fc region of the antibody at a highly conserved N-glycosylation site. N-glycans typically attach to asparagine. Antibodies may be glycosylated at other positions as well.

Optionally, the antibody portion of the compound of the present invention contains an Fc portion which is derived from human IgG_4 Fc region because of a reduced ability to engage Fc receptor-mediated inflammatory mechanisms or to activate complement resulting in reduced effector function.

Further, the antibody portion of certain compounds of the present invention contains an IgG_4-PAA Fc portion. The IgG_4-PAA Fc portion has a serine to proline mutation at position 231, a phenylalanine to alanine mutation at position 237, and a leucine to alanine mutation at position 238. The S231P mutation is a hinge mutation that prevents half-antibody formation (phenomenon of dynamic exchange of half-molecules in IgG_4 antibodies). The F237A and L238A mutations further reduce effector function of the already low human IgG_4 isotype.

An isolated DNA molecule encoding a HCVR region can be converted to a full-length heavy chain gene by operably linking the HCVR-encoding DNA to another DNA molecule encoding heavy chain constant regions. The sequences of human, as well as other mammalian, heavy chain constant region genes are known in the art. DNA fragments encompassing these regions can be obtained *e.g.*, by standard PCR amplification.

An isolated DNA encoding a LCVR region may be converted to a full-length light chain gene by operably linking the LCVR-encoding DNA to another DNA molecule encoding a light chain constant region. The sequences of human, as well as other

mammalian, light chain constant region genes are known in the art. DNA fragments encompassing these regions can be obtained by standard PCR amplification. The light chain constant region can be a kappa or lambda constant region.

A "single chain fragment variable" or "scFv" or "scFv polypeptide" refers to an engineered, non-naturally occurring single folded polypeptide comprising the LCVR and the HCVR of an antibody linked through a scFv linker molecule. The scFv polypeptide portion of the compound of the present invention is an engineered, non-naturally occurring scFv that has been designed to have engineered CDRs and have some portions of the scFv (all or parts of the frameworks) to be of human origin that are identical with or substantially identical (substantially human) with frameworks derived from human genomic sequences. Fully human frameworks are those human germline sequences as well as sequences with naturally-occurring somatic mutations and those with engineered mutations. The scFv polypeptide portion of the compound of the present invention may comprise framework derived from a fully human framework containing one or more amino acid substitutions, deletions, or additions therein. Further, the scFv polypeptide portion of the compound of the present invention is preferably substantially non-immunogenic in humans. Optionally, the scFv polypeptide portion of the compound can have disulfides from cysteine 44 in HCVR and cysteine 100 in LCVR (Cys44 and Cys100 numbering corresponds with amino acid numbering of HCVR and LCVR of the scFv polypeptide; for the numbering in the HC of antibody + linker + scFv polypeptide, it is Cys507 and Cys709). In such a scFv polypeptide, the HCVR and LCVR domains can be either in the HCVR – scFv linker - LCVR or LCVR – scFv linker - HCVR order. The scFv linker can be a flexible glycine-rich peptide linker which enables the HCVR and LCVR chains to be folded as a functional monomeric unit for recognizing an antigen. Optionally, the scFv linker is a glycine-rich linker such as a 2x G4S linker, a 3x G4S linker, a 4x G4S linker, or a 5x G4S linker.

The term "linker" and "scFv linker" both refer to glycine-rich peptide linkers. The "linkers" are utilized in certain embodiments of the invention to link the antibody to the scFv, and the "scFv linkers" are utilized in certain embodiments of the invention to link the LCVR of the scFv to the HCVR of the scFv. Preferably, the peptide linkers are glycine-rich peptides with at least 5 amino acids, preferably of at least 10 amino acids,

more preferably between 10 and 50 amino acids. In some embodiments of the present invention, said glycine-rich peptide linker is $(G_xS)_n$ with G=glycine, S=serine, (x=3 and n=3, 4, 5 or 6) or (x=4 and n=2, 3, 4 or 5). For example, in some embodiments of the present invention, said glycine-rich peptide linker is $(G_xS)_n$ with G=glycine, S=serine, x=4 and n=2, 3, 4 or 5 (i.e., GGGGSGGGGS (SEQ ID NO: 13), GGGGSGGGGSGGGGS (SEQ ID NO: 14), GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 15), or GGGGSGGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 16), respectively. In some embodiments of the present invention where the last amino acid in the antibody constant region is a glycine, said glycine-rich peptide linker is GGGSGGGGSGGGGS (SEQ ID NO:17).

The polynucleotides of the present invention will be expressed in a host cell after the sequences have been operably linked to an expression control sequence. The expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline, neomycin, and dihydrofolate reductase, to permit detection of those cells transformed with the desired DNA sequences.

The compound of the present invention may readily be produced in mammalian cells such as CHO, NS0, HEK293 or COS cells. The host cells are cultured using techniques well known in the art.

The vectors containing the polynucleotide sequences of interest (e.g., the polynucleotides encoding the polypeptides of the compound and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host.

Various methods of protein purification may be employed and such methods are known in the art and described, for example, in Deutscher, *Methods in Enzymology* 182: 83-89 (1990) and Scopes, *Protein Purification: Principles and Practice*, 3rd Edition, Springer, NY (1994).

In another embodiment of the present invention, the compound, or the nucleic acids encoding the same, is provided in isolated form. As used herein, the term "isolated" refers to a protein, peptide, or nucleic acid which is free or substantially free from any

other macromolecular species found in a cellular environment. "Substantially free" as used herein means the protein, peptide, or nucleic acid of interest comprises more than 80% (on a molar basis) of the macromolecular species present, preferably more than 90%, and more preferably more than 95%.

The compound of the present invention, or pharmaceutical compositions comprising the same, may be administered parenterally (e.g., intravitreal, intraocular, subconjunctival, subcutaneously or via intravenous injection or implant). A compound of the present invention may be administered to a patient alone with pharmaceutically acceptable carriers, diluents, or excipients in single or multiple doses. Pharmaceutical compositions of the present invention can be prepared by methods well known in the art (e.g., *Remington: The Science and Practice of Pharmacy*, 19th ed. (1995), A. Gennaro et al., Mack Publishing Co.) and comprise a compound, as disclosed herein, and one or more pharmaceutically acceptable carriers, diluents, or excipients.

The term "treating" (or "treat" or "treatment") refers to slowing, interrupting, arresting, alleviating, stopping, reducing, or reversing the progression or severity of an existing symptom, disorder, condition, or disease. A patient refers to a mammal, preferably a human with a disease, disorder, or condition that would benefit from inhibition of VEGFA and/or Ang2.

"Binds" as used herein in reference to the affinity of a compound, antibody, or scFv polypeptide for human VEGFA or human Ang2 is intended to mean, unless indicated otherwise, a K_D of less than about 1 x 10^{-8} M, preferably, less than about 1 x 10^{-9} M as determined by common methods known in the art, including by use of a surface plasmon resonance (SPR) biosensor at 25°C or 37°C essentially as described herein. The term "selective" or "selectivity" used herein in reference to a compound of the present invention refers to a compound that binds a target, such as human Ang2, with a K_D about 1000-, 500-, 200-, 100-, 50-, or about 10-fold lower than the compound binds other proteins, including member of the target family such as human Ang1, as measured by surface plasmon resonance at 25°C or 37°C. Additionally, or alternatively, an Ang2 selective compound of the present invention binds human Ang2 but does not bind or only minimally binds human Ang1 when assayed by the methods described in the Example herein below.

"Effective amount" means the amount of a compound of the present invention or pharmaceutical composition comprising a compound of the present invention that will elicit the biological or medical response of or desired therapeutic effect on a tissue, system, animal, mammal or human that is being sought by the researcher, medical doctor, or other clinician. An effective amount of the compound may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the compound to elicit a desired response in the individual. An effective amount is also one in which any toxic or detrimental effect of the compound is outweighed by the therapeutically beneficial effects.

This invention is further illustrated by the following non-limiting examples.

Example 1: Compound expression and purification

For Example 1 (Compound A) as shown in Table 1, the two first polypeptides (HC of antibody-linker-scFv of Compound A) have the amino acid sequence of SEQ ID NO: 3, and the two second polypeptides (LC of antibody of Compound A) have the amino acid sequence of SEQ ID NO: 5.

The polypeptide of the antibody portion, the scFv portion, and the antibody-linker-scFv of Compound A, and the nucleotide sequences encoding the same, are listed below in the section entitled "Amino Acid and Nucleotide Sequences."

The compounds of the present invention, including, but not limited to Compound A, can be made and purified essentially as follows. An appropriate host cell, such as HEK 293 or CHO, can be either transiently or stably transfected with an expression system for secreting compounds using an optimal predetermined HC-linker-scFv:LC vector ratio (such as 1:3 or 1:2) or a single vector system encoding both HC-linker-scFv and LC. Clarified media, into which the compound has been secreted, may be purified using any of many commonly-used techniques. For example, the medium may be conveniently applied to a MabSelect column (GE Healthcare), or KappaSelect column (GE Healthcare) for Fab fragment, that has been equilibrated with a compatible buffer, such as phosphate buffered saline (pH 7.4). The column may be washed to remove nonspecific binding components. The bound compound may be eluted, for example, by pH gradient (such as 20 mM Tris buffer pH 7 to 10 mM sodium citrate buffer pH 3.0, or

phosphate buffered saline pH 7.4 to 100 mM glycine buffer pH 3.0). Compound fractions may be detected, such as by SDS-PAGE, and then may be pooled. Further purification is optional, depending on the intended use. The compound may be concentrated and/or sterile filtered using common techniques. Soluble aggregate and multimers may be effectively removed by common techniques, including size exclusion, hydrophobic interaction, ion exchange, multimodal, or hydroxyapatite chromatography. The purity of the compound after these chromatography steps is greater than 95%. The product may be immediately frozen at -70°C or may be lyophilized.

Table 1

	Compound A
HCVR of antibody	1
HC of antibody	2
HC of antibody + linker + scFv polypeptide	3
LCVR of antibody	4
LC of antibody	5
scFv polypeptide	6
HCVR of scFv polypeptide	7
LCVR of scFv polypeptide	8

Assays

Binding kinetics, affinity, and selectivity

The binding kinetics, affinity, and selectivity to human Ang2 and to human VEGFA, for compounds of the present invention, are determined by use of a surface plasmon resonance (SPR) biosensor such as a Biacore® 2000, Biacore® 3000, or a Biacore® T100 (GE HealthCare), or alternatively by kinetic exclusion assay methods

associated with Kinexa 3000 or 3200 (Sapidyne Instruments) according to methods known in the art.

Affinity Measurement Using Biacore SPR

The kinetics and equilibrium dissociation constant (K_D) for soluble full length human Ang2 and human VEGFA is determined for compounds of the present invention at 25°C using or Biacore surface plasmon resonance assay methods. Human Ang2 is from R&D Systems (#623-AN-01M/CF) and human VEGFA165 is from R&D systems (#293-VE-001MG/CF), Peprotech (#00-20), or prepared by recombinant expression methods. Protein A surface for capture of antibodies is prepared using the following methods. Immobilization of soluble Protein A (Calbiochem #539202) on a CM4 (GE Healthcare #BR-1005-34) or CM5 (GE Healthcare #BR-1000-99) is prepared using EDC/NHS amine coupling method (GE Healthcare #BR-1000-50). Briefly, the surfaces of all four flow cells is activated by injecting a 1:1 mixture of EDC/NHS for seven minutes at 10 µL/min. After which, soluble protein A is diluted to 50-100 µg/mL in 10 mM acetate buffer, pH 4.5, and immobilized for seven minutes onto flow cell (Fc) 2, 3 or 4 at a flow rate of 10 µL/min. Unreacted sites still remaining on the chip surface are blocked with a seven minute injection of ethanolamine at 10 µL/min. Running buffer is HBS-EP+ (GE Healthcare #BR-1006-69) with addition of 100mM NaCl. Compound samples are prepared at 1 µg/mL by dilution into running buffer. Human Ang2 or human VEGFA165 ranging from 50 nM to 1.56 nM is prepared in running buffer using a two-fold serial dilution. Each analysis cycle consists of a series of five separate steps: (1) capture of compound onto separate flow cells (Fc2, Fc3, and Fc4), (2) injection (using kinject) of 250 µL (300-second surface contact time) of discrete concentrations of human Ang2 or human VEGFA165 over all Fc at 50 µL/min, (3) return to buffer flow for 20 minutes to monitor dissociation phase, (4) regeneration of chip surfaces with a 10 µL (30-second contact time) injection of 10 mM glycine, pH1.5, (5) equilibration of chip surface with a 15 µL (45-second contact time) injection of HBS-EP+ running buffer. Resultant data is processed using standard double-referencing and fit to a 1:1 binding model using Biacore 2000 Evaluation software, version 4.1, to determine the association rate (k_{on}, $M^{-1}s^{-1}$ units), dissociation rate (k_{off}, s^{-1} units). Calculation of the equilibrium dissociation constant (K_D)

is calculated from the following relationship, $K_D = k_{off}/k_{on}$, and is presented in molar units.

In experiments performed essentially as described in this assay, the parental anti-VEGFA antibody of Compound A exhibits a K_D of 13.2 pM for human VEGFA165. Compound A, a Mab-scFv fusion which combines a parental anti-VEGFA antibody with a C-terminal heavy chain scFv composed of HCVR and LCVR from an Ang2 Mab, exhibits a K_D of 83.2 pM (Table 2). The binding affinity (K_D) of Compound A for human Ang2 evaluated using Biacore SPR at 25°C is 136 pM (Table 3).

Table 2: Biacore SPR hVEGFA165 at 25°C

Compound	k_{on} (10^6 1/Ms)	k_{off} (10^{-4} 1/s)	K_D (pM)
Parental VEGFA Ab	2.93	0.39	13.2
Compound A	2.44	2.03	83.2

The values presented in Table 2 are an upper limit for the reported affinity as the off-rate is too slow to accurately quantitate.

Table 3: Biacore SPR hAng2 at 25°C

Compound	k_{on} (10^6 1/Ms)	k_{off} (10^{-4} 1/s)	K_D (pM)
Compound A	0.24	0.32	136

Affinity measurement with KinExA KEA

A KinExA 3200 instrument is used to measure binding kinetics to human VEGFA165. Briefly, human VEGFA165 is covalently coupled to NHS-activated sepharose beads (GE Healthcare #17-0906-01) and the binding of free Mab to the conjugated beads is detected on the KinExA 3200. To measure K_D, individual tubes containing a fixed concentration (typically 1-5 pM) of the compound are mixed with decreasing concentrations of serially diluted human VEGFA165 and pre-incubated for greater than 24 hours at either 25°C or 37°C (depending on the temperature analyzed) in Casein Blocking Buffer (ThermoFisher #37528). Following pre-incubation to reach steady state equilibrium, each sample is subjected to the following five step analysis cycle: 1) a small column of VEGFA165-conjugated beads is packed into a capillary to a

predefined height, 2) defined volumes of each discrete human VEGFA165/compound mixture is injected over the column for a defined time period, 3) defined volume of an appropriate fluorescently labeled antibody (I.E. CY5-labeled anti-human Fc gamma specific, Jackson Immunoresearch #309-175-008) is injected over the column, 4) column is washed with 1X PBS to remove excess detection antibody and any non-specifically bound materials and 5) detection of specifically bound materials is measured by excitation and subsequent emission monitoring of the bound secondary antibody. It follows that the relative intensity of signal generated in these steps is proportional to the degree of free/uncomplexed compound present in each solution tested. The resultant composite set of fluorescent intensities is plotted as a function of the concentration of human VEGFA165 present in each discrete sample and fit using the N-curve analysis software (KinExA) to a standard two state binding model to determine K_D for the given MOI. Statistical confidence is reported by calculation of the 95% confidence intervals.

In experiments performed essentially as described, the binding at 37°C for Compound A was 84 pM (Table 4), and the binding at 25°C for Compound A was 26.6 pM (Table 5).

Table 4: Kinexa KEA hVEGFA165 at 37°C

Compound	K_D (pM)	95% CI Range (pM)
Parental VEGFA Ab	183	98, 311
Compound A	84	117, 50

Table 5: Kinexa KEA hVEGFA165 at 25°C

Compound	K_D (pM)	95% CI Range (pM)
Parental VEGFA Ab	27	14, 47

Inhibition of human Ang2 to human Tie2 interaction via Solid Phase Elisa

The blocking of human Ang2 binding to its receptor human Tie 2 by a compound of the present invention is measured in a solid phase *in vitro* ELISA assay. The *in vitro*

cell-based assay is used to establish comparable blocking activity between a compound of the present invention and an Ang2 antibody with the same HCDRs and LCDRs sequences as the scFv polypeptide portion of the compound.

For this assay, high binding 96-well ELISA plates (Costar #2592) are coated with 4 μg/ml (in 100 μl) recombinant human Tie2-Fc (R&D Systems #313-TI), overnight at room temperature. The plates are washed 3X with TBST (Tris buffered saline containing 0.05% Tween 20) and then blocked with 300 μl per well of blocking buffer (0.5% BSA/D-PBS) (BSA: Jackson ImmunoResearch #001-000-162; IgG-free, protease-free) for 1-2 hours at room temperature on an orbital shaker. During the blocking step, in separate polypropylene multiwell plates, 75 μl of 2X test compounds (serially diluted 1:3 in blocking buffer) is added with 75 μl of 2X biotinylated human Ang2 (R&D Systems #BT623) (also diluted in blocking buffer). The compound/biotinylated Ang2 mixtures are then incubated for 1 hour at 37°C (final biotinylated Ang2 concentration was 100 ng/ml). The blocking solution is removed from the Tie2-Fc coated ELISA plates, after which 50 μl per well of the compound/biotinylated Ang2 mixtures is added (in duplicate wells). The plates are then incubated for 2 hours at room temperature, covered with plate sealers, on an orbital shaker. Plates are then washed 3X, after which 100 μl per well of streptavidin-HRP (R&D Systems #DY998), diluted 1:200 in blocking buffer is added. Plates are then incubated for 35 minutes at room temperature, covered with plate sealers, on an orbital shaker. Plates are then washed again 3X.

Plates are developed by adding 100 μl per well of One Component TMB substrate (Surmodics/BioFX Labs #TMBW-1000-01) which is warmed to room temperature. Development is allowed to progress for 10 minutes at room temperature, plates are covered with aluminum foil. Development is stopped with 100 μl per well of stop solution (Surmodics/BioFX Labs #LSTP-1000-01). Plates are mixed on an orbital shaker after which they are read at 450 nM on an ELISA reader (Molecular Devices SpectraMax 190), using SOFTmax PRO 5.4.1 software (Molecular Devices Corp.). The A450 values reflect the amount of biotinylated Ang2 that remained bound to Tie-2-Fc. Reduction of A450 values reflect blocking of biotinylated Ang2 binding to Tie-2-Fc.

IC50 values for inhibition of Ang2 binding to Tie-2 are calculated with SigmaPlot 9.0, using the "Pharmacology" menu, "Standard Curves Analysis" function. The curve is fit using a 4-parameter logistic fit (Hillslope method).

In experiments performed essentially as described in this assay, Compound A and the Ang2 parental antibody of Compound A result in geometric mean IC50 values (n=1) of 0.118 nM and 0.087 nM respectively. Compound A dose dependently blocks human Ang2 binding to human Tie-2 comparably to the Ang2 parental antibody. This data indicates that the Ang2 scFv polypeptide portion of Compound A has potency in this assay that is comparable to that of the parental Ang2 antibody.

Neutralization of Ang2 mediated phosphorylation of Tie2, but not Ang1 mediated phosphorylation.

The *in vitro* cell-based inhibition of human Ang2 by a compound of the present invention is measured in a cell-based assay where Ang1 and Ang2 bind to and induce human Tie2 phosphorylation in a dose-dependent manner. The *in vitro* cell-based assay is used to evaluate the ability of compounds of the present invention to selectively neutralize Ang2 and not Ang1 mediated phosphorylation of the Tie-2 receptor in a dose-dependent manner. An Ang2 antibody, an Ang2/Ang1 antibody, and a control human IgG4 PAA isotype antibody are included as positive and negative controls, respectively.

The CHO-Tie2 cell line is generated by stable transfection of a full-length human Tie2 receptor (with a 3X FLAG tag at the C-terminus). CHO-Tie2 cells are maintained in complete medium of Hams F-12 (CellGro/Mediatech #10-080-CV), 10% heat inactivated FBS (Life Technologies/Invitrogen #10082-147), 1X antibiotic-antimycotic (Life Technologies/Invitrogen #15240-062), 1.25 mg/ml G418 (Corning Cellgro #30-234-CI), 10 μg/ml puromycin (Calbiochem #540411), and 0.078% sodium bicarbonate (Thermo Hyclone #SH30033.01).

For this assay, CHO-Tie2 cells are resuspended to 10,000 cells per well (in 100 μl growth medium), into the inner 60 wells of poly-lysine coated 96-well plates (BD Biocoat #356640). 200 μl of D-PBS are placed into the edge wells to reduce evaporation. Cells are incubated overnight at 37°C, 95% RH, 5% CO_2. The next day, cells are washed once and medium is replaced with 100 μl serum-free growth medium containing 0.1% BSA

(Sigma #A7979, low endotoxin). Cells are then starved for 7 to 24 hours in serum-free medium at 37°C, 95% RH, 5% CO_2. During the starvation period, compounds (at 6X the final concentrations) are serially diluted 1:2 in polypropylene plates in serum-free growth medium containing 0.1% BSA. Human Ang2 (R&D Systems #623-AN, reconstituted in D-PBS/0.1% BSA) and human Ang1 (R&D Systems #923-AN, reconstituted in D-PBS/0.1% BSA) are also diluted to 6X the final concentration in serum-free growth medium containing 0.1% BSA. Test compounds and the Ang2 or Ang1 ligand are then mixed at a 1:1 ratio (v/v) in polypropylene plates and incubated for 60-80 minutes at 37°C. The compound/ligand mixtures are then added at 50 µl per well to the cells (in triplicate wells per treatment) and incubated for 13 minutes to 21 hours at 37°C, 95% RH, 5% CO_2. The final concentration range of compounds are 0.125 – 383 nM, and the final concentration of human Ang2 and Ang1 is 0.3 µg/ml (approx. 6 nM) and 0.5 µg/ml (approx. 8.9 nM), respectively. After the incubation time, medium is quickly and fully removed from the cells, and cells lysed in 60 µl per well of cold 1X Tris Lysis Buffer (Meso Scale Discovery #R60TX; 150 mM NaCl, 20 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) which contains freshly added protease and phosphatase inhibitors (1X protease inhibitor cocktail, Sigma #P8340; 1X phosphatase inhibitor cocktail 2, Sigma #P5726; 1X phosphatase inhibitor cocktail 3, Sigma #P0044; 1 mM final activated sodium orthovanadate EMD Chemicals #567540). Plates are then placed on ice for 10 minutes, after which they may be placed on an orbital shaker at low speed for 25 minutes at 4°C. The plates are then sealed and frozen at -80°C.

The day before analysis for phospho-Tie2 (with a human phospho-Tie2 DuoSet ELISA kit from R&D Systems, #DYC2720), high binding ELISA plates (Greiner BioOne, #655081) are coated overnight at 4°C with 4 µg/ml mouse anti-human total Tie2 capture antibody in 1X ELISA coating buffer (Surmodics/BioFX Labs #COAT-1000-01).

The day of phospho-Tie2 measurement, plates containing lysates are thawed on ice. The coated ELISA plates are washed with wash buffer (1X TBST containing 0.05% Tween 20) and blocked with 300 µl per well of blocking buffer (1% BSA (Jackson ImmunoResearch #001-000-162; IgG-free, protease-free), 0.01% sodium azide) for a minimum of 1 hour at room temperature on an orbital shaker (while covered with plate sealers). During blocking, lysates are diluted 1:5 or 1:10 in polypropylene plates in cold

lysis buffer containing protease and phosphatase inhibitors. ELISA plates are washed 4X, and 100 µl per well of diluted lysates or phospho-Tie2 ELISA standards is added and incubated for 2 hours at room temperature, covered with sealers, on an orbital shaker. Plates are washed 4X and 100 µl per well of HRP conjugated mouse anti-phospho tyrosine (diluted as recommended on the vial, in TBST/0.1% BSA) is added. Plates may then be covered with sealers, and incubated for 2 hours at room temperature on an orbital shaker. Plates are then washed 6X and removal of liquid from the wells is ensured. Plates are then developed by adding 100 µl per well of One Component TMB substrate (Surmodics/BioFX Labs #TMBW-1000-01). Plates are allowed to develop for 30 minutes at room temperature covered with aluminum foil. Development is stopped with 100 µl per well of stop solution (Surmodics/BioFX Labs #LSTP-1000-01). Plates are then mixed on an orbital shaker. The ELISA plates are read at 450 nm on an ELISA reader (Molecular Devices SpectraMax 190), using SOFTmax PRO 5.4.1 software (Molecular Devices Corp.). Phospho-Tie2 values for the samples are obtained from the standard curve (4-parameter logistic fit), and multiplied by the dilution factor of 5 or 10.

IC50 values for inhibition of Ang2 induced phospho-Tie2 are calculated with GraphPad Prism 4, using Log-transformed X values. Nonlinear regression (curve fit) analysis (sigmoidal dose response, variable slope) is performed on the log-transformed data to obtain IC_{50} values.

In experiments performed essentially as described in this assay, Compound A dose-dependently neutralizes human Ang2 induced phospho-Tie2 in CHO-Tie2 cells with an IC50 of 0.87 nM (n=1) while the parental Ang2 antibody has an IC50 of 1.01 nM. The results indicate that Compound A neutralizes Ang2 induced phospho-Tie2, but does not neutralize human Ang1 induced phospho-Tie2 in CHO-Tie-2 cells when compared to the positive control Ang2/Ang1 antibody. Moreover, this data indicates that the Ang2 scFv polypeptide portion of Compound A has maintained potency in this assay that is comparable to that of the parental Ang2 antibody.

Neutralization of human VEGFA induced phosphorylation of human VEGFR2

The *in vitro* cell-based inhibition of human VEGFA is measured in a cell-based assay where binding of VEGFA165 to VEGFR2 on a VEGFR2 expressing cell line,

induces VEGFR2 phosphorylation in a dose-dependent manner. The assay is used to evaluate the ability of a compound of the present invention to selectively neutralize VEGFA mediated phosphorylation of the VEGFR2 receptor in a dose-dependent manner. A VEGFA antibody and an irrelevant antibody human IgG4 PAA isotype are included as a positive and negative control, respectively.

For the assay, VEGFR2 expressing human ECFC (endothelial colony forming cells, derived from umbilical cord blood endothelial progenitors) (Endgenitor Technologies, Lot 100506-14-P4, passages 8-10) are seeded at 14,000 cells per well (in 100 µl growth medium), into the inner 60 wells of collagen I coated 96-well plates (BD Biocoat #35-4407) in growth medium: EGM-2MV BulletKit (Lonza #CC-4147). Components of the included EGM-2MV Singlequot bag may be added to 500 ml of EBM-2 basal medium, adjusted to 10% final FBS concentration (Life Technologies/Invitrogen #10082-147, heat inactivated). 250 µl of growth medium are placed into the edge wells to reduce evaporation. Cells are incubated ON at 37°C, 95% RH, 5% CO_2. The next day, medium is removed and replaced with 100 µl serum-free EBM-2 basal medium containing 0.1% BSA (Sigma #A7979, low endotoxin). Cells are starved for 6.5 hours at 37°C, 95% RH, 5% CO_2. During the starvation period, compounds (at 6X the final concentrations) are serially diluted 1:4 in polypropylene plates in EBM-2/0.1% BSA. Human VEGFA165 is diluted to 6X the final concentration in EBM-2/ 0.1% BSA. Compounds and VEGFA165 are then mixed at a 1:1 ratio (v/v) in polypropylene plates and incubated for 1 hour at 37°C. The compound/VEGFA165 mixtures are then added at 50 µl per well to the cells (in triplicate wells per treatment) and incubated for 5 minutes at 37°C, 95% RH, 5% CO_2. (The final concentration range of compounds is 0.018 – 300 nM, and the final concentration of human VEGFA165 is 0.16 nM). Medium is removed from the cells, and cells lysed in 60 µl per well of cold 1X Tris Lysis Buffer (Meso Scale Discovery #R60TX; 150 mM NaCl, 20 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) containing freshly added 1X protease and phosphatase inhibitors (included with the phospho-VEGFR2 assay kit). Plates are placed on ice for 10 minutes, then on an orbital shaker at low speed for 20 minutes at 4°C. Plates are then sealed and frozen at -80°C.

The day of phospho-VEGFR2 measurement, plates containing lysates are thawed on ice. Phospho-VEGFR2 levels are measured using a phospho-VEGFR2 (Tyr1054) whole cell lysate kit, (Meso Scale Discovery #K151DJD). Meso Scale assay plates, pre-coated with an antibody against phospho-VEGFR2, are blocked with 150 µl per well of blocking buffer (3% blocker A in TBST) for a minimum of 1 hour at room temperature on an orbital shaker (while covered with plate sealers). The plates are washed 3X with 1X Meso Scale wash buffer, and 50 µl of lysates is added per well (incubated for 1 hour at room temperature, covered with sealers, on an orbital shaker). The plates are washed 3X and 25 µl per well of 1X MSD SULFO-TAG™ conjugated anti-total VEGFR2 (diluted in the manufacturer's recommended antibody diluent) is added, and incubated for 1 hour at room temperature, covered with sealers, on an orbital shaker. Plates are washed 3X and liquid from the wells is removed. 150 µl per well of 1X Read Buffer T is added to the plates, and read immediately on a Meso Scale Discovery SECTOR Imager MA6000.

IC50 values for inhibition of VEGFA165 induced phospho-VEGFR2 are calculated with GraphPad Prism 4, using Log-transformed X values. Nonlinear regression (curve fit) analysis (sigmoidal dose response, variable slope) are performed on the log-transformed data to obtain IC50 values. If an experiment was performed more than once, the geometric mean IC50 value between experiments is calculated.

In experiments performed essentially as described in this assay, Compound A has an IC50 of 0.252 nM (n=2) that is comparable to the IC50 of 0.194 nM (n=2) for parental VEGFA antibody. The results indicate that the VEGFA antibody portion of Compound A has maintained potency. The results also show that Compound A has comparable VEGFA165 neutralization activity to bevacizumab (purchased from Myoderm Medical Supply, Norristown, PA), which has an IC50 of 0.290 nM.

Neutralization of VEGFA induced cell proliferation.

The *in vitro* cell-based inhibition of human VEGFA by a compound of the present invention is measured in a cell-based assay where VEGFA165 induces proliferation in a dose-dependent manner. The ability of a compound of the present invention to neutralize human VEGFA165 induced proliferation is measured in human ECFC (endothelial

colony forming cells, derived from umbilical cord blood endothelial progenitors). VEGFA antibodies and an irrelevant human IgG4 PAA antibody are included as positive and negative controls.

Human ECFC (Endgenitor Technologies, Lot 100506-14-P4) are maintained in collagen coated flasks in EGM-2MV BulletKit (Lonza #CC-4147). Components of the included EGM-2MV Singlequot bag are added to 500 ml of EBM-2 basal medium, adjusted to 10% final FBS concentration (Life Technologies/Invitrogen #10082-147, heat inactivated).

For the assay, ECFC at passages 7-12 is washed twice in pre-warmed cell plating medium, and plated into the inner 60 wells of collagen I coated 96-well plates (BD Biocoat #35-4407) at 4,200 cells per 150 µl/well of cell plating media. 250 µl of assay diluent is added to the edge wells to reduce evaporation. Cell plating medium consists of Medium 199 (M199) with Earle's Salts (Life Technologies/Invitrogen #11150-059) containing 3.3% FBS (Life Technologies/Invitrogen #10082-147, heat inactivated), 10 mM HEPES (Thermo Scientific/HyClone #SH30237.01) and 1X penicillin-streptomycin (Thermo Scientific/HyClone #SV30010). Assay diluent consists of serum-free M199/HEPES/penicillin-streptomycin, containing 0.1% BSA (Sigma #A7979, low endotoxin). Cells are incubated 60-90 minutes at 37°C, 95% RH, 5% CO_2 before treating. Compounds and human VEGFA165 are diluted in assay diluent at 8X the final concentrations. Compounds are serially diluted 1:4 in polypropylene plates in assay diluent. Compounds and VEGFA165 are then mixed at a 1:1 ratio (v/v) in polypropylene plates and incubated for 1-3 hours at 37°C. The compound/VEGFA165 mixtures are then added at 50 µl per well to the cells in triplicate wells per treatment and incubated for a total of 3 days at 37°C, 95% RH, 5% CO_2. The final concentration range of compounds is 0.049 – 800 nM, and the final concentration of human VEGFA165 is 0.16-0.5 nM.

Two days before the end of the incubation period, the cells are pulsed with 10 µl (1 µCi) of methyl-^{3}H thymidine (Perkin Elmer #NET027005MC, 6.7 Ci/mmol; 1 mCi/ml stock diluted 1:10 in PBS). At the end of the incubation period, the plates are frozen at -80°C and then thawed for 1-2 hours at 37°C. The cells are then harvested on 96-well glass fiber filter plates (Perkin Elmer UniFilter, GF/C, #6005174) with distilled water. After the filter plates are air dried, the incorporated ^{3}H thymidine is counted in 20 µl

scintillant per well (MicroScint 0, Perkin Elmer #6013611) on a Perkin Elmer-Packard TopCount microplate scintillation counter.

IC_{50} values for inhibition of VEGFA165 induced proliferation are calculated with GraphPad Prism 4, using Log-transformed X values. Medium alone values are included as the highest point of curves; the X-value (concentration) for medium alone are set to 100X higher than the highest X value. Also, the VEGFA alone values are included as the lowest point of the curves; the concentration for VEGFA alone is set to 100X lower than the lowest X value. Nonlinear regression (curve fit) analysis (sigmoidal dose response, variable slope) is performed on the log-transformed data to obtain IC50 values. If an experiment was performed more than once, the geometric mean IC50 value between experiments is calculated.

In experiments performed essentially as described in this assay, Compound A dose dependently neutralizes human VEGFA165-induced proliferation of ECFC to levels similar to both the parental VEGFA antibody and bevacizumab (n= 2-3 experiments) at IC50's of 1.18 nM, 1.38 nM and 2.68 nM, respectively.

Repression of pathological angiogenesis in oxygen-induced retinopathy model.

The *in vivo* repression of pathological angiogenesis by a VEGFA-Ang2 bispecific molecule, such as Compound A, is measured in a model of oxygen-induced retinopathy in the mouse retina. The assay is used to study the ability of compounds of the present invention to repress pathological angiogenesis in the mouse retina.

For this assay, the day of mouse pup delivery by the pregnant females is marked P0 (postnatal day 0). Following delivery, at day 7 (P7) pups are placed in a chamber at 75% oxygen. At P12, pups are moved back at room air (20% oxygen) and injected with vehicle control (PBS) or 10 mg/kg of Ang2 or VEGFA antibodies or 13.5 mg/kg of the test compound to maintain comparable molar amounts of the molecules. At P15, pups are injected a second time at the same doses. At P17, mice are sacrificed and eyes harvested and fixed in formalin for 5 hours and washed with PBS.

Retinas are then dissected, and stained with anti-CD31 diluted at 1:200 (BD Pharmingen; clone MEC 13.3; #553370), and anti-SMA-FTIC diluted at 1:200 (Sigma; Clone1A4 #F3777). For the anti-CD31 treated retinas, an anti-Rat Alexa-647 antibody

diluted at 1:400 (Jackson Immuno Research; #712-606-153) is used as a secondary antibody. Acquisition of the retinas is done by using Nikon Ti, and quantification of area enclosing pathological blood endothelial cell structures, i.e., glomeruloid microvascular proliferation (GMP) of full retina is performed by using FIJI software. High magnification images are acquired using a confocal Nikon A1.

In experiments performed essentially as described in this assay, Compound A and the combination treatment of the parental Ang2 antibody and the parental VEGFA antibody comparably repress GMP area of full retina with a Mean(%) of 17.20% (std error 3.853) and 14.10% (std. error 3.822), respectively, with p=0.9778. Furthermore, Compound A shows increased repression of GMP area of full retina compared to the parental Ang2 antibody or the parental VEGFA antibody treatment alone, which showed a Mean(%) of 83.03% (std error 4.542) p<0.0001 and 40.14% (std. error 2.189) p=0.029, respectively. These results indicate that Compound A has not only maintained function and potency equivalent to the combination treatment of Ang2 antibody plus VEGFA antibody, but also has a superior treatment effect when compared to each agent alone.

Table 6

GMP area of full retina					
Parameters	Vehicle	Ang2 parental mAb	VEGFA parental mAb	Ang2 mAb + VEGFA mAb	Compound A
Mean (%)	100	83.03	40.14	14.10	17.20
Std. Error of Mean	9.526	4.542	2.199	3.822	3.853
P value (Vehicle vs. Compound) (Dunnett's test)		0.102	< 0.0001	< 0.0001	< 0.0001
P value (Ang2 mAb vs. Compound) (Dunnett's test)			< 0.0001	< 0.0001	< 0.0001
P value (VEGFA mAb vs. Compound) (Dunnett's test)				0.012	0.029
P value (Ang2 mAb + VEGFA					0.9778 (non-

mAb vs. Compound) (Dunnett's test)					significant)

Inhibition of VEGFA Induced Cord Formation

The *in vitro* inhibition of VEGFA induced cord formation is measured in an *in vitro* co-culture system. The assay is used to measure inhibition of VEGFA induced cord formation by a compound of the present invention.

For this assay, adipose derived stem cells (ADSC; Lonza #PT5006, lot#OF4505-01) are cultured on Corning culture flasks (Corning #431082) in EGM-2MV medium (Lonza #CC3202). Endothelial colony forming cells (ECFC; Lonza, lot#EGT-ECFC100506r) are cultured on Collagen I coated flasks (BD Biosciences #356486) in EGM-2MV medium supplemented with 5% heat inactivated FBS (Gibco #10082-147). ADSC at passages 4-6 are harvested from culture flasks which are rinsed with DPBS (Hyclone #SH30028.03) followed by TrypLE Express (Gibco #12605-010). ADSC cells are suspended in Basal Medium (MCDC-131 (Gibco #10372-019) supplemented with 10 µg/ml insulin, 1 µM dexamethasone, 30 µg/ml ascorbic acid, 10 µg/ml human transferrin and 50 µg/ml tobramycin). Viable cell count is determined and cells are seeded onto black, clear bottomed 96-well plates (BD Falcon #353219) at 4 x 10^4 cells per well in 100 µl Basal Medium. Cells are incubated at 37°C in 5% CO_2 overnight to allow attachment. Next day, ECFC at passages 7-10 is harvested in Basal Medium as above and viable cell count is adjusted to 4 x 10^4 cells per ml. Medium is removed from ADSC cells and 100 µl ECFC cell suspension is added to each well. Plates are incubated at 37°C in 5% CO_2 for 2-3 hours to allow cells to settle on top of the ADSC monolayer. Compounds of the present invention are diluted to 80 µg/ml in Basal Medium, and then serially diluted 1:3 with Basal Medium to produce a nine point dose response series. 50 µl of each dilution of compound is added to the co-culture. 50 µl of an 80 ng/ml solution of rhVEGFA (R&D #293-VE/CF, 50 µg/ml in DPBS) prepared in Basal Medium is added to the co-culture plus compound combination. Final concentrations for compounds and rhVEGFA are 20 µg/ml and 20 ng/ml, respectively. Positive control for the assay is 20 ng/ml rhVEGFA in the absence of compound. Negative control for the assay is Basal Medium

without rhVEGFA. Plates are then incubated at 37°C in 5% CO_2 for 3 days to allow cords to form.

At the end of the incubation period, medium is aspirated from each well and 100 µl room temperature 80% ethanol is carefully added. Plates are incubated at room temperature for 20 minutes. Ethanol solution is aspirated and wells washed twice with 150 µl DPBS. Anti-huCD31 (R&D #AF806 Affinity purified sheep IgG, 200ug/ml) and MAB Anti-Actin, alpha-Smooth Muscle-Cy3 (Sigma #C6198) are each diluted 1:250 in 2.5% FBS/DPBS. A 100 µl antibody mixture is added to wells and plates are incubated at 37°C in 5% CO_2 for 2 hours. Plates are then aspirated and wells washed twice with 150 µl DPBS. Alexa Fluor 488 donkey anti-sheep IgG (H+L) (Life Technologies #A11015) is diluted 1:400 and Hoescht 33342 (Life Technologies #H3570) is diluted 1:1000 in 2.5% FBS/DPBS and 100 µl per well is added to plates. Plates are incubated at room temperature protected from light for 30 minutes. Wells are then washed twice with 150 µl DPBS. 150 µl DPBS is added to each well and plates sealed with black adhesive seals (PerkinElmer #6050173).

Plates are read on the ArrayScan VTI HCS Reader (Cellomics-Thermo Fisher) using the Tube Formation Bio-application. Total Tube Area data is plotted against compound concentrations in nM in GraphPad Prism 6. Compound concentrations are transformed into log data and IC_{50} values for inhibition are calculated by nonlinear regression (sigmoidal dose response, variable slope). Each experiment represents the mean of triplicates and triplicate experiments are expressed as the geometric means and 95% confidence intervals calculated.

In experiments performed essentially as described in this assay, Compound A dose dependently inhibits human VEGFA-induced cord formation in the ADSC/ECFC co-culture system, comparably to bevacizumab and parental VEGFA antibody with mean IC_{50} of 1.680 nM, 1.578 and 1.570 nM, respectively (n=3). This indicates that the VEGFA antibody portion of Compound A has maintained potency that is comparable to the parental VEGFA antibody in this cell based assay.

Compound A inhibits *in vivo* tumor growth

The efficacy of the compounds of the present invention is measured via *in vivo* xenograft models. The antitumor efficacy of the parental VEGFA antibody, Compound A, and its combination is assessed in the subcutaneous triple negative patient derived breast cancer model (EL1997) and the subcutaneous ovarian xenograft model (SKOV3x.1). Mice bearing tumors are treated with compounds diluted in PBS, on a twice weekly basis via intra-peritoneal injection. Tumor growth is determined by three dimensional caliper measurements of tumor volumes twice weekly during the course of treatment.

EL1997 triple negative breast patient derived xenografts: Immuno-deficient mice bearing EL1997 triple negative breast patient derived xenografts (TNBC PDX) at approximately 350mm^3 volume randomized at n=7 mice/group are treated with vehicle control, parental VEGFA antibody at 20 mg/kg, parental Ang2 antibody at 20 mg/kg, or Antibody A dosed at 26.7 mg/kg. Treatments are administered twice a week for 4 consecutive weeks.

In experiments performed essentially as described, parental VEGFA antibody and parental Ang2 antibody treatment groups exhibits a %T/C (change in tumor volume) of 12% and 56.7%, respectively. Compound A results in a % regression of -15.4% with a p=0.0151 when compared to the parental VEGFA antibody. These results indicate that Compound A has greater efficacy than treatment with either the parental VEGFA antibody or the parental Ang2 antibody alone.

SKOV3x.1 ovarian xenografts: Immuno-deficient mice bearing SKOV3x.1 ovarian xenografts at approximately 250mm^3 volume, randomized at n=10 mice/group, are treated with vehicle control, parental VEGFA antibody at 10 mg/kg, parental Ang2 antibody at 10 mg/kg, combination of parental VEGFA antibody and parental Ang2 antibody at 10 mg/kg each, or 13.3 mg/kg (equimolar to 10 mg/kg of antibody) Compound A. Treatments may be administered twice a week for 4 weeks.

In experiments performed essentially as described, monotherapy treatment of parental VEGFA antibody and of parental Ang2 antibody resulted in a %T/C of 9.6% and 38.5%, respectively. The combination of parental VEGFA antibody and parental Ang2 antibody or Compound A results in tumor regressions of -8.1% and -2.7% respectively.

These results indicate in the xenograft model that Compound A has a potential for greater efficacy than parental VEGFA antibody or parental Ang2 antibody alone.

Amino Acid and Nucleotide Sequences

SEQ ID NO: 1 (HCVR of antibody – Compound A)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGS
GGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDPSDSSSWYF
AFDIWGQGTTVTVSS

SEQ ID NO: 2 (HC of antibody – Compound A)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGS
GGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDPSDSSSWYF
AFDIWGQGTTVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVS
WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVD
KRVESKYGPPCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDP
EVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALH
NHYTQKSLSLSLG

SEQ ID NO: 3 (HC of antibody/linker/scFv polypeptide – Compound A)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGS
GGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDPSDSSSWYF
AFDIWGQGTTVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVS
WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVD
KRVESKYGPPCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDP
EVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALH
NHYTQKSLSLSLGGGGSGGGGSGGGGSQVQLVQSGAEVKKPGASVKVSCKASG
YSFTDYNMVWVRQAPGQCLEWMGYIDPYNGGTGYNQKFEGRVTMTTDTSTST
AYMELRSLRSDDTAVYYCARTRDRYDVWYFDVWGQGTLVTVSSGGGGSGGGG
SGGGGSGGGGSGGGGSDIQMTQSPSSVSASVGDRVTITCKASQDVYIAVAWYQQ

KPGKAPKLLIYWASTRDTGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCHQYSSY
PPTFGCGTKVEIK

SEQ ID NO: 4 (LCVR of antibody – Compound A)
DIVMTQSPATLSVSPGQRATLSCRASQNIRNNLAWYQQKRGQAPRLLIYGASTRA
TGIPDRFSGSGSGADFTLTISKLEPEDFAVYYCQQYGSSPRTFGQGTKVDIK

SEQ ID NO: 5 (LC of antibody – Compound A)
DIVMTQSPATLSVSPGQRATLSCRASQNIRNNLAWYQQKRGQAPRLLIYGASTRA
TGIPDRFSGSGSGADFTLTISKLEPEDFAVYYCQQYGSSPRTFGQGTKVDIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO: 6 (scFv polypeptide – Compound A)
QVQLVQSGAEVKKPGASVKVSCKASGYSFTDYNMVWVRQAPGQCLEWMGYID
PYNGGTGYNQKFEGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCARTRDRYDV
WYFDVWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSDIQMTQSPSSVS
ASVGDRVTITCKASQDVYIAVAWYQQKPGKAPKLLIYWASTRDTGVPSRFSGSG
SGTDFTLTISSLQPEDFATYYCHQYSSYPPTFGCGTKVEIK

SEQ ID NO: 7 (HCVR of scFv polypeptide – Compound A)
QVQLVQSGAEVKKPGASVKVSCKASGYSFTDYNMVWVRQAPGQCLEWMGYID
PYNGGTGYNQKFEGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCARTRDRYDV
WYFDVWGQGTLVTVSS

SEQ ID NO: 8 (LCVR of scFv polypeptide – Compound A)
DIQMTQSPSSVSASVGDRVTITCKASQDVYIAVAWYQQKPGKAPKLLIYWASTR
DTGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCHQYSSYPPTFGCGTKVEIK

SEQ ID NO: 9 (DNA of HC of antibody/linker/scFv polypeptide – Compound A)

GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCC
TGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGC
TGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTG
GTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCAT
CTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGA
GCCGAGGACACGGCCGTATATTACTGTGCAAGAGATCCCTCGGATAGCAGCA
GCTGGTACTTTGCTTTTGATATCTGGGGCCAAGGGACCACGGTCACCGTCTCC
TCAGCCTCTACCAAGGGCCCATCGGTCTTCCCGCTAGCGCCCTGCTCCAGGAG
CACCTCCGAGAGCACAGCCGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCC
GAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCACA
CCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTG
ACCGTGCCCTCCAGCAGCTTGGGCACGAAGACCTACACCTGCAACGTAGATC
ACAAGCCCAGCAACACCAAGGTGGACAAGAGAGTTGAGTCCAAATATGGTCC
CCCATGCCCACCCTGCCCAGCACCTGAGGCCGCCGGGGGACCATCAGTCTTCC
TGTTCCCCCCAAAACCCAAGGACACTCTCATGATCTCCCGGACCCCTGAGGTC
ACGTGCGTGGTGGTGGACGTGAGCCAGGAAGACCCCGAGGTCCAGTTCAACT
GGTACGTGGATGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGG
AGCAGTTCAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAG
GACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGGCCTCC
CGTCCTCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAGCC
ACAGGTGTACACCCTGCCCCCATCCCAGGAGGAGATGACCAAGAACCAGGTC
AGCCTGACCTGCCTGGTCAAAGGCTTCTACCCCAGCGACATCGCCGTGGAGT
GGGAAAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCT
GGACTCCGACGGCTCCTTCTTCCTCTACAGCAGGCTAACCGTGGACAAGAGC
AGGTGGCAGGAGGGGAATGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGC
ACAACCACTACACACAGAAGAGCCTCTCCCTGTCTCTGGGTGGCGGAGGCTC
CGGGGGAGGGGGTAGCGGAGGAGGGGGATCCCAGGTTCAGCTGGTGCAGTC
TGGAGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAAGGTCTCCTGCAAGGCT
TCTGGTTACTCATTCACTGACTACAACATGGTGTGGGTGCGACAGGCCCCTGG
ACAATGCCTTGAGTGGATGGGATATATTGATCCTTACAATGGTGGTACTGGCT
ACAACCAGAAGTTCGAGGGCAGAGTCACCATGACCACAGACACATCCACGAG

CACAGCCTACATGGAGCTGAGGAGCCTGAGATCTGACGACACGGCCGTGTAT
TACTGTGCGAGAACGAGGGATAGGTACGACGTCTGGTACTTCGATGTCTGGG
GCCAGGGAACCCTGGTCACCGTCTCCTCAGGAGGCGGAGGTTCCGGGGGAGG
GGGCAGCGGAGGAGGCGGATCGGGCGGAGGAGGAAGTGGAGGCGGAGGATC
TGACATCCAGATGACCCAGTCTCCATCTTCCGTGTCTGCATCTGTAGGAGACA
GAGTCACCATCACTTGTAAGGCCAGTCAGGATGTGTATATTGCTGTAGCCTGG
TATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATTGGGCATCCA
CCCGGGACACTGGGGTCCCATCAAGGTTCAGCGGCAGTGGATCTGGGACAGA
TTTCACTCTCACCATCAGCAGCCTGCAGCCTGAAGATTTTGCAACTTACTATT
GTCACCAATATAGCAGCTATCCTCCTACGTTCGGCTGCGGGACCAAGGTGGA
GATCAAA

SEQ ID NO: 10 (DNA of LC of antibody-- Compound A)
GATATTGTGATGACTCAGTCTCCAGCCACCCTGTCTGTGTCTCCAGGGCAAAG
AGCCACCCTCTCCTGCAGGGCCAGTCAAAATATTAGGAATAACTTAGCCTGGT
ACCAGCAGAAACGTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCGTCCACT
CGGGCCACAGGTATCCCAGACAGGTTCAGTGGCAGTGGGTCTGGGGCGGACT
TCACTCTCACCATCAGCAAACTGGAGCCTGAAGATTTTGCAGTTTATTACTGT
CAGCAATATGGTAGCTCACCTCGGACGTTCGGCCAAGGGACCAAAGTGGATA
TCAAAAGAACTGTGGCGGCGCCATCTGTCTTCATCTTCCCGCCATCTGATGAG
CAGTTGAAATCCGGAACTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCC
CAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAAC
TCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCA
GCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTACGC
CTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAAC
AGGGGAGAGTGC

SEQ ID NO: 11 (human VEGFA165)
APMAEGGGQNHHEVVKFMDVYQRSYCHPIETLVDIFQEYPDEIEYIFKPSCVPLM
RCGGCCNDEGLECVPTEESNITMQIMRIKPHQGQHIGEMSFLQHNKCECRPKKDR

ARQENPCGPCSERRKHLFVQDPQTCKCSCKNTDSRCKARQLELNERTCRCDKPR
R

SEQ ID NO: 12 (human Ang2)

YNNFRKSMDSIGKKQYQVQHGSCSYTFLLPEMDNCRSSSSPYVSNAVQRDAPLE
YDDSVQRLQVLENIMENNTQWLMKLENYIQDNMKKEMVEIQQNAVQNQTAVM
IEIGTNLLNQTAEQTRKLTDVEAQVLNQTTRLELQLLEHSLSTNKLEKQILDQTSEI
NKLQDKNSFLEKKVLAMEDKHIIQLQSIKEEKDQLQVLVSKQNSIIEELEKKIVTA
TVNNSVLQKQQHDLMETVNNLLTMMSTSNSAKDPTVAKEEQISFRDCAEVFKSG
HTTNGIYTLTFPNSTEEIKAYCDMEAGGGGWTIIQRREDGSVDFQRTWKEYKVGF
GNPSGEYWLGNEFVSQLTNQQRYVLKIHLKDWEGNEAYSLYEHFYLSSEELNYR
IHLKGLTGTAGKISSISQPGNDFSTKDGDNDKCICKCSQMLTGGWWFDACGPSNL
NGMYYPQRQNTNKFNGIKWYYWKGSGYSLKATTMMIRPADF

SEQ ID NO: 13
GGGGSGGGGS

SEQ ID NO: 14
GGGGSGGGGSGGGGS

SEQ ID NO: 15
GGGGSGGGGSGGGGSGGGGS

SEQ ID NO: 16
GGGGSGGGGSGGGGSGGGGSGGGGS

SEQ ID NO: 17
GGGSGGGGSGGGGS

WE CLAIM:

1. A compound, comprising an antibody fused by two linkers to two single chain fragment variable (scFv) polypeptides, wherein:
   a) the antibody comprises two identical heavy chains (HCs) and two identical light chains (LCs), wherein each HC comprises a heavy chain variable region (HCVR) whose amino acid sequence is given in SEQ ID NO: 1, and wherein each LC comprises a light chain variable region (LCVR) whose amino acid sequence is given in SEQ ID NO: 4,
   b) the two scFv polypeptides are identical and each comprise an HCVR operably linked to an LCVR, wherein each HCVR has the amino acid sequence given in SEQ ID NO: 7, and wherein each LCVR has the amino acid sequence given in SEQ ID NO: 8, and
   c) the two linkers are identical glycine-rich linkers that each operably link the carboxy-terminus of one HC of the antibody to the amino-terminus of one of the scFv polypeptides.
2. The compound of Claim 1, wherein the two scFv polypeptides each comprise the carboxy-terminus of the HCVR of one scFv polypeptide operably linked to the amino-terminus of the LCVR of one scFv polypeptide.
3. The compound of Claims 1 or 2, wherein the antibody comprises two heavy chains (HCs) and two light chains (LCs), wherein each HC has the amino acid sequence given in one of SEQ ID NO: 2, and each LC has the amino acid sequence given in one of SEQ ID NO: 5.
4. The compound of any one of Claims 1-3, wherein each scFv polypeptide has the identical amino acid sequence given in SEQ ID NO: 6.
5. A compound comprising two first polypeptides and two second polypeptides wherein each of the first polypeptides has the amino acid sequence of SEQ ID NO: 3, and each of the second polypeptides has the amino acid sequence of SEQ ID NO: 5.

6. The compound of Claim 5, wherein each of the first polypeptides forms an inter-chain disulfide bond with each of the second polypeptides, and the first polypeptide forms two inter-chain disulfide bonds with the other first polypeptide, and each of the first polypeptides forms seven intra-chain disulfide bonds.
7. A mammalian cell comprising a DNA molecule comprising a polynucleotide sequence encoding a first polypeptide given by SEQ ID NO: 3 and a polynucleotide sequence encoding a second polypeptide given by SEQ ID NO: 5, wherein the cell is capable of expressing a compound comprising the first polypeptide and the second polypeptide.
8. A process for producing a compound comprising two first polypeptides given by SEQ ID NO: 3 and two second polypeptides given by SEQ ID NO: 5, comprising cultivating the mammalian cell of Claim 7 under conditions such that the compound is expressed, and recovering the expressed compound.
9. A compound obtainable by the process of Claim 8.
10. A pharmaceutical composition, comprising the compound of any one of Claims 1-6, and an acceptable carrier, diluent, or excipient.
11. A method of treating cancer, comprising administering to a patient in need thereof, an effective amount of the compound of any one of Claims 1-6.
12. The method of Claim 11, wherein the cancer is breast cancer, lung cancer, ovarian cancer, gastric cancer, colorectal cancer, or hepatocellular carcinoma.
13. A method of treating proliferative retinopathy, comprising administering to a patient in need thereof, an effective amount of the compound of any one of Claims 1-6.
14. The method of Claim 13, wherein the proliferative retinopathy is diabetic retinopathy, or retinopathy of prematurity.
15. A method of treating angiogenic eye disease, comprising administering to a patient in need thereof, an effective amount of the compound of any one of Claims 1-6.

16. The method of Claim 15, wherein the angiogenic eye disease is neovascular glaucoma, age-related macular degeneration, diabetic macular edema, corneal neovascularization, corneal graft neovascularization, corneal graft rejection, retinal/choroidal neovascularization, neovascularization of the angle (rubeosis), ocular neovascular disease, vascular restenosis, or arteriovenous malformations (AVM).
17. The compound of any one of Claims 1-6, for use in therapy.
18. The compound of any one of Claims 1-6, for use in the treatment of cancer.
19. The compound for use of Claim 18, wherein the cancer is breast cancer, lung cancer, ovarian cancer, gastric cancer, colorectal cancer, or hepatocellular carcinoma.
20. The compound of any one of Claims 1-6, for use in the treatment of proliferative retinopathy.
21. The compound for use of Claim 20, wherein the proliferative retinopathy is diabetic retinopathy, or retinopathy of prematurity.
22. The compound of any one of Claims 1-6, for use in the treatment of angiogenic eye disease.
23. The compound for use of Claim 22, wherein the angiogenic eye disease is neovascular glaucoma, age-related macular degeneration, diabetic macular edema, corneal neovascularization, corneal graft neovascularization, corneal graft rejection, retinal/choroidal neovascularization, neovascularization of the angle (rubeosis), ocular neovascular disease, vascular restenosis, or arteriovenous malformations (AVM).

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/014667

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/22 A61P35/00 A61K39/395 A61P27/02
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/040508 A1 (HOFFMANN LA ROCHE [CH]; BAEHNER MONIKA [DE]; BRINKMANN ULRICH [DE]; GE) 15 April 2010 (2010-04-15) cited in the application page 49, line 1 - page 51, line 11; claims; figure 1B; examples; table 3	1-23
A	WO 2012/131078 A1 (BOEHRINGER INGELHEIM INT [DE]; GSCHWIND ANDREAS [AT]; OTT RENE GEORG [) 4 October 2012 (2012-10-04) cited in the application page 22, line 12 - page 23, line 20; examples	1-23
A	WO 2010/145793 A1 (HOFFMANN LA ROCHE [CH]; IMHOF-JUNG SABINE [DE]; KLEIN CHRISTIAN [CH];) 23 December 2010 (2010-12-23) examples	1-23

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 April 2016	03/05/2016

Name and mailing address of the ISA/	Authorized officer
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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2011/117329 A1 (HOFFMANN LA ROCHE [CH]; BAEHNER MONIKA [DE]; IMHOF-JUNG SABINE [DE]; K) 29 September 2011 (2011-09-29) cited in the application examples	1-23
A	WO 2014/137961 A1 (LILLY CO ELI [US]) 12 September 2014 (2014-09-12) examples	1-23
A,P	WO 2015/179168 A1 (LILLY CO ELI [US]) 26 November 2015 (2015-11-26) examples; sequence 19	1-23

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2016/014667

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