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(71) Applicants: **FUSIMAB LTD.** [IL/IL]; 14 Shenkar Street, P.O. Box 4093, 4614002 Herzlia (IL). **RAMOT AT TEL-AVIV UNIVERSITY LTD.** [IL/IL]; P.O. Box 39296, 6139201 Tel-Aviv (IL).

(72) Inventors: **BENHAR, Itai**; 38 Bareket Street, 7622785 Rehovot (IL). **HAKIM, Rahel**; 4493000 Kibbutz Ramat HaKovesh (IL). **NOIMAN, Silvia**; 12A Kibbush HaAvoda Street, 4632214 Herzlia (IL).

(74) Agents: **G.E. EHRLICH (1995) LTD.** et al.; 11 Menachem Begin Road, 5268104 Ramat Gan (IL).

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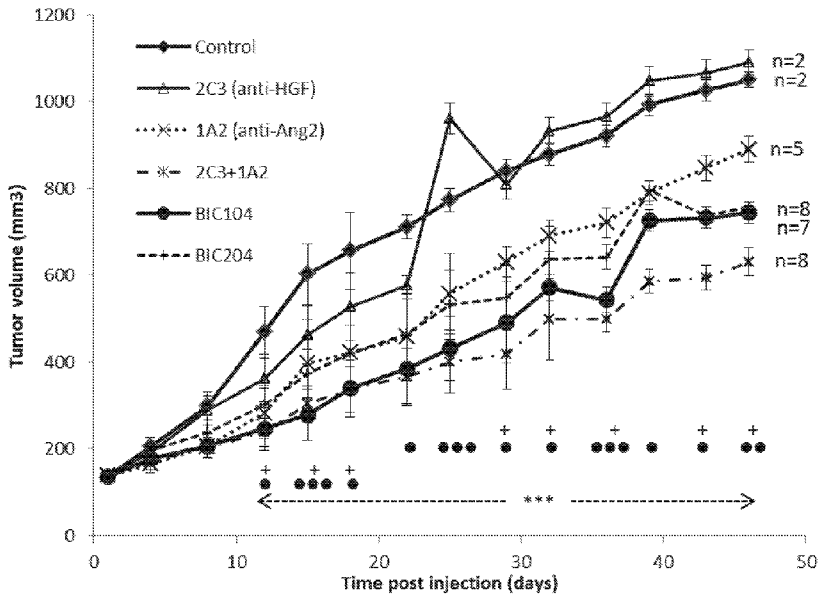
[Continued on next page]

(54) Title: MONOSPECIFIC ANTI-HGF AND ANTI-ANG2 ANTIBODIES AND BISPECIFIC ANTI-HGF/ANTI-ANG2 ANTIBODIES

FIG. 17A



(57) Abstract: An antibody comprising a human angiopoietin-2 (ANG2) or human hepatocyte growth factor (HGF) recognition region is disclosed. Bispecific antibodies are also disclosed which comprises a first antigen-binding site that specifically binds to HGF and a second antigen-binding site that specifically binds to ANG2. Uses thereof and compositions comprising same are also disclosed.

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MONOSPECIFIC ANTI-HGF AND ANTI-ANG2 ANTIBODIES AND BISPECIFIC ANTI-HGF/ANTI-ANG2 ANTIBODIES

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to monospecific and bispecific antibodies and, more particularly, but not exclusively, to bispecific antibodies which target hepatocyte growth factor (HGF) and angiopoietin-2 (ANG2).

Angiogenesis is implicated in the pathogenesis of a variety of disorders which include solid tumors, intraocular neovascular syndromes such as proliferative retinopathies or age-related macular degeneration (AMD), rheumatoid arthritis, and psoriasis. In the case of solid tumors, the neovascularization allows the tumor cells to acquire a growth advantage and proliferative autonomy compared to the normal cells. Accordingly, a correlation has been observed between density of microvessels in tumor sections and patient survival in breast cancer as well as in several other tumors.

Human angiopoietin-2 (ANG2) (alternatively abbreviated with ANGPT2 or ANG2) is described in Maisonpierre, P. C., et al, Science 277 (1997) 55-60 and Cheung, A. H., et al., Genomics 48 (1998) 389-91. The angiopoietins-1 and -2 (ANG-1 and ANG2) were discovered as ligands for the Tie2 Receptor, a tyrosine kinase receptor that is selectively expressed within the vascular endothelium. Whereas Ang-1 was shown to support EC survival and to promote endothelium integrity, ANG2 was shown to promote angiogenesis in tumors by destabilizing the blood vessels (e.g. by decreasing pericyte coverage) and sensitizing ECs to proliferation signals mediated by other proangiogenic factors, namely VEGF. ANG2 mediated blood vessel destabilization facilitates the infiltration of proteases, cytokines and angiogenic myeloid cells — which primes the vasculature for a robust angiogenic response in the presence of growth factors such as VEGFA. Moreover, the endothelial cells of unstable vessels die in the absence of VEGFA.

High levels of ANG2 correlate with increased metastatic and invasive potential in breast cancer [Imanishi, Y. et al. Angiopoietin-2 stimulates breast cancer metastasis through the $\alpha 5\beta 1$ integrin-mediated pathway. Cancer Res. 67, 4254–4263 (2007)], metastatic melanoma [Helfrich, I. et al. Angiopoietin-2 levels are associated with disease progression in metastatic malignant melanoma. Clin. Cancer Res. 15, 1384–1392

(2009)] and lung cancer [Park, J. H. et al. Serum angiopoietin-2 as a clinical marker for lung cancer. Chest 132, 200–206 (2007)]. Consistent with its role in angiogenesis and metastasis, ANG2 is also often expressed at the invasive fronts of human tumours, such as metastatic melanoma [Helfrich, I. et al. Angiopoietin-2 levels are associated with disease progression in metastatic malignant melanoma. Clin. Cancer Res. 15, 1384–1392 (2009)], invasive ductal breast carcinoma [Tsutsui, S. et al. Angiopoietin 2 expression in invasive ductal carcinoma of the breast: its relationship to the VeGF expression and microvessel density. Breast Cancer Res. Treat 98, 261–266 (2006)], and glioma [Hu, B. et al. Angiopoietin-2 induces human glioma invasion through the activation of matrix metalloprotease-2. Proc. Natl Acad. Sci. USA 100, 8904–8909 (2003)]. Using B16/F10 melanoma xenograft models, treatment with recombinant ANG2 disrupted pulmonary vascular integrity and led to increased vascular permeability and extravasation of circulating tumour cells [Huang, Y. et al. Pulmonary vascular destabilization in the premetastatic phase facilitates lung metastasis. Cancer Res. 69, 7529–7537 (2009)]. Intrapleural injection of lenti viruses encoding siRNA against ANG2 knocked down its expression in the lung and inhibited metastasis in the lung of orthotopically injected MDA-MB231-Luc-D3H1 breast cancer cells [Huang, Y. et al. Pulmonary vascular destabilization in the premetastatic phase facilitates lung metastasis. Cancer Res. 69, 7529–7537 (2009)]. These data strongly suggest that inhibiting ANG2 will not only have an effect on tumour angiogenesis, but may also decrease metastatic potential.

In recent years Angiopoietin-1, Angiopoietin-2 and/or Tie-2 have been proposed as possible anti-cancer therapeutic targets. Various strategies to target Tie-2 and block Tie-2 signaling pathways have been reported. Small molecule tyrosine kinase inhibitors ARRY-614 (Array BioPharma) and CEP-11981 (Cephalon) are found in Phase 1 clinical development. However, both are not Tie-2 selective, ARRY-614 inhibits also p38 and CEP-11981 inhibits VEGFR. Monoclonal antibodies which target the extracellular region of Tie-2 or ANG2, block ligand binding and thereby inhibit downstream events. Monoclonal antibodies to the extracellular domain of Tie-2 will inhibit ligand binding of both ANG2 and Ang-1. For example U.S. Pat. No. 6,166,185, U.S. Pat. No. 5,650,490 and U.S. Pat. No. 5,814,464 each discloses anti-Tie-2 ligand and receptor antibodies. In addition some groups have reported the use of anti-peptide and peptides that bind to Angiopoietin-2. AMG386 (Amgen) is an anti-Ang1/2 peptide fused to the Fc portion of

an antibody which is found in Phase 3 clinical development. PF-04856884 (Pfizer) is an engineered protein which targets ANG2 and is found in Phase 2 clinical development. See, for example, U.S. Pat. No. 6,166,185 and US 2003/10124129. WO 03/030833, WO 2006/068953, WO 03/057134 or US 2006/0122370.

Hepatocyte growth factor (HGF) is a multifunctional heterodimeric polypeptide produced by mesenchymal cells. HGF is composed of an alpha-chain containing an N-terminal domain and four kringle domains (NK1-4) covalently linked to a serine protease-like beta-chain C-terminal domain. Mammalian HGF is synthesized as a biologically inactive single chain precursor consisting of 728 amino acids with a 29 amino acid signal peptide which is not present in the mature protein. Biologically active HGF is achieved through cleavage at the R494 residue by a specific, extracellular serum serine protease. The active HGF thus achieved is a fully active heterodimer which is composed of disulfide linked 69 kDa alpha-chain and 34 kDa beta-chain.

The binding of HGF to its tyrosine kinase receptor, Met, induces the growth and scattering of various cell types, mediates epithelial mesenchymal transition (EMT) and the formation of tubules and lumens, and promotes angiogenesis. Both Met and HGF knockout mice are embryonic lethal and show developmental defects in placenta, fetal liver and limb/muscle formation. Aberrant paracrine or autocrine HGF production is seen in various epithelial and mesenchymal tumors. HGF/c-Met also play a role in angiogenesis, since downstream signaling results in endothelial cell proliferation and promotion of angiogenesis. Met signaling can induce the production of Vascular-Endothelial growth factor a (VEGFA) which is a recognized pro-angiogenic agent.

Met was originally isolated as a product of a human oncogene, trp-met, which encodes a constitutively active altered protein kinase with transforming activity. Met activation has also been shown to remarkably enhance the metastatic spread of cancer stemming from its stimulatory influence of processes such as angiogenesis, cell motility, and cell surface protease regulation (Wielenga et al., American Journal of Pathology 157(5): 1563-1573, 2000). Since Met was reported to be over-expressed in various human cancers of liver, prostate, colon, breast, brain and skin (Maulik et al., Cytokine & Growth Factor Reviews 13(1): 1-59, 2002), it has been regarded as an important target factor for the prevention and treatment of cancer.

HGF/c-Met are a recognized target for cancer (Yap et. al. Molecular Cancer Therapeutics 9: 1077-1079, 2012). Antibodies that recognize HGF for the treatment of cancer have been disclosed for example in U.S. Patent Application Numbers 20090155284, 20090023894 and 20070036789. Three anti-HGF antibodies were found in clinical development and have reached Phase 2. AV-299 (Aveo Pharma) and TAK-701 (Takeda) have failed to show efficacy in Phase 2 studies and their development has ceased. AMG102 (Amgen) has shown efficacy in Phase 2 only in a subset of patients which show high expression of HGF in the tumor.

Combinations of HGF and ANG2 inhibitors have been disclosed in U.S. Patent application Nos. 20050118643, 20120065380, 20120052073, 20110311546 and United States Patent 8,129,331.

More specifically, U.S. Patent application No. 20050118643 teaches the use of an antibody which recognizes HGF in combination with an inhibitor of ANG2.

U.S. Patent Application No. 20120065380 teaches bispecific antibodies where one arm of the antibody recognizes VEGFR-1, VEGFR-2, VEGFR-3, FLT3, c-FMS/CSF1R, RET, c-Met, EGFR, Her2/neu, HER3, HER4, FGFRs, IGFR, PDGFR, c-KIT, BCR, integrin or an MMP, whereas the other arm of the antibody recognizes VEGF, EGF, PlGF, FGF, PDGF, HGF, Tie-2 or angiopoietin.

U.S. Patent Application No. 20120052073 teaches anti-ang 2 antibodies in combination with HGF inhibitors.

U.S. Patent 8,129,331 teaches anti-ang 2 peptibodies in combination with HGF inhibitors.

U.S. Patent Application No. 20110311546 teaches antibodies which recognize ANG2 in combination with an antagonist of HGF.

U.S. Patent Application No. 20120100166 teaches bispecific antibodies which recognize ANG2 and c-MET.

Additional background art includes U.S. Patent Application Publication Nos. 2010/115589, 2012/123949 and 2010/0256340, and U.S. Patent Nos. 5,747,654, 6,147,203, 6,558,672 and 7,183,076.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a bispecific antibody comprising a first antigen-binding site that specifically binds to human hepatocyte growth factor (HGF) and a second antigen-binding site that specifically binds to human angiopoietin-2 (ANG2).

According to an aspect of some embodiments of the present invention there is provided an antibody comprising an HGF recognition region which comprises CDR sequences selected from the group consisting of:

(i) SEQ ID NOs: 1-6;

(ii) SEQ ID NOs: 17-22;

(iii) SEQ ID NOs 23-28;

(iv) SEQ ID NOs 29-34;

(v) SEQ ID NOs 35-40;

(vi) SEQ ID NOs 41-46;

(vii) SEQ ID NOs 47-52; and

(viii) SEQ ID NOs 53-58.

According to an aspect of some embodiments of the present invention there is provided an antibody comprising an ANG2 recognition region which comprises CDR amino acid sequences as set forth:

(i) SEQ ID NOs: 7-12; or

(ii) SEQ ID NOs: 107-112.

According to an aspect of some embodiments of the present invention there is provided a method of treating cancer in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of at least one of the antibodies described herein.

According to an aspect of some embodiments of the present invention there is provided a pharmaceutical composition comprising the antibody described herein as the active agent and a pharmaceutically acceptable carrier.

According to an aspect of some embodiments of the present invention there is provided an isolated nucleic acid encoding a heavy chain of the first antigen binding site of the antibody described herein.

According to an aspect of some embodiments of the present invention there is provided an isolated nucleic acid encoding a light chain of the first antigen binding site of any one of the antibodies described herein.

According to an aspect of some embodiments of the present invention there is provided an isolated nucleic acid encoding a heavy chain of the second antigen binding site of any one of the antibodies described herein.

According to an aspect of some embodiments of the present invention there is provided an isolated nucleic acid encoding a light chain of the second antigen binding site of any one of the antibodies described herein.

According to an aspect of some embodiments of the present invention there is provided an expression vector comprising the nucleic acid described herein.

According to an aspect of some embodiments of the present invention there is provided a prokaryotic or eukaryotic host cell comprising the nucleic acid described herein.

According to an aspect of some embodiments of the present invention there is provided a method of preparing the antibody described herein, comprising:

(a) providing the nucleic acids described herein;

(b) culturing host cells comprising the nucleic acids under conditions that permit expression of the nucleic acids; and

(c) recovering the antibody.

According to some embodiments of the invention,

(i) the first antigen binding site comprises CDR amino acid sequences as set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3; SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6; and

(ii) the second antigen binding site comprises CDR amino acid sequences as set forth in SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9; SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 12.

According to some embodiments of the invention, the antibody is monospecific.

According to some embodiments of the invention, the antibody is bispecific.

According to some embodiments of the invention, the VH region of the first antigen binding domain comprises a sequence as set forth in SEQ ID NO: 135.

According to some embodiments of the invention, the VL region of the first antigen binding domain comprises a sequence as set forth in SEQ ID NO: 136.

According to some embodiments of the invention, the VH region of the second antigen binding domain comprises a sequence as set forth in SEQ ID NO: 157.

According to some embodiments of the invention, the VL region of the second antigen binding domain comprises a sequence as set forth in SEQ ID NO: 158.

According to some embodiments of the invention, the first heavy chain of the first antigen binding domain is non-identical to a second heavy chain of the second antigen binding domain in the Fc region of the antibody, wherein at least one of the heavy chains comprises an amino acid modification so as to form complementation between two non-identical heavy chains thereby increasing the probability of forming heterodimers of the non-identical heavy chains and decreasing the probability of forming homodimers of identical heavy chains.

According to some embodiments of the invention, the bispecific antibody comprises a first disulfide bond between a first heavy chain and a first light chain of an Fab region of the first antigen binding site and a second disulfide bond between a second heavy chain and a second light chain of an Fab region of the second antigen binding site, wherein a position of the first disulfide bond relative to the first heavy chain is different to a position of the second disulfide bond relative to the second heavy chain.

According to some embodiments of the invention, the complementation comprises a steric complementation.

According to some embodiments of the invention, the complementation comprises a charge complementation.

According to some embodiments of the invention, the Fc region comprises a protuberance of one heavy chain of the Fc region and a sterically compensatory cavity on a second heavy chain of the Fc region, the protuberance protruding into the compensatory cavity.

According to some embodiments of the invention, the protuberance is generated by substituting an amino acid at one position on a CH3 domain of the one heavy chain with another amino acid having a larger side chain volume than the original amino acid.

According to some embodiments of the invention, the compensatory cavity is generated by substituting an amino acid at one position on a CH3 domain of the second

heavy chain with another amino acid having a smaller side chain volume than the original amino acid.

According to some embodiments of the invention, the first disulfide bond is between a V_H domain of the first heavy chain and a V_L domain of the first light chain and the second disulfide bond is between a CH1 domain of the second heavy chain and a CL domain of the second light chain.

According to some embodiments of the invention, the at least one antibody comprises a combination of the antibody comprising an ANG2 recognition region which comprises CDR amino acid sequences as set forth:

(i) SEQ ID NOs: 7-12; or

(ii) SEQ ID NOs: 107-112.

and the antibody comprising an HGF recognition region which comprises CDR sequences selected from the group consisting of:

(i) SEQ ID NOs: 1-6;

(ii) SEQ ID NOs: 17-22;

(iii) SEQ ID NOs 23-28;

(iv) SEQ ID NOs 29-34;

(v) SEQ ID NOs 35-40;

(vi) SEQ ID NOs 41-46;

(vii) SEQ ID NOs 47-52; and

(viii) SEQ ID NOs 53-58.

According to some embodiments of the invention, the amino acid having a larger side chain volume than the original amino acid is selected from the group consisting of tyrosine, arginine, phenylalanine, isoleucine and tryptophan.

According to some embodiments of the invention, the amino acid having a smaller side chain volume than the original amino acid is selected from the group consisting of alanine, glycine, valine and threonine.

According to some embodiments of the invention, the bispecific antibody is selected from the group consisting of a chimeric antibody, a humanized antibody and a fully human antibody.

According to some embodiments of the invention, the CH3 domain of a first heavy chain of the first antigen binding site is covalently linked to a CH3 domain of a second heavy chain of the second antigen binding site.

According to some embodiments of the invention, each light chain of the first and second antigen binding site is linked to its cognate heavy chain via a single disulfide bond.

According to some embodiments of the invention, the bispecific antibody is an intact antibody.

According to some embodiments of the invention, the antibody is selected from the group consisting of IgA, IgD, IgE and IgG.

According to some embodiments of the invention, the IgG comprises IgG1, IgG2, IgG3 or IgG4.

According to some embodiments of the invention, the first heavy chain comprises a T366W mutation; and the second heavy chain comprises T366S, L368A, Y407V mutations.

According to some embodiments of the invention, the first heavy chain comprises an S354C mutation and the second heavy chain comprises a Y349C mutation.

According to some embodiments of the invention, at least one heavy chain of the antibody is attached to a therapeutic moiety.

According to some embodiments of the invention, at least one heavy chain of the antibody is attached to an identifiable moiety.

According to some embodiments of the invention, the bispecific antibody is selected from the group consisting of a primate antibody, a porcine antibody, a murine antibody, a bovine antibody, a goat antibody and an equine antibody.

According to some embodiments of the invention, the cancer is a solid tumor or lymphoid tumor.

According to some embodiments of the invention, the cancer is selected from the group consisting of colorectal cancer, lung cancer, breast cancer, renal cancer, ovarian cancer, gastric cancer, bladder cancer, liver cancer, ovarian cancer, fallopian cancer, glioblastoma, Mesothelioma and leukemia.

According to some embodiments of the invention, the host cells comprise bacterial cells.

According to some embodiments of the invention, the host cells comprise mammalian cells.

According to some embodiments of the invention, the expression takes place in inclusion bodies of the bacterial cells.

According to some embodiments of the invention, each of the nucleic acid molecules are transfected into different host cells.

According to some embodiments of the invention, each of the nucleic acid molecules are transfected into the same host cell.

According to some embodiments of the invention, the bacterial cells comprise gram negative bacterial cells.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIG. 1 is an example of an anti-HGF heavy chain DNA sequence (SEQ ID NO: 165). The VH domain coding sequence is highlighted in yellow. The codon of the engineered cysteine for disulfide stabilization (Cys 44 according to the Kabat numbering scheme) is highlighted in red. The codon for elimination of the native

disulfide bond between CH1 and CL (Ala 222 according to the EU index, Kabat numbering scheme) is highlighted in grey. The "knob" mutation (Trp 366 according to the EU index, Kabat numbering scheme) is highlighted in blue. The mutation to generate the additional disulfide bond Cys 354 is highlighted in green.

FIG. 2 is an example of an anti-HGF light chain DNA sequence (SEQ ID NO: 166). The VL (lambda) domain is highlighted in green. The engineered cysteine for disulfide stabilization (Cys100 according to the Kabat numbering scheme) is highlighted in red. The codon for elimination of the native disulfide bond between CH1 and CL (Ala 214 according to the Kabat numbering scheme) is highlighted in grey.

FIG. 3 is an example of an anti-ANG2 heavy chain DNA sequence (SEQ ID NO: 167).The VH domain coding sequence is highlighted in yellow. The "hole" mutations (Ser366, Ala 368 and Val 407 according to the EU index, Kabat numbering scheme) are highlighted in blue. Cys 349, the mutation which generates the additional disulfide bond is highlighted in green.

FIG. 4 is an example of an anti-ANG2 light chain DNA sequence (SEQ ID NO: 16). The VL (lambda) domain is highlighted in green.

FIGs. 5A-B are bar graphs illustrating specific binding by phage ELISA. ELISA plates were coated with HGF (A), Ang2 (B) and control antigens (*Pseudomonas* exotoxin A (PE), Erbitux and BSA) at 5 μg/ml. About 10^{10} freshly rescued scFv displaying phages were added per well and detected with HRP-conjugated anti-phage antibody.

FIGs. 6A-C are graphs illustrating SDS/PAGE analysis of *E.coli* produced 2C3 monospecific (A), 1A2 monospecific (B) and BIC104 bispecific (C) antibodies. Lanes A1-5, B1-5 and C1-6 were analyzed under reducing conditions. Lane A1, purified inclusion bodies of 2C3 heavy chain knob. Lane A2, purified inclusion bodies of 2C3 heavy chain hole. Lane A3, purified inclusion bodies of 2C3 light chain. Lane A4, commercial Avastin. Lane A5, Protein-A purified 2C3 monospecific IgG. Lane A6, MW marker. Lane A7, Protein-A purified 2C3 monospecific IgG. Lane A8, commercial Avastin. Lane B1, purified inclusion bodies of 1A2 heavy chain knob. Lane B2, purified inclusion bodies of 1A2 heavy chain hole. Lane B3, purified inclusion bodies of 1A2 light chain. Lane B4, Protein-A purified 1A2 monospecific IgG. Lane B5, commercial Avastin. Lane B6, MW marker. Lane B7, commercial Avastin. Lane B8, Protein-A

purified 1A2 monospecific IgG. Lane C1, purified inclusion bodies of mutated disulfide stabilized 2C3 heavy chain knob. Lane C2, purified inclusion bodies of 2C3 mutated disulfide stabilized light chain. Lane C3, purified inclusion bodies of 1A2 heavy chain hole. Lane C4, purified inclusion bodies of 1A2 light chain. Lane C5, Protein-A purified BIC 104 IgG. Lane C6, commercial Avastin. Lane C7, MW marker. Lane C8, commercial Avastin. Lane C9, Protein-A purified BIC 104 bispecific IgG.

FIG. 7 is a graph illustrating ELISA binding analysis of anti-HGF 2C3 monospecific IgG, anti-Ang2 monospecific IgG and bispecific BIC 104 IgG. ELISA plates were coated with human HGF or human Ang2 at 2.5 µg/ml. serial dilutions of *E. coli* produced IgG antibodies were added. Binding was detected with HRP-conjugated anti-human antibody. The EC_{50} values (determined as the half maximal binding signal) are 0.2 nM for 2C3 IgG on HGF, 0.1 nM for 1A2 IgG on Ang2 and 2 nM and 0.25 nM for BIC 104 IgG on HGF and Ang2 respectively.

FIGs. 8A-C are graphs illustrating kinetics of HGF binding to bispecific and monospecific antibodies by SPR analysis. BIC 104 bispecific (A) and 2C3 monospecific (B) Antibodies are captured by an anti-human Fc immobilized onto an SPR chip. Increasing concentrations of HGF were flowed on the chip (0, 0.3125, 0.625, 1.25, 2.5 and 5 nM). (C) The (monovalent) affinities for HGF of the bispecific compound BIC 104 and the parental antibody 2C3 determined by surface plasmon resonance.

FIG. 9 is a graph illustrating binding of 2C3 IgG to mouse HGF. ELISA plates were coated with human HGF or mouse HGF at 2.5 µg/ml. Serial dilutions of 2C3 monospecific IgG antibody were added. Binding was detected with HRP-conjugated anti-human antibody. The EC_{50} values (determined as the half maximal binding signal) are 0.2 nM and 0.7 nM for 2C3 IgG on human HGF and mouse HGF respectively.

FIG. 10 is a graph illustrating binding of BIC104 IgG to human and to mouse Ang2. ELISA plates were coated with human Ang2 or mouse Ang2 at 2.5 µg/ml. Serial dilutions of BIC104 bispecific IgG antibody were added. Binding was detected with HRP-conjugated anti-human antibody.

FIG. 11 is a graph illustrating competition of c-Met binding to HGF by antibody 2C3 as evaluated by ELISA. ELISA plates were coated with human HGF at 2.5 µg/ml. Serial dilutions of 2C3 IgG antibody mixed with 1 nM of c-Met Fc were added. c-MET

binding was detected with biotinylated anti-human c-Met mixed with HRP-conjugated streptavidin.

FIG. 12 is a graph illustrating competition with Tie2 by ELISA. ELISA plates were coated with 4 μg/ml human Tie2 Fc. Serial dilutions of BIC 104 antibody mixed with 100 ng/ml of human Ang2 were added. Ang2 binding was detected with HRP-conjugated streptavidin.

FIGs. 13A-B are photographs of purified BIC104 and BIC204. SDS/PAGE analysis (A) and Immunoblot (B) of BIC104 and BIC204. (A) Samples in lanes A1-3 were analyzed under reducing conditions. Lane A1, commercial Avastin. Lane A2, BIC 204. Lane A3, BIC 104. Lane A4, MW marker. Lane A5, commercial Avastin. Lane A6, BIC 204. Lane A7, BIC 104. (B) immunoblot using HRP-conjugated anti-kappa and HRP-conjugated anti-lambda antibodies and ECL development. Lane B1, commercial Avastin. Lane B2, BIC 204. Lane B3, BIC 104.

FIGs. 14A-B are graphs illustrating binding of *E. coli* produced BIC 104 IgG or CHO produced BIC 204. ELISA plates were coated with human HGF or human Ang2 at 2.5 μg/ml. Serial dilutions of *E. coli*-produced BIC 104 IgG or CHO-produced BIC 204 IgG antibodies were added. Binding was detected with HRP-conjugated anti-human antibody. The EC_{50} values (determined as the half maximal binding signal) are 3 nM and 0.4 nM for BIC 104 IgG and 2 nM and 0.6 nM for BIC 204 IgG on HGF and Ang2 respectively.

FIGs. 15A-B are graphs illustrating bispecific binding of BIC104 and BIC204 by ELISA. ELISA plates were coated with human HGF or human Ang2 at 2.5 μg/ml. serial dilutions of BIC104, BIC204 antibodies and 2C3 monospecific (as a control) mixed with 1 μg/ml of biotinylated Ang2 were added. Detection was with HRP-conjugated streptavidin.

FIGs. 16A-B provide the amino acid sequence alignment of selected anti-human HGF – SEQ ID NOs: 125-140 (A) and anti Ang2 – SEQ ID NOs: 157-160 (B) scFv binding clones.

FIGs. 17A-B are graphs illustrating the anti-tumor growth inhibition activity of c-Met expressing Colo205 colorectal tumors grown as xenografts in nude mice by bispecific anti-ANG2/anti-HGF antibody molecules (BIC 104 and BIC 204) and their parent monospecific antibodies.

FIG. 18 is a graph illustrating the anti-tumor growth inhibition activity of SKOV-3 human ovary tumors grown as xenografts in nude mice cells by anti-ANG2 1A2 and a control antibody directed against CD20 (Rituximab).

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to monospecific and bispecific antibodies and, more particularly, but not exclusively, to antibodies that target hepatocyte growth factor (HGF) and/or angiopoietin-2 (ANG2).

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

The present inventors have unexpectedly shown that simultaneous inhibition of binding of the two ligand proteins - Hepatocyte growth factor (HGF) and human Angiopoietin-2 (ANG2) to their receptors cMET and Tie2, respectively, has a synergistic effect and enhances the efficacy of cancer treatment as compared to a simple additive effect of the two activities or only inhibition activity of only one of these target proteins. As illustrated in Figure 17A mice injected with c-Met expressing Colo205 colorectal cells demonstrated an enhanced decrease in tumor size following antibody treatment directed against both ANG2 and HGF as compared with antibody treatment directed against only one of these proteins. Further, as illustrated in Figure 17B mice injected with c-Met expressing Colo205 colorectal tumor cells survived longer following antibody treatment directed against both ANG2 and HGF as compared with antibody treatment directed against only one of these proteins and surprisingly survival showed a synergistic activity as it is more than just an additive effect of the two inhibitory pathways. The present inventors contemplate inhibition of the activity of two proteins using a single bifunctional antibody or alternatively by simultaneous administration of one antibody directed against HGF and another directed against ANG2.

To identify scFvs that specifically bind to human HGF or human ANG2, a human synthetic combinatorial library of arrayable single-chain antibodies was screened. After their isolation by antibody phage display, the coding sequences for the VH and VL domain were prepared as synthetic genes with optimization for expression in *E. coli* or in mammalian CHO cells.

The sequence of the bispecific antibody was modified to ensure correct assembly of the bispecific antibody. This modification involved heterodimerization of the two heavy chains by applying the knobs into holes approach, combined with facilitation of pairing of each heavy chain with only its cognate light chain by addition of an engineered disulfide bond on one arm of the antibody (between the VL and VH domain) and elimination of a naturally occurring disulfide bond on the same arm (between the CH1 and CL domains).

As illustrated in Figures 5-15 the generated recombinant bispecific antibodies were capable of specifically binding to both HGF and ANG2.

Thus, according to one aspect of the present invention, there is provided a bispecific antibody comprising a first antigen-binding site that specifically binds to human hepatocyte growth factor (HGF) and a second antigen-binding site that specifically binds to human angiopoietin-2 (ANG2).

As used herein, the term "bispecific antibody" refers to an antibody which comprises two antigen binding sites, each binding to a different epitope of a different antigen. The bispecific antibodies of this aspect of the present invention do not share common light chains or common heavy chains.

Bispecific antibodies can be produced by many methods known in the art including for example chemical cross-linkage, genetic engineering, or somatic hybridization, as further described herein below.

The bispecific antibodies according to the invention may be "bivalent" – denoting the presence of two binding sites.

According to another embodiment, the antibodies of this aspect of the present invention are trivalent or tetravalent.

The term "hepatocyte growth factor" or "HGF" refers to a human polypeptide (Swiss prot No. P14210, REFSeq NP_000592; SEQ ID NO: 13) which is described in Nakamura et al., Nature 342: 440-443 (1989) or fragments thereof, as well as related

polypeptides, which include, but are not limited to, allelic variants, splice variants, derivative variants, substitution variants, deletion variants, and/or insertion variants, fusion polypeptides, and interspecies homologs. According to one embodiment, the antibody of this aspect of the present invention also recognizes the mouse HGF. In certain embodiments, an HGF polypeptide includes terminal residues, such as, but not limited to, leader sequence residues, targeting residues, amino terminal methionine residues, lysine residues, tag residues and/or fusion protein residues. Other synonyms for HGF include hepapoietin A and scatter factor (SF).

The term "ANG2" as used herein refers to human angiopoietin-2 (ANG2) (alternatively abbreviated with ANGPT2 or ANG-2) (SEQ ID NO: 14) which is described e.g. in Maisonpierre, P. C., et al, Science 277 (1997) 55-60 and Cheung, A. H., et al., Genomics 48 (1998) 389-91. The angiopoietins-1 and -2 were discovered as ligands for the Ties, a family of tyrosine kinases that is selectively expressed within the vascular endothelium. Yancopoulos, G. D., et al., Nature 407 (2000) 242-48. There are now four definitive members of the angiopoietin family. Angiopoietin-3 and -4 (ANG3 and ANG4) may represent widely diverged counterparts of the same gene locus in mouse and man. Kim, I., et al., FEBS Let, 443 (1999) 353-56; Kim, I., et al., J Biol Chem 274 (1999) 26523-28. ANG1 and ANG2 were originally identified in tissue culture experiments as agonist and antagonist, respectively (see for ANG-1: Davis, S., et al., Cell 87 (1996) 1161-69; and for ANG2: Maisonpierre, P. C., et al., Science 277 (1997) 55-60) All of the known angiopoietins bind primarily to Tie2, and both ANG1 and -2 bind to Tie2 with an affinity of 3 nM (Kd). Maisonpierre, P. C., et al., Science 277 (1997) 55-60.

According to one embodiment, the antibody of this aspect of the present invention also recognizes mouse ANG2.

ANG2 also refers to fragments thereof, as well as related polypeptides, which include, but are not limited to, allelic variants, splice variants, derivative variants, substitution variants, deletion variants, and/or insertion variants, fusion polypeptides, and interspecies homologs. In certain embodiments, an ANG2 polypeptide includes terminal residues, such as, but not limited to, leader sequence residues, targeting residues, amino terminal methionine residues, lysine residues, tag residues and/or fusion protein residues.

As used herein, the term "binding" or "specifically binding" refers to the binding of the antibody to an epitope of the antigen (either HGF or ANG2) in an in-vitro assay, e.g. in an ELISA assay or in an plasmon resonance assay (BIAcore, GE-Healthcare Uppsala, Sweden) with purified wild-type antigen. The affinity of the binding is defined by the terms Ka (rate constant for the association of the antibody from the antibody/antigen complex), kD (dissociation constant), and KD (kD/ka). In one embodiment binding or specifically binding means a binding affinity (KD) of 10^{-8} mol/l or less, preferably 10^{-9} M to 10^{-13} mol/1.

Preferably, the affinity of each of the antigen binding sites of the antibody for its target is not substantially reduced as compared with one arm of its corresponding monoclonal antibody for the identical target. According to a specific embodiment, the affinity is not reduced more than 100 fold, more preferably is not reduced more than 50 fold, more preferably is not reduced more than 20 fold, more preferably is not reduced more than 10 fold and even more preferably is not reduced more than 5 fold.

The term "epitope" includes any polypeptide determinant capable of specific binding to an antibody. In certain embodiments, epitope determinant include chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl, or sulfonyl, and, in certain embodiments, may have specific three dimensional structural characteristics, and or specific charge characteristics. An epitope is a region of an antigen that is bound by an antibody.

In certain embodiments, an antibody is said to specifically bind an antigen when it preferentially recognizes its target antigen in a complex mixture of proteins and/or macromolecules.

According to one embodiment, the antibody is an intact antibody i.e. full length antibody. The term "full length antibody" denotes an antibody consisting of two "full length antibody heavy chains" and two "full length antibody light chains". A "full length antibody heavy chain" is a polypeptide consisting in N-terminal to C-terminal direction of an antibody heavy chain variable domain (VH), an antibody constant heavy chain domain 1 (CH1), an antibody hinge region (HR), an antibody heavy chain constant domain 2 (CH2), and an antibody heavy chain constant domain 3 (CH3), abbreviated as VH-CH1-HR--CH2-CH_3; and optionally an antibody heavy chain constant domain 4 (CH4) in case of an antibody of the subclass IgE. Preferably the "full length antibody

heavy chain" is a polypeptide consisting in N-terminal to C-terminal direction of VH, CH1, HR, CH2 and CH3. A "full length antibody light chain" is a polypeptide consisting in N-terminal to C-terminal direction of an antibody light chain variable domain (VL), and an antibody light chain constant domain (CL), abbreviated as VL-CL. The two full length antibody chains are linked together as further described herein below.

Examples of typical full length antibodies are natural antibodies like IgG, IgM, IgA, IgD, and IgE. The full length antibodies according to the invention can be from a single species e.g. human, or they can be chimerized or humanized antibodies. The full length antibodies according to the invention comprise two antigen binding sites each formed by a pair of VH and VL, which both specifically bind to the same antigen. The C-terminus of the heavy or light chain of the full length antibody denotes the last amino acid at the C-terminus of the heavy or light chain. The N-terminus of the heavy or light chain of the full length antibody denotes the first amino acid at the N-terminus of the heavy or light chain.

The "variable domain" (variable domain of a light chain (VL), variable domain of a heavy chain (VH) as used herein denotes each of the pair of light and heavy chains which is involved directly in binding the antibody to the antigen. The domains of variable human light and heavy chains have the same general structure and each domain comprises four framework (FR) regions whose sequences are widely conserved, connected by three "hypervariable regions" (or complementarity determining regions, CDRs). The framework regions adopt a β-sheet conformation and the CDRs may form loops connecting the β-sheet structure. The CDRs in each chain are held in their three-dimensional structure by the framework regions and form together with the CDRs from the other chain the antigen binding site. The antibody heavy and light chain CDR3 regions play a particularly important role in the binding specificity/affinity of the antibodies according to the invention and therefore provide a further object of the invention.

The antigen-binding sites of the bispecific antibody of the invention contain six complementarity determining regions (CDRs) which contribute in varying degrees to the affinity of the binding site for the particular antigen. There are three heavy chain variable domain CDRs (CDRH1, CDRH2 and CDRH3) and three light chain variable

domain CDRs (CDRL1, CDRL2 and CDRL3). The extent of CDR and framework regions (FRs) is determined by comparison to a compiled database of amino acid sequences in which those regions have been defined according to variability among the sequences. Also included within the scope of the invention are functional antigen binding sites comprised of fewer CDRs (i.e., where binding specificity is determined by one, two, three, four or five CDRs). For example, less than a complete set of 6 CDRs may be sufficient for binding. In some cases, a VH or a VL domain will be sufficient.

Exemplary CDRs which may be present in the arm of the bispecific antibody that recognizes HGF are set forth in Table 1, herein below.

Table 1

DNA sequence	Amino acid sequence	CDR	Clone
SEQ ID NO: 59	SEQ ID NO: 17	VH- CDR1	2F8
SEQ ID NO: 60	SEQ ID NO: 18	VH- CDR2	
SEQ ID NO: 61	SEQ ID NO: 19	VH- CDR3	
SEQ ID NO: 62	SEQ ID NO: 20	VL- CDR1	
SEQ ID NO: 63	SEQ ID NO: 21	VL-CDR2	
SEQ ID NO: 64	SEQ ID NO: 22	VL- CDR3	
SEQ ID NO: 65	SEQ ID NO: 23	VH- CDR1	2B6
SEQ ID NO: 66	SEQ ID NO: 24	VH- CDR2	
SEQ ID NO: 67	SEQ ID NO: 25	VH- CDR3	
SEQ ID NO: 68	SEQ ID NO: 26	VL- CDR1	
SEQ ID NO: 69	SEQ ID NO: 27	VL-CDR2	
SEQ ID NO: 70	SEQ ID NO: 28	VL- CDR3	
SEQ ID NO: 71	SEQ ID NO: 29	VH- CDR1	1D2
SEQ ID NO: 72	SEQ ID NO: 30	VH- CDR2	
SEQ ID NO: 73	SEQ ID NO: 31	VH- CDR3	
SEQ ID NO: 74	SEQ ID NO: 32	VL- CDR1	
SEQ ID NO: 75	SEQ ID NO: 33	VL-CDR2	
SEQ ID NO: 76	SEQ ID NO: 34	VL- CDR3	
SEQ ID NO: 77	SEQ ID NO: 35	VH- CDR1	1A11
SEQ ID NO: 78	SEQ ID NO: 36	VH- CDR2	
SEQ ID NO: 79	SEQ ID NO: 37	VH- CDR3	
SEQ ID NO: 80	SEQ ID NO: 38	VL- CDR1	
SEQ ID NO: 81	SEQ ID NO: 39	VL-CDR2	
SEQ ID NO: 82	SEQ ID NO: 40	VL- CDR3	
SEQ ID NO: 83	SEQ ID NO: 41	VH- CDR1	2H12
SEQ ID NO: 84	SEQ ID NO: 42	VH- CDR2	
SEQ ID NO: 85	SEQ ID NO: 43	VH- CDR3	
SEQ ID NO: 86	SEQ ID NO: 44	VL- CDR1	
SEQ ID NO: 87	SEQ ID NO: 45	VL-CDR2	
SEQ ID NO: 88	SEQ ID NO: 46	VL- CDR3	

SEQ ID NO: 89	SEQ ID NO: 1	VH- CDR1	2C3
SEQ ID NO: 90	SEQ ID NO: 2	VH- CDR2	
SEQ ID NO: 91	SEQ ID NO: 3	VH- CDR3	
SEQ ID NO: 92	SEQ ID NO: 4	VL- CDR1	
SEQ ID NO: 93	SEQ ID NO: 5	VL-CDR2	
SEQ ID NO: 94	SEQ ID NO: 6	VL- CDR3	
SEQ ID NO: 95	SEQ ID NO: 47	VH- CDR1	1G8
SEQ ID NO: 96	SEQ ID NO: 48	VH- CDR2	
SEQ ID NO: 97	SEQ ID NO: 49	VH- CDR3	
SEQ ID NO: 98	SEQ ID NO: 50	VL- CDR1	
SEQ ID NO: 99	SEQ ID NO: 51	VL-CDR2	
SEQ ID NO: 100	SEQ ID NO: 52	VL- CDR3	
SEQ ID NO: 101	SEQ ID NO: 53	VH- CDR1	2E2
SEQ ID NO: 102	SEQ ID NO: 54	VH- CDR2	
SEQ ID NO: 103	SEQ ID NO: 55	VH- CDR3	
SEQ ID NO: 104	SEQ ID NO: 56	VL- CDR1	
SEQ ID NO: 105	SEQ ID NO: 57	VL-CDR2	
SEQ ID NO: 106	SEQ ID NO: 58	VL- CDR3	

Exemplary CDRs which may be present in the arm of the bispecific antibody that recognizes ANG2 are set forth in Table 2, herein below.

Table 2

DNA sequence	**Amino acid sequence**	**CDR**	**Clone**
SEQ ID NO: 113	SEQ ID NO: 7	VH- CDR1	1A2
SEQ ID NO: 114	SEQ ID NO: 8	VH- CDR2	
SEQ ID NO: 115	SEQ ID NO: 9	VH- CDR3	
SEQ ID NO: 116	SEQ ID NO: 10	VL- CDR1	
SEQ ID NO: 117	SEQ ID NO: 11	VL-CDR2	
SEQ ID NO: 118	SEQ ID NO: 12	VL- CDR3	
SEQ ID NO: 119	SEQ ID NO: 107	VH- CDR1	1A12
SEQ ID NO: 120	SEQ ID NO: 108	VH- CDR2	
SEQ ID NO: 121	SEQ ID NO: 109	VH- CDR3	
SEQ ID NO: 122	SEQ ID NO: 110	VL- CDR1	
SEQ ID NO: 123	SEQ ID NO: 111	VL-CDR2	
SEQ ID NO: 124	SEQ ID NO: 112	VL- CDR3	

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Exemplary variable domains which may be present in the arm of the bispecific antibody that recognizes HGF are set forth in Table 3, herein below.

Table 3

DNA Sequence	**Amino acid sequence**	**Variable domain**	**Clone**
SEQ ID NO: 141	SEQ ID NO: 125	VH	2F8
SEQ ID NO: 142	SEQ ID NO: 126	VL	
SEQ ID NO: 143	SEQ ID NO: 127	VH	2B6

SEQ ID NO: 144	SEQ ID NO: 128	VL	
SEQ ID NO: 145	SEQ ID NO: 129	VH	1D2
SEQ ID NO: 146	SEQ ID NO: 130	VL	
SEQ ID NO: 147	SEQ ID NO: 131	VH	1A11
SEQ ID NO: 148	SEQ ID NO: 132	VL	
SEQ ID NO: 149	SEQ ID NO: 133	VH	2H12
SEQ ID NO: 150	SEQ ID NO: 134	VL	
SEQ ID NO: 151	SEQ ID NO: 135	VH	2C3
SEQ ID NO: 152	SEQ ID NO: 136	VL	
SEQ ID NO: 153	SEQ ID NO: 137	VH	1G8
SEQ ID NO: 154	SEQ ID NO: 138	VL	
SEQ ID NO: 155	SEQ ID NO: 139	VH	2E2
SEQ ID NO: 156	SEQ ID NO: 140	VL	

Exemplary variable domains which may be present in the arm of the bispecific antibody that recognizes ANG2 are set forth in Table 4, herein below.

Table 4

DNA sequence	**Amino acid sequence**	**Variable domain**	**Clone**
SEQ ID NO: 161	SEQ ID NO: 157	VH	1A2
SEQ ID NO: 162	SEQ ID NO: 158	VL	
SEQ ID NO: 163	SEQ ID NO: 159	VH	1A12
SEQ ID NO: 164	SEQ ID NO: 160	VL	

Although the CDR sequences and variable sequences have been disclosed for a particular clone, it will be appreciated that the contemplated bifunctional antibody may comprise any combination of the above disclosed CDRs, or and VL sequences so long as the first antigen binding site recognizes HGF and the second antigen binding site recognizes ANG2.

According to a specific embodiment, the first antigen binding site comprises the CDR sequences as set forth in SEQ ID NOs: 1-6 and the second antigen binding site comprises the CDR sequences as set forth in SEQ ID NOs: 7-12.

According to yet another embodiment, the VH region of the arm that recognizes the first antigen binding site is set forth in SEQ ID NO: 135, the VL region of the arm that recognizes the first antigen binding site is set forth in SEQ ID NO: 136, the VH region of the arm that recognizes the second antigen binding site is set forth in SEQ ID NO: 157, and the VL region of the arm that recognizes the first antigen binding site is set forth in SEQ ID NO: 158.

The antibodies of the invention further comprise immunoglobulin constant regions of one or more immunoglobulin classes. Immunoglobulin classes include IgG, IgM, IgA, IgD, and IgE isotypes and, in the case of IgG and IgA, their subtypes.

According to one embodiment, the Fc region is an IgG Fc region.

According to one embodiment, the Fc region of the antibodies described herein comprises two non-identical heavy chains (e.g. that differ in the sequence of the variable domains), wherein at least one of the two non-identical heavy chains comprises an amino acid modification so as to increase the probability of forming a stable heterodimer of the non-identical heavy chains and decrease the probability of forming a stable homodimer of identical heavy chains.

According to one embodiment, at least one heavy chain is genetically modified such that an altered charge polarity across the interface is created. As a consequence, a stable heterodimer between electrostatically matched Fc chains is promoted, and unwanted Fc homodimer formation is suppressed due to unfavorable repulsive charge interactions.

Determination of which amino acids to modify and to which amino acids is further explained in Gunasekaran K, Pentony M, Shen M, Garrett L, Forte C, Woodward A, Ng SB, Born T, Retter M, Manchulenko K, Sweet H, Foltz IN, Wittekind M, Yan W. Enhancing antibody Fc heterodimer formation through electrostatic steering effects: applications to bispecific molecules and monovalent IgG. J Biol Chem. 2010 Jun 18;285(25):19637-46. Epub 2010 Apr 16, incorporated herein by reference.

According to one embodiment, the amino acid modifications (that affect charge complementarity) are effected at the rim of the interface between the two heavy chains and not in structurally conserved buried residues at the hydrophobic core of the interface.

According to another embodiment, at least one heavy chain is genetically modified, to generate a heavy chain with a 3D structure which binds more efficiently to the non-identical heavy chain (i.e. a heterodimer) as opposed to an identical heavy chain (i.e. a homodimer). The generation of heterodimers is encouraged due to steric complementation and the generation of homodimers is discouraged due to steric hindrance.

According to this embodiment, one heavy chain is genetically modified to generate a protuberance and the second heavy chain is genetically modified to generate a sterically compensatory cavity, the protuberance protruding into the compensatory cavity.

"Proturbances" are constructed by replacing small amino acid side chains from the interface of the first heavy chains with larger side chains (e.g. tyrosine, arginine, phenylalanine, isoleucine, leucine or tryptophan). Compensatory "cavities" of identical or similar size to the protuberances are optionally created on the interface of the second heavy chain by replacing large amino acid side chains with smaller ones (e.g. alanine, glycine, serine, valine, or threonine).

The protuberance or cavity can be "introduced" into the interface of the first or second heavy chain by synthetic means, e.g. by recombinant techniques, in vitro peptide synthesis, those techniques for introducing non-naturally occurring amino acid residues previously described, by enzymatic or chemical coupling of peptides or some combination of these techniques. According, the protuberance, or cavity which is "introduced" is "non-naturally occurring" or "non-native", which means that it does not exist in nature or in the original polypeptide (e.g. a humanized monoclonal antibody).

Preferably the import amino acid residue for forming the protuberance has a relatively small number of "rotamers" (e.g. about 3 6). A "rotamer" is an energetically favorable conformation of an amino acid side chain. The number of rotamers of the various amino acid residues are reviewed in Ponders and Richards, J. Mol. Biol. 193:775 791 (1987).

As a first step to selecting original residues for forming the protuberance and/or cavity, the three-dimensional structure of the antibodies are obtained using techniques which are well known in the art such as X-ray crystallography or NMR. Based on the three-dimensional structure, those skilled in the art will be able to identify the interface residues.

The preferred interface is the C_{H3} domain of an immunoglobulin constant domain. It is preferable to select "buried" residues to be replaced. The interface residues of the CH3 domains of IgG, IgA, IgD, IgE and IgM have been identified (see, for example, PCT/US96/01598, herein incorporated by reference in its entirety), including those which are optimal for replacing with import residues; as were the interface

residues of various IgG subtypes and "buried" residues. The preferred C_{H3} domain is derived from an IgG antibody, such as an human IgG_1.

The C_{H3}/C_{H3} interface of human IgG_1 involves sixteen residues on each domain located on four anti-parallel β-strands which buries 1090 ANG^2 from each surface. Mutations are preferably targeted to residues located on the two central anti-parallel β-strands. The aim is to minimize the risk that the protuberances which are created can be accommodated by protruding into surrounding solvent rather than by compensatory cavities in the partner C_{H3} domain. Methods of selection particular sites on the heavy chains have been disclosed in U.S. Patent No. 7,183,076, incorporated herein by reference.

According to a specific embodiment, the first heavy chain comprises a T366W mutation (i.e. threonine to tryptophan); and the second heavy chain comprises T366S, L368A, Y407V mutations (i.e. threonine to serine; leucine to alanine; and tyrosine to valine).

Preferably, the heavy chain which comprises the T366W mutation is the one that forms the HGF arm of the bispecific antibody – see for example Figure 1.

Preferably, the heavy chain which comprises the T366S, L368A, Y407V mutation is the one that forms the ANG2 arm of the bispecific antibody – see for example Figure 3.

According to one embodiment, the amino acid modifications (that affect structural complementarity) are effected at structurally conserved buried residues at the hydrophobic core of the interface, and not in at the rim of the interface between the two heavy chains.

The effect of replacing residues on the heavy chains can be studied using a molecular graphics modeling program such as the Insight™ program (Biosym Technologies).

Once the preferred original/import residues are identified by molecular modeling, the amino acid replacements may be introduced into the heavy chains using techniques which are well known in the art.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution variants of the DNA encoding the first or second heavy chain. This technique is well known in the art as described by Adelman et al., DNA, 2:183 (1983).

Briefly, first or second polypeptide-coding DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of heteromultimer. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the heteromultimer DNA.

Cassette mutagenesis can be performed as described Wells et al. Gene 34:315 (1985) by replacing a region of the DNA of interest with a synthetic mutant fragment generated by annealing complimentary oligonucleotides. PCR mutagenesis is also suitable for making variants of the first or second polypeptide DNA. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR technique generally refers to the following procedure (see Erlich, Science, 252:1643 1650 (1991), the chapter by R. Higuchi, p. 61 70).

It will be appreciated that a gene encoding the antibody of this aspect of the present invention (containing mutations or not), can be synthesized by total gene synthesis (as illustrated in the Examples section below) by transferring the variable domains of the anti HGF and anti ANG2 antibodies from the phage clones into the IgG expression vectors. Importantly, such gene synthesis can involve gene optimization for expression in a particular host (for example for *E. coli* or for CHO cells).

Additional modifications are also contemplated to further enhance the specificity of interaction between the two heavy chains. Accordingly, the present invention incorporates a covalent link between the two heavy chains (e.g. on the CH3 domains).

Examples of covalent links contemplated by the present invention include amide links and disulfide links.

Thus, for example the present invention contemplates introduction of a free thiol which forms an intermolecular disulfide bond between the two heavy chains of the antibody. The free thiol may be introduced into the interface of one of the heavy chains by substituting a naturally occurring residue of the heavy chain with, for example, a cysteine at a position allowing for the formation of a disulfide bond between the heavy chains.

The phrase "free thiol-containing compound" as used herein refers to a compound that can be incorporated into or reacted with an amino acid of a polypeptide interface of the invention such that the free thiol moiety of the compound is positioned to interact with a free thiol of moiety at the interface of additional polypeptide of the invention to form a disulfide bond. Preferably, the free thiol-containing compound is cysteine.

According to a specific embodiment, the first heavy chain comprises a S354C mutation (i.e. serine to cysteine), according to the EU index, Kabat numbering scheme; and the second heavy chain comprises a Y349C mutation (tyrosine to cysteine).

Preferably, the heavy chain which comprises the S354C mutation is the one that forms the HGF arm of the bispecific antibody - see for example Figure 1.

Preferably, the heavy chain which comprises the Y349C mutation is the one that forms the ANG2 arm of the bispecific antibody – see for example Figure 3.

As well as having modifications in their heavy chains, at least one light chain of the antibodies described herein may also be modified such that there is a first covalent link between a first heavy chain and a first light chain and a second covalent link between a second heavy chain and a second light chain, wherein a position of the first covalent link relative to the first heavy chain is different to a position of the second covalent link relative to the second heavy chain.

The positioning of the first and second covalent link is selected such that pairing between a heavy chain with its cognate light chain is facilitated, whilst the specificity and stability of the antibody is not reduced by more than 20 % or preferably by more than 10 % or even more preferably by more than 5 % as compared to the individual antibodies from which it is generated.

According to another embodiment, the covalent link between the first heavy chain (e.g. the HGF arm of the bispecific antibody) to its cognate light chain is positioned between the V_H and the V_L region and the covalent link between the second heavy chain (e.g. the ANG2 arm of the bispecific antibody) to its cognate light chain is positioned between the C_{H1} and the C_L region.

Examples of covalent links contemplated by the present invention include for example amide links, disulfide links and additional forms of covalent bonds occurring between site-specifically inserted amino acid residues, including non-natural amino

acids (see Wu, X., Schultz, P.G. "Synthesis at the Interface of Chemistry and Biology." J. Am. Chem. Soc., 131(35):12497-515, 2009; Hutchins BM, Kazane SA, Staflin K, Forsyth JS, Felding-Habermann B, Schultz PG, Smider VV. Site-specific coupling and sterically controlled formation of multimeric antibody fab fragments with unnatural amino acids J Mol Biol. 2011 Mar 4;406(4):595-603. Epub 2011 Jan 13; Liu CC, Schultz PG. Adding new chemistries to the genetic code. Annu Rev Biochem. 2010;79:413-44. Review, all of which are incorporated herein by reference).

Accordingly, the present invention contemplates mutating at least one of the heavy chains and its cognate light chain such that at least one naturally occurring (i.e. native) disulfide bond that connects the two molecules can no longer be generated. Typically, this is effected by deleting (or substituting) the cysteines at the positions described herein above.

As used herein, the phrase "native disulfide bond" refers to the interchain disulfide bond that connects a heavy chain to its cognate light chain (typically between the constant region of the light chain and the CH1 region of the heavy chain) encoded in a naturally occurring germline antibody gene.

Substitution of the cysteine is typically effected by replacing the amino acid with one similar in size and charge (i.e. a conservative amino acid, such as cysteine to alanine).

The present invention contemplates that the first covalent link is a naturally occurring disulfide bond (e.g. in the anti ANG2 arm of the antibody) and the second covalent link is a non-naturally occurring covalent bond, (e.g. an engineered disulfide bond), wherein at least one cysteine amino acid residue has been inserted into the chain – i.e. an engineered cysteine (in the anti HGF arm of the antibody).

The term "engineered cysteine" as used herein, refers to a cysteine which has been introduced into the antibody fragment sequence at a position where a cysteine does not occur in the natural germline antibody sequence.

Alternatively, both the first and second covalent links may be non-naturally occurring and the cysteines (which in the non-modified antibody serve as amino acid residues to generate disulfide bonds) may be replaced by other amino acids that are not capable of serving as amino acid residues to generate covalent bonds.

Information regarding the antibody of interest is required in order to produce proper placement of the disulfide bond. The amino acid sequences of the variable regions that are of interest are compared by alignment with those analogous sequences in the well-known publication by Kabat and Wu [Sequences of Proteins of Immunological Interest," E. Kabat, et al., U.S. Government Printing Office, NIH Publication No. 91-3242 (1991)], incorporated herein by reference, to determine which sequences can be mutated so that cysteine is encoded for in the proper position of each heavy and light chain variable region to provide a disulfide bond in the framework regions of the desired antibody.

After the sequences are aligned, the amino acid positions in the sequence of interest that align with the following positions in the numbering system used by Kabat and Wu are identified: positions 43, 44, 45, 46, and 47 (group 1) and positions 103, 104, 105, and 106 (group 2) of the heavy chain variable region; and positions 42, 43, 44, 45, and 46 (group 3) and positions 98, 99, 100, and 101 (group 4) of the light chain variable region. In some cases, some of these positions may be missing, representing a gap in the alignment.

Then, the nucleic acid sequences encoding the amino acids at two of these identified positions are changed such that these two amino acids are mutated to cysteine residues. Contemplated pairs of amino acids to be selected are,: V_H44-V_L100, V_H105-V_L43, V_H105-V_L42, V_H44-V_L101, V_H106-V_L43, V_H104-V_L43, V_H44-V_L99, V_H45-V_L98, V_H46-V_L98, V_H103-V_L43, V_H103-V_L44, V_H103-V_L45.

Most preferably, substitutions of cysteine are made at the positions: V_H44-V_L100; or V_H105-V_L43. (The notation V_H44-V_L100, for example, refers to a polypeptide with a V_H having a cysteine at position 44 and a cysteine in V_L at position 100; the positions being in accordance with the numbering given by Kabat and Wu.)

Note that with the assignment of positions according to Kabat and Wu, the numbering of positions refers to defined conserved residues and not to actual sequentially numbered amino acid positions in a given antibody. For example, CysL100 (of Kabat and Wu) which is used to generate ds(Fv)B3 as described in the example below, actually corresponds to position 105 of B3(V_L).

According to one embodiment, selection of which amino acid to mutate may be effected according to the rules set out in U.S. Patent No. 5,747,654, incorporated herein

by reference. The sites of mutation to the cysteine residues can be identified by review of either the actual antibody or the model antibody of interest as exemplified below. Computer programs to create models of proteins such as antibodies are generally available and well-known to those skilled in the art (see Kabat and Wu; Loew, et al., Int. J. Quant. Chem., Quant. Biol. Symp., 15:55-66 (1988); Bruccoleri, et al., Nature, 335:564-568 (1988); Chothia, et al., Science, 233:755-758 (1986), all of which are incorporated herein by reference. Commercially available computer programs can be used to display these models on a computer monitor, to calculate the distance between atoms, and to estimate the likelihood of different amino acids interacting (see, Ferrin, et al., J. Mol. Graphics, 6:13-27 (1988), incorporated by reference herein). For example, computer models can predict charged amino acid residues that are accessible and relevant in binding and then conformationally restricted organic molecules can be synthesized. See, for example, Saragovi, et al., Science, 253:792 (1991), incorporated by referenced herein. In other cases, an experimentally determined actual structure of the antibody may be available.

According to one embodiment, a pair of suitable amino acid residues should (1) have a C_α-C_α distance between the two residues less than or equal to 8 ANG, preferably less than or equal to 6.5 ANG (determined from the crystal structure of antibodies which are available such as those from the Brookhaven Protein Data Bank) and (2) be as far away from the CDR region as possible. Once they are identified, they can be substituted with cysteins.

Modifications of the genes to encode cysteine at the target point may be readily accomplished by well-known techniques, such as oligonucleotides-directed mutagenesis (as described herein above), site-directed mutagenesis (see, Gillman and Smith, Gene, 8:81-97 (1979) and Roberts, S., et al, Nature, 328:731-734 (1987), both of which are incorporated herein by reference), by the method described in Kunkel, Proc. Natl. Acad. Sci. USA 82:488-492 (1985), incorporated by reference herein, by total gene synthesis (Hughes, R.A. et al, , Methods in Enzymology, Volume 498 p. 277-309 (2011)) or by any other means known in the art.

Exemplary mutations which allow for the formation of novel disulfide bridges between the heavy chain and its cognate light chain and deletion of naturally occurring

disulfide bridges between the heavy chain and its cognate light chain are illustrated in Figure 2.

As mentioned, the present invention contemplates antibodies which recognize either HGF and/or ANG2. Thus, the antibodies may be monospecific or bispecific. Monospecific antibodies of this aspect may or may not be modified to comprise mutations. According to one embodiment, the monospecific antibodies comprise the "knobs into hole" mutation described herein above.

Thus, according to another aspect of the present invention there is provided an antibody (either monospecific or bispecific) comprising an HGF recognition region which comprises six CDR amino acid sequences selected from the group consisting of SEQ ID NOs: 1-6, 17-22, 23-28, 29-34, 35-40, 41-46, 47-52, 53-58.

According to an additional aspect of the present invention there is provided an antibody (either monospecific or bispecific) comprising an ANG2 recognition region which comprises six CDR amino acid sequences selected from the group consisting of SEQ ID NOs: 7-12 and 107-112.

According to a particular embodiment, the antibody which recognizes ANG2 has the CDR sequences as set forth in SEQ ID NOs: 7-12.

Bispecific antibodies which comprise a first antigen binding site which recognizes ANG2 (and comprise the CDRs disclosed herein) may have a second antigen binding site which recognizes a second target which is not directed to HGF, but a different protein.

Similarly, bispecific antibodies which comprise a first antigen binding site which recognizes HGF (and comprise the CDRs disclosed herein) may have a second antigen binding site which recognizes a second target which is not directed to ANG2, but a different protein.

Antibodies of some embodiments of the present invention may be from any mammalian origin including human, porcine, murine, bovine, goat, equine, canine, feline, ovine and the like. The antibody may be a heterologous antibody.

As used herein a "heterologous antibody" is defined in relation to a transgenic host such as a plant expressing the antibody.

According to some embodiments of the invention, the antibody is an isolated intact antibody (i.e., substantially free of cellular material other antibodies having different antigenic specificities and/or other chemicals).

As used herein "recombinant antibody" refers to intact antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (e.g., mouse) that is transgenic for immunoglobulin genes (e.g., human immunoglobulin genes) or hybridoma prepared therefrom; (b) antibodies isolated from a host cell (e.g. prokaryotic cells) transformed to express the antibody; (c) antibodies isolated from a recombinant antibody library; and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of immunoglobulin gene sequences to other DNA sequences. In certain embodiments immunoglobulin of the present invention may have variable and constant regions derived from human germline immunoglobulin sequences. In other embodiments, such recombinant human antibodies can be subjected to in vitro mutagenesis and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies comprise sequences that while derived from and related to human germline V_H and V_L sequences, may not naturally exist within the human antibody germline repertoire in vivo.

The following exemplary embodiments of antibodies are encompassed by the scope of the invention.

As used herein "human antibody" refers to intact antibodies having variable regions in which both the framework and CDR regions are derived from human immunoglobulin sequences as described, for example, by Kabat et al. (see Kabat 1991, Sequences of proteins of immunological Interest, 5th Ed. NIH Publication No. 91-3242). The constant region of the human antibody is also described from human immunoglobulin sequences. The human antibodies may include amino residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site directed mutagenesis in vitro or somatic mutation in vivo). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

As used herein, a "chimeric antibody" refers to an intact antibody in which the variable regions derive from a first species and the constant regions are derived from a

second species. Chimeric immunoglobulins can be constructed by genetic engineering from immunoglobulin gene segments belonging to different species (e.g., VH and VL domains from a mouse antibody with constant domains of human origin).

As used herein "humanized immunoglobulin" refers to an intact antibody in which the minimum mouse part from a non-human (e.g., murine) antibody is transplanted onto a human antibody; generally humanized antibodies are 5-10 % mouse and 90-95 % human.

In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cole et al. and

Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)]. Similarly, human antibodies can be made by introduction of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., Bio/Technology 10,: 779-783 (1992); Lonberg et al., Nature 368: 856-859 (1994); Morrison, Nature 368 812-13 (1994); Fishwild et al., Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14: 826 (1996); and Lonberg and Huszar, Intern. Rev. Immunol. 13, 65-93 (1995).

The antibodies of the present invention may be conjugated to a functional moiety such as a detectable or a therapeutic moiety.

Various types of detectable or reporter moieties may be conjugated to the antibody of the invention. These include, but not are limited to, a radioactive isotope (such as $^{[125]}$iodine), a phosphorescent chemical, a chemiluminescent chemical, a fluorescent chemical (fluorophore), an enzyme, a fluorescent polypeptide, an affinity tag, and molecules (contrast agents) detectable by Positron Emission Tomagraphy (PET) or Magnetic Resonance Imaging (MRI).

Table 5 provides non-limiting examples of identifiable moieties which can be conjugated to the antibody of the invention.

Table 5

Nucleic Acid sequence (GenBank Accession No.)	***Amino Acid sequence (GenBank Accession No.)***	***Identifiable Moiety***
AF435427	AAL33912	Green Fluorescent protein
AY042185	AAK73766	Alkaline phosphatase
A00740	CAA00083	Peroxidase
Nucleotides 790-807 of GenBank Accession No. AF329457	Amino acids 264-269 of GenBank Accession No. AAK09208	Histidine tag

Nucleic Acid sequence (GenBank Accession No.)	*Amino Acid sequence (GenBank Accession No.)*	*Identifiable Moiety*
Nucleotides 817-849 of GenBank Accession No. AF329457	Amino acids 273-283 of GenBank Accession No. AAK09208	Myc tag
	LHHILDAQKMVWNHR /SEQ ID NO: 15	Biotin lygase tag
AF435432	AAL33917	orange fluorescent protein
EU626139	ACH42114	Beta galactosidase
AF283893	AAM49066	Streptavidin

As mentioned, the antibody may be conjugated to a therapeutic moiety. The therapeutic moiety can be, for example, a cytotoxic moiety, a toxic moiety, a cytokine moiety and a second antibody moiety comprising a different specificity to the antibodies of the invention.

Non-limiting examples of therapeutic moieties which can be conjugated to the antibody of the invention are provided in Table 6, hereinbelow.

Table 6

Nucleic acid sequence (GenBank Accession No.)	*Amino acid sequence (GenBank Accession No.)*	*Therapeutic moiety*
EU090068	ABU63124	Pseudomonas exotoxin
AY820132.1	AAV70486	Diphtheria toxin
A02159	CAA00227	interleukin 2
X03884	P07766	CD3
NM_000569.6	NP_000560.5	CD16
NM_000589.2	NP_000580.1	interleukin 4
K02883	P01892	HLA-A2
M57627	P22301	interleukin 10
EQ975183	EEF27734	Ricin toxin

The functional moiety may be conjugated to the V_H or the V_L sequence at either the N- or C-terminus or be inserted into other protein sequences in a suitable position.

It will be appreciated that such fusions can also be effected using chemical conjugation (i.e., not by recombinant DNA technology).

The V_H and V_L sequences for application in this invention can be obtained from antibodies produced by any one of a variety of techniques known in the art.

Methods of producing polyclonal and monoclonal antibodies are well known in the art (See for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

According to one embodiment, antibodies are provided by immunization of a non-human animal, preferably a mouse, with an immunogen comprising a desired antigen or immunogen. Alternatively, antibodies may be provided by selection of combinatorial libraries of immunoglobulins, as disclosed for instance in Ward et al (Nature 341 (1989) 544).

The step of immunizing a non-human mammal with an antigen may be carried out in any manner well known in the art for stimulating the production of antibodies in a mouse (see, for example, E. Harlow and D. Lane, Antibodies: A Laboratory Manual., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1988)). In a preferred embodiment, the non-human animal is a mammal, such as a rodent (e.g., mouse, rat, etc.), bovine, porcine, horse, rabbit, goat, sheep, etc. As mentioned, the non-human mammal may be genetically modified or engineered to produce "human" antibodies. Typically, the immunogen is suspended or dissolved in a buffer, optionally with an adjuvant, such as complete Freund's adjuvant. Methods for determining the amount of immunogen, types of buffers and amounts of adjuvant are well known to those of skill in the art and are not limiting in any way on the present invention. These parameters may be different for different immunogens, but are easily elucidated.

Similarly, the location and frequency of immunization sufficient to stimulate the production of antibodies is also well known in the art. In a typical immunization protocol, the non-human animals are injected intraperitoneally with antigen on day 1 and again about a week later. This is followed by recall injections of the antigen around day 20, optionally with adjuvant such as incomplete Freund's adjuvant. The recall injections are performed intravenously or intraperitoneally and may be repeated for

several consecutive days. This is followed by a booster injection at day 40, either intravenously or intraperitoneally, typically without adjuvant. This protocol results in the production of antigen-specific antibody-producing B cells after about 40 days. Other protocols may also be utilized as long as they result in the production of B cells expressing an antibody directed to the antigen used in immunization.

In an alternate embodiment, lymphocytes from a non-immunized non-human mammal are isolated, grown in vitro, and then exposed to the immunogen in cell culture. The lymphocytes are then harvested and the fusion step described below is carried out.

For monoclonal antibodies, the next step is the isolation of splenocytes from the immunized non-human mammal and the subsequent fusion of those splenocytes with an immortalized cell in order to form an antibody-producing hybridoma. The isolation of splenocytes from a non-human mammal is well-known in the art and typically involves removing the spleen from an anesthetized non-human mammal, cutting it into small pieces and squeezing the splenocytes from the splenic capsule and through a nylon mesh of a cell strainer into an appropriate buffer so as to produce a single cell suspension. The cells are washed, centrifuged and re-suspended in a buffer that lyses any red blood cells. The solution is again centrifuged and remaining lymphocytes in the pellet are finally re-suspended in fresh buffer.

Once isolated and present in single cell suspension, the lymphocytes are fused to an immortal cell line. This is typically a mouse myeloma cell line, although many other immortal cell lines useful for creating hybridomas are known in the art. Preferred murine myeloma lines include, but are not limited to, those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. U.S.A., X63 Ag8653 and SP-2 cells available from the American Type Culture Collection, Rockville, Md. U.S.A. The fusion is effected using polyethylene glycol or the like. The resulting hybridomas are then grown in selective media that contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and

thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

The hybridomas are typically grown on a feeder layer of macrophages. The macrophages are preferably from littermates of the non-human mammal used to isolate splenocytes and are typically primed with incomplete Freund's adjuvant or the like several days before plating the hybridomas. Fusion methods are described in (Goding, "Monoclonal Antibodies: Principles and Practice," pp. 59-103, Academic Press, 1986).

The cells are allowed to grow in the selection media for sufficient time for colony formation and antibody production. This is usually between 7 and 14 days. The hybridoma colonies are then assayed for the production of antibodies that bind the immunogen/antigen. The assay is typically a colorimetric ELISA-type assay, although any assay may be employed that can be adapted to the wells that the hybridomas are grown in. Other assays include immunoprecipitation and radioimmunoassay. The wells positive for the desired antibody production are examined to determine if one or more distinct colonies are present. If more than one colony is present, the cells may be re-cloned and grown to ensure that only a single cell has given rise to the colony producing the desired antibody. Positive wells with a single apparent colony are typically recloned and re-assayed to insure only one monoclonal antibody is being detected and produced.

Hybridomas that are confirmed to be producing a monoclonal antibody are then grown up in larger amounts in an appropriate medium, such as DMEM or RPMI-1640. Alternatively, the hybridoma cells can be grown in vivo as ascites tumors in an animal.

After sufficient growth to produce the desired monoclonal antibody, the growth media containing monoclonal antibody (or the ascites fluid) is separated away from the cells and the monoclonal antibody present therein is purified. Purification is typically achieved by gel electrophoresis, dialysis, chromatography using protein A or protein G-Sepharose, or an anti-mouse Ig linked to a solid support such as agarose or Sepharose beads (all described, for example, in the Antibody Purification Handbook, Amersham Biosciences, publication No. 18-1037-46, Edition AC, the disclosure of which is hereby incorporated by reference). The bound antibody is typically eluted from protein A, protein G or protein L columns by using low pH buffers (glycine or acetate buffers of pH 3.0 or less) with immediate neutralization of antibody-containing fractions. These fractions are pooled, dialyzed, and concentrated as needed.

DNA encoding the heavy and light chains of the antibody may be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of antibodies such as murine or human). Once isolated, the DNA can be ligated into expression vectors, which are then transfected into host cells.

The antibodies according to the invention are typically produced by recombinant means.

The DNA sequences encoding the immunoglobulin light chain and heavy chain polypeptides may be independently inserted into separate recombinant vectors or one single vector, which may be any vector, which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced.

Methods for recombinant production are widely known in the state of the art and comprise protein expression in prokaryotic and eukaryotic cells with subsequent isolation of the antibody and usually purification to a pharmaceutically acceptable purity.

For the expression of the antibodies as aforementioned in a host cell, nucleic acids encoding the respective modified light and heavy chains are inserted into expression vectors by standard methods.

The procedures used to ligate the DNA sequences coding for the polypeptides, the promoter (e.g., constitutive or inducible) and optionally the terminator sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (see, for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y., 1989).

Expression is performed in appropriate prokaryotic or eukaryotic host cells like CHO cells, NSO cells, SP2/0 cells, HEK293 cells, COS cells, PER.C6 cells, yeast, or bacterial cells, and the antibody is recovered from the cells (supernatant or cells after lysis).

The present invention contemplates expressing each component of the antibody in its own individual host cell, or various combinations of the antibody components in their own host cells. Thus for example, the light chains may be expressed in one host

cell and the heavy chains in another host cell. Alternatively, one light chain and one heavy chain is expressed in one host cell and the second light chain and the second heavy chain is expressed in another host cell. Still alternatively, both the heavy chains and both the light chains may be expressed in the same host cell.

It will be appreciated that when both the heavy chains and both the light chains are expressed in the same host cell, in vitro assembly of the chains is not necessary and only purification of the antibodies form the conditioned medium i.e. by protein A chromatography is required (See for example: Jackman J, J Biol Chem. 2010 Jul 2;285(27):20850-9. Epub 2010 May 5).

When at least one of the chains is expressed in a different host cell to the other three chains, in vitro assembly of the chains is required.

According to a specific embodiment, the host cell comprises bacterial cells.

According to another embodiment the antibodies are generated as inclonals as described in WO2009/107129 incorporated herein by reference.

The bacterial host may be selected capable of producing the recombinant proteins (i.e., heavy and light chains) as inclusion bodies (i.e., nuclear or cytoplasmic aggregates of stainable substances).

The host cells (e.g., first host cell and second host cell) used can be of identical species or different species.

According to specific embodiments of the present invention the host cells are selected from a Gram-negative bacterium/bacteria.

Examples of Gram-negative bacteria which can be used in accordance with the present teachings include, but are not limited to, *Escherichia coli Pseudomonas*, *erwinia* and *Serratia*. It should be noted that the use of such Gram-negative bacteria other than *E. coli* such as *Pseudomonas* as a host cell would provide great economic value owing to both the metabolic and physiologic properties of pseudomonas. Under certain conditions, pseudomonas, for example, can be grown to higher cell culture densities than *E. coli* thus providing potentially greater product yields.

Examples of bacterial expression vectors suitable for use in accordance with the present teachings include, but are not limited to, pET™ systems, the T7 systems and the pBAD™ system, which are well known in the art.

Methods of introducing expression vectors into bacterial host cells are well known in the art and mainly depend on the host system used.

The host cells can either be co-cultured in the same medium, or cultured separately.

Host cells are cultured under effective conditions, which allow for the expression of high amounts of recombinant heavy and light chain. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit recombinant protein production. An effective medium refers to any medium in which a bacterium is cultured to produce the recombinant protein of the present invention. Such a medium typically includes an aqueous solution having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Bacterial hosts of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes, and petri plates, dependent on the desired amount. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant host. Such culturing conditions are within the expertise of one of ordinary skill in the art.

Once appropriate expression levels of immunoglobulin heavy and light chains are obtained, the polypeptides are recovered from the inclusion bodies. Methods of recovering recombinant proteins from bacterial inclusion bodies are well known in the art and typically involve cell lysis followed by solubilization in denaturant [e.g., De Bernardez-Clark and Georgiou, "Inclusion bodies and recovery of proteins from the aggregated state" Protein Refolding Chapter 1:1-20 (1991). See also Examples section which follows, under *"Expression of Inclonals in E. coli"*].

Briefly, the inclusion bodies can be separated from the bulk of cytoplasmic proteins by simple centrifugation giving an effective purification strategy. They can then be solubilized by strong denaturing agents like urea (e.g., 8 M) or guanidinium hydrochloride and sometimes with extremes of pH or temperature. The denaturant concentration, time and temperature of exposure should be standardized for each protein. Before complete solubilization, inclusion bodies can be washed with diluted solutions of denaturant and detergent to remove some of the contaminating proteins.

Finally, the solubilized inclusion bodies can be directly subjected to further purification through chromatographic techniques under denaturing conditions or the heavy and light chains may be refolded to native conformation before purification.

Thus, further purification of the reconstituted/refolded heavy and light chain polypeptides (i.e., solubilized reduced polypeptides) can be effected prior to, and alternatively or additionally, following refolding.

Methods of antibody purification are well known in the art and are described hereinabove and in the Examples section which follows. Other methods for purification of IgG are described in "Purification of IgG and insulin on supports grafted by sialic acid developing "thiophilic-like" interactions Hamid Lakhiaria and Daniel Mullerb, Journal of Chromatography B Volume 818, Issue 1, 15 April 2005, Pages 53-59.

Alternatively or additionally, purification can be affinity-based through the identifiable or therapeutic moiety (e.g., using affinity columns which bind thereto).

Further purification of antibodies may be performed in order to eliminate cellular components or other contaminants, e.g. other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis, and others well known in the art. See Ausubel, F., et al., ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1987). Different methods are well established and widespread used for protein purification, such as affinity chromatography with microbial proteins (e.g. protein A or protein G affinity chromatography), ion exchange chromatography (e.g. cation exchange (carboxymethyl resins), anion exchange (amino ethyl resins) and mixed-mode exchange), thiophilic adsorption (e.g. with beta-mercaptoethanol and other SH ligands), hydrophobic interaction or aromatic adsorption chromatography (e.g. with phenyl-sepharose, aza-arenophilic resins, or m-aminophenylboronic acid), metal chelate affinity chromatography (e.g. with Ni(II)- and Cu(II)-affinity material), size exclusion chromatography, and electrophoretical methods (such as gel electrophoresis, capillary electrophoresis) (Vijayalakshmi, M.A., Appl. Biochem. Biotech. 75 (1998) 93-102).

Exemplary matrices useful for purifying the antibodies of this aspect of the present invention include Lambda-select (GE Healthcare, USA).

To improve the refolding yield, the reconstituted heavy chains and reconstituted light chains are provided at a ratio selected to maximize the formation of an intact antibody. To this end, a heavy to light chain molar ratio of about 1:1 to 1:3, 1:1.5 to 1:3, 1:2 to 1:3 is. In an exemplary embodiment the heavy to light chain molar ratio is about 1:1.

When desired the immunoglobulin may be subjected to directed *in vitro* glycosylation, which can be done according to the method described by Isabelle Meynial-salles and Didier Combes. In vitro glycosylation of proteins: An enzymatic approach. Journal of Biotechnology Volume 46, Issue 1, 18 April 1996, Pages 1-14.

One aspect of the invention is a pharmaceutical composition comprising an antibody according to the invention. Another aspect of the invention is the use of an antibody according to the invention for the manufacture of a pharmaceutical composition. A further aspect of the invention is a method for the manufacture of a pharmaceutical composition comprising an antibody according to the invention. In another aspect, the present invention provides a composition, e.g. a pharmaceutical composition, containing an antibody according to the present invention, formulated together with a pharmaceutical carrier.

Antibodies and compositions (e.g., pharmaceutical composition) comprising same may be used in diagnostic and therapeutic applications and as such may be included in therapeutic or diagnostic kits.

Thus, compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient i.e., antibody. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared,

placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

It will be appreciated that a monospecific antibody which is directed against HGF and a monospecific antibody which is directed against ANG2 may be packaged in a single article of manufacture, each one being packaged separately. Alternatively, the monospecific antibody which is directed against HGF and the monospecific antibody which is directed against ANG2 may be combined in a single formulation.

According to another aspect of the present invention there is a provided a method of treating an angiogenesis related disorder. The method comprises administering to a subject in need thereof a therapeutically effective amount of an antibody (or antibodies) that targets either ANG2 and/or HGF. The antibody may be a bispecific antibody such as described herein or may be a combination of two monospecifics i.e. one targeting ANG2 and the other targeting HGF.

Alternatively, the antibody may be a single monospecific antibody that targets ANG2 or HGF, the CDRs of which have been described herein above. Together with administration of such monospecific antibodies, the present invention contemplates administering additional agents that downregulate tumor microenvironment and/or immunomodulatory targets. Examples of tumor microenvironment targets include, but are not limited to bone morphogenic protein 9 (BMP9) or its receptor activin receptor-like kinase 1 (Alk1), Delta like ligand 4 (DLL4) (one of the Notch ligands), TNF-related weak inducer of apoptosis (TWEAK), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) or epidermal growth factor receptor (EGFR), ErbB2, ErbB3, integrin-alpha-9-beta1, integerin-alpha-5-beta1, Tumor endothelial marker 8 protein (TEM8), Tumor endothelial marker 1 protein (TEM1), Galectin3, CD24 Examples of immunomodulatory targets include, but are not limited to Programmed cell death 1 ligand (PD-L1) or its receptor PD-1, CD47, Macrophage colony-stimulating factor 1 (CSF1) or its receptor CSF1R, CD137, CTLA-4, LAG3, TIM3, SPARC and CD124.

Exemplary agents that may be used to downregulate these additional targets include antibodies, polynucleotide agents (e.g. siRNAs), receptor antagonists, small molecules etc.

As mentioned, the antibodies of the present invention are useful for treating angiogenesis related diseases.

Examples of angiogenesis related diseases include diabetic retinopathy, ischemic chronic wounds, age-related macular degeneration, cardiovascular diseases and cancer.

Examples of cancer include but are not limited to carcinoma, lymphoma, blastoma, sarcoma, and leukemia. Particular examples of cancerous diseases but are not limited to: Myeloid leukemia such as Chronic myelogenous leukemia. Acute myelogenous leukemia with maturation. Acute promyelocytic leukemia, Acute nonlymphocytic leukemia with increased basophils, Acute monocytic leukemia. Acute myelomonocytic leukemia with eosinophilia; Malignant lymphoma, such as Birkitt's Non-Hodgkin's; Lymphoctyic leukemia, such as Acute lymphoblastic leukemia. Chronic lymphocytic leukemia; Myeloproliferative diseases, such as Solid tumors Benign Meningioma, Mixed tumors of salivary gland, Colonic adenomas; Adenocarcinomas, such as Small cell lung cancer, Kidney, Uterus, Prostate, Bladder, Ovary, Colon, Sarcomas, Liposarcoma, myxoid, Synovial sarcoma, Rhabdomyosarcoma (alveolar), Extraskeletel myxoid chonodrosarcoma, Ewing's tumor; other include Testicular and ovarian dysgerminoma, Retinoblastoma, Wilms' tumor, Neuroblastoma, Malignant melanoma, Mesothelioma, breast, skin, prostate, and ovarian.

According to a particular embodiment, the cancer is colorectal cancer, lung cancer, breast cancer, renal cancer, ovarian cancer, gastric cancer, bladder cancer, liver cancer, ovarian cancer, fallopian cancer, glioblastoma, leukemia, including acute myeloid leukemia and lymphoma.

Treatment of diseases may be effected by administering the antibody alone, or together with a carrier as a pharmaceutical composition.

As used herein, "pharmaceutical 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. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (e.g. by injection or infusion).

A composition of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. To administer a

compound of the invention by certain routes of administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent.

Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Pharmaceutical carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intra-arterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intra-articular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, supra, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions.

In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the

patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical art.

The composition must be sterile and fluid to the extent that the composition is deliverable by syringe. In addition to water, the carrier preferably is an isotonic buffered saline solution.

Proper fluidity can be maintained, for example, by use of coating such as lecithin, by maintenance of required particle size in the case of dispersion and by use of surfactants. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol or sorbitol, and sodium chloride in the composition.

Other contemplated uses of the bispecific antibodies of the present invention include purification of analytes; in immunohistochemistry and enzyme immunoassays; for radioimaging and radioimmunotherapy and for drug delivery.

Other contemplated uses are set forth in Cao Y, Suresh MR. Bispecific antibodies as novel bioconjugates. Bioconjug Chem. 1998 Nov-Dec;9(6):635-44, incorporated herein by reference.

As used herein the term “about” refers to ± 10 %.

The terms "comprises", "comprising", "includes", "including", “having” and their conjugates mean "including but not limited to".

The term “consisting of” means “including and limited to”.

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known

manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed.

(1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

GENERAL MATERIALS AND METHODS

Library screening

To identify scFvs that specifically bind to human Hepatocyte growth factor (HGF) or human Angiopoietin 2 (ANG2), a human synthetic combinatorial library of arrayable single-chain antibodies was screened. The construction and properties of the library is described in Azriel-Rosenfeld, 2004 (J Mol Biol. 2004 Jan 2;335(1):177-92. PubMed PMID: 14659749).

An aliquot of the bacterial library glycerol stock (~$1x10^{11}$ clones) was diluted into 2xYT medium supplemented with 100 µg/ml ampicillin and 1% glucose (YTAG) and grown with shaking at 37 °C until reaching an OD_{600} between 600 to 800 nm.

M13KO7 helper phage was added to 500 ml of the culture at a ratio of 1:20. For infection, the culture was incubated without shaking at 37°C for 30 min and then transferred to a shaking incubator at 37°C and 100 RPM for 30 min. The infected cells were collected by centrifugation and resuspended in 2xYT medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin. The culture was incubated with shaking (250 RPM) at 30°C overnight for phage proliferation. On the following day, the culture was spun down and the supernatant was filtered through 0.45 μm filter to eliminate remaining bacteria. PEG/NaCl (20% PEG6000, 2.5 M NaCl) was added (fifth of the volume) to the supernatant, mixed well and incubated for >1 hr on ice. Phage-precipitates were collected by centrifugation at 10,800 x g for 30 min at 4°C and the supernatant was carefully aspirated off. The phages were resuspended in sterile and filtered PBS.

To titer the phage stock, 10-fold serial dilutions of the phages were made in sterile 2xYT medium. A logarithmic TG-1 *E. coli* culture was infected with the diluted phages by mixing and incubating at 37°C for 60 min. Infected cells were plated on 2xYT plates supplemented with 100 μg/ml ampicillin and 1% glucose and grown overnight at 37°C.

Four panning (affinity selection) cycles were applied. About 10^{12} phage-particles were used at the first round. 24 wells plate were coated with human HGF or human Ang2 (R&D systems) in PBS overnight at 4ºC. The wells were blocked with 3% milk in PBS (in the first and the second panning cycles) or 1% BSA in PBS (in the third and the fourth panning cycles) for 1 hour at room temperature. Phages were pre-incubated for 1 hour at 37ºC with 3% milk or 1% BSA in PBS to deplete and avoid anti-milk/BSA binders. The phages were added to the blocked wells and incubated for 1 hour at room temperature on a rotator. Wells were washed 10 times with PBS and 10 times with PBST. The bound phages were eluted from wells by the addition of 1 ml of 100 mM Triethylamine, followed by 20 min incubation at room temperature on a rotator (round 1 completed). Immediately after elution, the phage solution was neutralized by the addition of 500 μl of 1 M Tris (HCl) pH 7.4. TG1 cells were infected with round 1 output phages and incubated for 30 min at 37ºC followed by an additional 30 min at 37ºC with shaking. For output phage enrichment, the selected phages were used for re-infection of TG-1 cells. The enriched output phages were used for another round of

selection, 0.75 ml of the neutralized TEA-eluate were mixed with 5 ml of logarithmic TG-1 cells. For infection, the culture was incubated without shaking at 37°C for 30 min and then transferred to a shaking 37°C incubator at 100 RPM for 30 min. The infected cells were spread on 2xYT plates supplemented with 100 μg/ml ampicillin and 1% glucose and grown overnight at 37°C.

Single clone panned phages were validated for binding to antigens by standard enzyme-linked immunosorbent assays. For infection and rescue of individual clones, *E. coli* TG-1 cells were infected with fourth panning output phages and plated to yield individual colonies. 96 individual colonies were picked into sterile 96-well plates containing 100 μl of 2xYT with 100 μg/ml ampicillin and 1% glucose medium per well and incubated in a 30°C shaker at 150 RPM overnight. 10 μl of inoculum was transferred to a second 96-well plate containing fresh 100 μl of YTAG per well and grown with shaking for 2 hours at 37ºC. Helper phage was added, incubated for 30 minutes at 37ºC without shaking and for an additional 30 min with shaking (150 rpm) at 37ºC. The infected cells were collected by centrifugation and resuspended in 100 μl of 2xYT medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin and grown overnight at 30ºC while shaking (150 rpm). The plates were centrifuged to pellet the cells. The supernatant containing the phages was used for analysis by ELISA.

Ninety six-well ELISA plates were coated with 5 μg/ml human HGF or human Ang2 or irrelevant proteins as a negative control in PBS at 4˚C overnight. Plate was washed once with PBST and then blocked for >1 hr at room temperature with blocking solution (3% skim milk powder in PBS). Plate was washed once with PBST. Phages displaying scFvs were added to wells directly or after dilution in PBST and incubated at room temperature for 1 hr. After washing 3 times with PBST, HRP conjugated anti M13 IgG diluted 1:5000 in PBST was added to the wells and incubated for 1 hr at room temperature. Plates were washed 3 times with PBST and TMB peroxidase-substrate solution used for detection. Absorbance was recorded at 450 nm.

Construction of vectors for expression of monospecific/bispecific IgG1 antibodies in E. coli and CHO cells

The antibodies described herein are composed of heavy chains that are dimerized by the knobs into holes (KIH) approach (as disclosed in PCT Patent Application No. IL2012/050093). In addition, bispecific antibodies were engineered by

disulfide stabilization between the VH and VL domains and are mutated in the CH1 and CL domains (as disclosed in PCT Patent Application No. IL2012/050093). The monospecific heavy chains were not engineered in the VH, VL CH1 or CL domains.

Construction of vectors for expression of monospecific IgG1antibodies in E. coli: Vectors for the expression of monospecific antibodies anti HGF 2C3 and anti Ang2 1A2 were constructed as follows. After their isolation by antibody phage display, the coding sequences for the VH and VL domain were prepared as synthetic genes with optimization for expression in *E. coli* by GeneArt (Germany). The synthetic genes contained the *NdeI* and *NheI* sites required for cloning into the pHAK-HC-knob vector or pHAK-HC-hole vector and the *NdeI* and *AvrII* sites required for cloning into the pHAK-IgLam vector (described herein below). The synthetic genes were subcloned into the pHAK expression vectors and the sequences were verified by DNA sequencing.

Construction of pHAK-IgLam plasmid: A lambda light chain gene fragment was constructed by overlap extension PCR from a lambda VL that was isolated from the Ronit 1 library and a lambda constant domain that was PCR-amplified from human lymphoid cDNA (commercial cDNA from human spleen, lymph nodes and peripheral blood lymphocytes (Clontech, USA)). The cloned fragment contained an *NdeI* site at the 5' end (CATATG (SEQ ID NO: 168) with the ATG serving as a translation initiation codon), an *AvrII* site (CCTAGG (SEQ ID NO: 169) with the first C being the last base of the penultimate codon of V-lambda, CTA being the last codon of V-lambda and GG being the first two bases of the first residue of C-lambda). At the 3' end a TAA stop codon was inserted followed by an *EcoRI* site.

Plasmid pHAK-IgL (Kappa light chain) was linearized with *NdeI* and *EcoRI* restriction enzymes that removed the entire kappa light chain and the Lambda light chain fragment was cloned into it as *NdeI-EcoRI* fragment. The resulting plasmid was named pHAK-IgLam and is used to express a wild-type lambda light chain.

Construction of vectors for expression of bispecific IgG1antibodies in E. coli

Vectors for the expression of bispecific antibody BIC 104 (composed of the anti HGF 2C3 and the anti ANG2 1A2) were constructed as follows.

The coding sequences for the VH and VL domains, after their isolation by antibody phage display, were prepared as synthetic genes with optimization for expression in *E. coli* by GeneArt (Germany). Anti HGF antibody 2C3 was synthesized

with mutations G44C in the VH domain and G100C in the VL domain to facilitate disulfide stabilization of the association between the two engineered chains. The variable domains of anti-Ang2 were not mutated since that side of the BIC104 molecule contains the native disulfide bond between CH1 and CL. The synthetic genes contained the *NdeI* and *NheI* sites required for cloning the heavy variable regions of desired antibody were cloned into pHAK-HC-knob (carrying mutations T366W, S354C) vector or pHAK-HC-hole (carrying mutations T366S, L368A, Y407V, Y349C) (PCT Patent Application No. IL2012/050093). The synthetic genes contained the *NdeI* and *AvrII* sites required for cloning the light variable regions of desired antibody were cloned into the pHAK-IgLam or into the pFUS12 (described below) vectors. The sequences were verified by DNA sequencing.

Construction of pFUS12 plasmid: To express engineered lambda light chains for association with engineered heavy chain using disulfide stabilization, pHAK-IgLam vector was modified; the constant lambda domain was amplified with primer CLam-AvrII-FOR (for introduction of C214A mutation) and primer CLam-C214A-EcoRI-REV (Table 7, herein below), digested with AvrII-EcoRI restriction enzymes and cloned to pHAK-IgLam previously digested with the same enzymes. The resulted vector named pFUS12.

Table 7

Notes	Sequence 5' to 3'	Primer name
Forward primer for PCR amplification of constant region of lambda light chain on pHAK-IgLam vector	ATATATCCTAGGTCAGCCCAAG GCTGCCC - SEQ ID NO: 170	CLam-AvrII-FOR
Reverse primer for C214A replacement in constant domain of lambda light chain on pHAK-IgLam vector	TATATAGAATTCTTATTAAGAA GCTTCTGCAGGGGCCACTGTC - SEQ ID NO: 171	CLam-C214A-EcoRI-REV

Construction of vectors for expression of bispecific IgG1antibodies in CHO: The variable domains from pHAK-HC-hole, pHAK-HC-Cys-knob, pHAK-IgLam and pFUS12 which encode 2C3 and 1A2 were synthesized after codon optimization for expression in mammalian cells. Subsequently, synthetic DNA was cloned into GPEx® (Catalent Pharma Solution, MO, USA) retroviral IgG1 vectors.

IgG production in E. coli: Heavy and light chains constructs were expressed in separate *E. coli* BL21 (DE3) pUBS500 bacterial cultures as inclusion bodies. The

inclusion bodies were purified, denatured, mixed and refolded according to the Hakim et al., MAbs. 2009 May-Jun;1(3):281-7.

Protein A purification: Following the refolding process, refolded IgGs were concentrated (Vivaflow200, Sartorius) followed by diafiltration against 50mM Tris, 50g/L Arginine, pH7.0. Subsequently the IgG was purified by protein A chromatography. The proteins were eluted with 0.1 M citric acid pH3.0, neutralized with 1M Tris-HCl, pH8.5 followed by buffer exchange against 20 mM phosphate buffer solution pH6.5 (PD-10 column, GE Healthcare). The protein final concentration was determined by absorbance at 280 nm.

SDS-PAGE analysis: Polyacrylamide gel electrophoresis of proteins was performed according to Laemmli (Laemmli, 1970). Half volume of 2x sample buffer was added to the protein samples followed by boiling for 5 min prior to the loading onto the gel. 7.5 %, 10 % and 12 % mini-gels were run at 120 V. For evaluation of full length IgG, the non-reduced samples (without β-mercaptoethanol) were loaded, while the reduced protein samples separated into heavy and light chains components. Gels were stained with Coomassie based staining solution (InstantBlue, expedeon) until protein bands could be clearly seen. Gels that were stained were loaded with 3-5 µg for purified proteins. Gels that were further processed by immunoblotting were loaded with 1/10 that quantity.

Western blot analysis: Proteins resolved by SDS-PAGE were electro-transferred onto the nitrocellulose membrane. The membrane was blocked for at least 1 hour with PBS containing 5 % non-fat milk powder at room temperature with slow agitation. The membrane was washed with PBS followed by incubation HRP conjugated secondary antibodies. After three washes with PBS containing 0.05 % Tween-20 (PBST) and one wash with PBS the nitrocellulose filter was developed with the EZ-ECL Chemiluminescence detection kit (Biological Industries, Israel) as described by the vendor.

ELISA analysis: 96-well ELISA plate was coated with 2-5 µg/ml of pure antigen in PBS overnight at 4°C. All subsequent steps were carried out at room temperature (25°C). The plates were blocked with 3 % skim milk (in PBS) for 1 hour at room temperature. Protein A purified antibodies, serially diluted in PBST, were added to wells and incubated for 1 hour and washed three times with PBST. Following a 50

min incubation with HRP conjugated secondary antibodies, the plates were washed in PBST and developed using chromogenic HRP substrate TMB and colour development was terminated with 1M H_2SO_4. The plated were read at 450 nm.

Bispecific binding ELISA: 96 wells plate was coated with 2.5 µg/ml human HGF (Sino Biologicals Inc.). Samples containing 1 µg/ml of biotinylated Ang2 (R&D Systems) and serial dilutions of antibodies were added for 1 hour incubation at room temperature. Following incubation with streptavidin-HRP (1:5000; Jackson), wells were washed with PBST. Reaction was developed using chromogenic HRP substrate TMB and colour development was terminated with 1M H_2SO_4. The plated were read at 450 nm.

Competition ELISA:

Competition with cMet- 96 wells plate was coated with 2.5 µg/ml human HGF (R&D Systems). Samples contained 1 nM soluble c-Met Fc (R&D Systems) and serial dilutions of 2C3 antibody were added. c-Met binding was detected with 1:1000 biotinylated anti-c-Met (R&D Systems) mixed with 1:5000 streptavidin-HRP (Jackson) in PBST, 1 hour, RT. Reaction was developed using chromogenic HRP substrate TMB and colour development was terminated with 1M H_2SO_4. The plated were read at 450 nm. The data were evaluated by calculating the percentage inhibition compared with the maximal signal (no antibody added).

Competition with Tie2: - 96 wells plate was coated with 4 µg/ml human Tie2 Fc (R&D Systems). Samples contained 100 ng/ml of human Ang2 (R&D Systems) and serial dilutions of BIC104 antibody were added. Ang2 binding was detected with 1:5000 streptavidin-HRP (Jackson) in PBST, 1 hour, RT. Reaction was developed using chromogenic HRP substrate TMB and colour development was terminated with 1M H_2SO_4. The plated were read at 450 nm.

Surface Plasmon Resonance (SPR) analysis: Experiments were performed on a ProteOn XPR36 instrument (Bio-Rad) using PBS, 0.05% polysorbate 20 as running and dilution buffer. Anti-human IgG polyclonal antibodies (Jackson ImmunoResearch Europe Ltd.) were immobilized to the surface of a CM5 sensorchip using standard amine-coupling chemistry to a surface density of ~200 response units (RU). After capturing the bispecific antibodies to the surface human HGF was injected at increasing concentrations at a flow rate of 80 µL/min. The contact time (association phase) was 5

min. The dissociation time (washing with running buffer) was 15 min. The derived curves were fit to a 1:1 Langmuir binding model using the ProteOn evaluation software.

Xenograft animal model: Colo205 human colorectal cancer cells were originally obtained from ATCC. $1x10^6$ Colo205 tumor cells in 50% Matrigel (0.2 ml per mouse) were injected subcutaneously in the flank to 8-12 weeks CR female NCr nu/nu mice. Pair match of the tumor size was done when tumors reached an average size of 100-150 mm^3, at which time antibody treatment was initiated. Antibodies were injected to a group of 9 animals (10 mg/kg for monospecific antibodies and 20 mg/kg for bispecific antibodies), once a week for 6 weeks. Non-relevant antibody was injected to a control group. Caliper measurements of tumors was performed twice a week. Tumor size analysis and survival analysis were used as an efficacy endpoint. Survival data contain duration times until occurrence of a specific event, (i.e. the death of an animal). Survival data was analyzed with specialized methods, because they have specialized non-normal distributions, line the exponential or Weibyll. Furthermore, the censored observations cannot be ignored without biasing the analysis. Kaplan-Meier curves give an estimation of the survival functions for one or more group of right-censored data.

RESULTS

Experimental results are provided in Figures 1-17.

EXAMPLE 2

Therapeutic effect of an antibody directed against Ang-2

MATERIALS AND METHODS

Tumor xenografts were initiated with SKOV3 human ovarian carcinomas maintained by serial subcutaneous transplantation in severe combined immunodeficient mice. On the day of tumor implant, each athymic nude test mouse received a 1-mm^3 SKOV3 fragment implanted subcutaneously in the right flank, and tumor growth was monitored until 80 to 120 mm^3. Twenty-one days after tumor implantation, designated as Day 1 of dosing, the animals were sorted into groups; treatment with antibodies was once a week.

RESULTS

As illustrated in Figure 18, Anti-ANG2 antibody (1A2 - CDRs SEQ ID NOs: 7-12) significantly inhibited xenograft tumor growth, whereas the control antibody anti-CD20 (Rituximab) did not.

WHAT IS CLAIMED IS:

1. An antibody comprising an ANG2 recognition region which comprises CDR amino acid sequences as set forth:

(i) SEQ ID NOs: 7-12; or

(ii) SEQ ID NOs: 107-112.

2. A bispecific antibody comprising a first antigen-binding site that specifically binds to human hepatocyte growth factor (HGF) and a second antigen-binding site that specifically binds to human angiopoietin-2 (ANG2).

3. An antibody comprising an HGF recognition region which comprises CDR sequences selected from the group consisting of:

(i) SEQ ID NOs: 1-6;

(ii) SEQ ID NOs: 17-22;

(iii) SEQ ID NOs: 23-28;

(iv) SEQ ID NOs: 29-34;

(v) SEQ ID NOs: 35-40;

(vi) SEQ ID NOs: 41-46;

(vii) SEQ ID NOs: 47-52; and

(viii) SEQ ID NOs: 53-58.

4. The antibody of claims 1 or 3 being monospecific.

5. The antibody of claims 1 or 3 being bispecific.

6. The bispecific antibody of claim 2, wherein,

(i) said first antigen binding site comprises CDR amino acid sequences as set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3; SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6; and

(ii) said second antigen binding site comprises CDR amino acid sequences as set forth in SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9; SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 12.

7. The bispecific antibody of claim 2, wherein said first antigen binding site comprises CDR sequences selected from the group consisting of:

(i) SEQ ID NOs: 1-6;

(ii) SEQ ID NOs: 17-22;

(iii) SEQ ID NOs: 23-28;

(iv) SEQ ID NOs: 29-34;

(v) SEQ ID NOs: 35-40;

(vi) SEQ ID NOs: 41-46;

(vii) SEQ ID NOs: 47-52; and

(viii) SEQ ID NOs: 53-58.

8. The bispecific antibody of claims 2 or 7, wherein said second antigen binding site comprises CDR amino acid sequences as set forth:

(i) SEQ ID NOs: 7-12; or

(ii) SEQ ID NOs: 107-112.

9. The bispecific antibody of claims 2 or 7, wherein said second antigen binding site comprises CDR amino acid sequences as set forth in SEQ ID NOs: 7-12.

10. The bispecific antibody of claim 1, wherein a VH region of said first antigen binding domain comprises a sequence as set forth in SEQ ID NO: 135.

11. The bispecific antibody of claim 1, wherein a VL region of said first antigen binding domain comprises a sequence as set forth in SEQ ID NO: 136.

12. The bispecific antibody of claim 2, wherein a VH region of said second antigen binding domain comprises a sequence as set forth in SEQ ID NO: 157.

13. The antibody of claim 1, wherein a VH region of said ANG2 binding domain comprises a sequence as set forth in SEQ ID NO: 157.

14. The bispecific antibody of claim 2, wherein a VL region of said second antigen binding domain comprises a sequence as set forth in SEQ ID NO: 158.

15. The antibody of claim 1, wherein a VL region of said second antigen binding domain comprises a sequence as set forth in SEQ ID NO: 158.

16. The bispecific antibody of claim 2, wherein a first heavy chain of said first antigen binding domain is non-identical to a second heavy chain of said second antigen binding domain in the Fc region of the antibody, wherein at least one of said heavy chains comprises an amino acid modification so as to form complementation between two non-identical heavy chains thereby increasing the probability of forming heterodimers of said non-identical heavy chains and decreasing the probability of forming homodimers of identical heavy chains.

17. The bispecific antibody of claim 2, comprising a first disulfide bond between a first heavy chain and a first light chain of an Fab region of said first antigen binding site and a second disulfide bond between a second heavy chain and a second light chain of an Fab region of said second antigen binding site, wherein a position of said first disulfide bond relative to said first heavy chain is different to a position of said second disulfide bond relative to said second heavy chain.

18. The bispecific antibody of claim 16, wherein said complementation comprises a steric complementation.

19. The bispecific antibody of claim 16, wherein said complementation comprises a charge complementation.

20. The bispecific antibody of claim 18, wherein said Fc region comprises a protuberance of one heavy chain of said Fc region and a sterically compensatory cavity on a second heavy chain of said Fc region, said protuberance protruding into said compensatory cavity.

21. The bispecific antibody of claim 18, wherein said protuberance is generated by substituting an amino acid at one position on a CH3 domain of said one heavy chain with another amino acid having a larger side chain volume than the original amino acid.

22. The bispecific antibody of claim 21, wherein said compensatory cavity is generated by substituting an amino acid at one position on a CH3 domain of said second heavy chain with another amino acid having a smaller side chain volume than the original amino acid.

23. The bispecific antibody of claim 17, wherein said first disulfide bond is between a V_H domain of said first heavy chain and a V_L domain of said first light chain and said second disulfide bond is between a CH1 domain of said second heavy chain and a CL domain of said second light chain.

24. The bispecific antibody of claim 21, wherein said amino acid having a larger side chain volume than the original amino acid is selected from the group consisting of tyrosine, arginine, phenylalanine, isoleucine and tryptophan.

25. The bispecific antibody of claim 22, wherein said amino acid having a smaller side chain volume than the original amino acid is selected from the group consisting of alanine, glycine, valine and threonine.

26. The antibody of any of claims 1-3, being selected from the group consisting of a chimeric antibody, a humanized antibody and a fully human antibody.

27. The bispecific antibody of claim 2, wherein a CH3 domain of a first heavy chain of said first antigen binding site is covalently linked to a CH3 domain of a second heavy chain of said second antigen binding site.

28. The bispecific antibody of claim 2, wherein each light chain of said first and second antigen binding site is linked to its cognate heavy chain via a single disulfide bond.

29. The antibody of any of claims 1-3 being an intact antibody.

30. The antibody of any of claims 1-3, wherein the antibody is selected from the group consisting of IgA, IgD, IgE and IgG.

31. The antibody of any of claims 1-3, wherein said IgG comprises IgG1, IgG2, IgG3 or IgG4.

32. The bispecific antibody of claim 16, wherein said first heavy chain comprises a T366W mutation and said second heavy chain comprises T366S, L368A, Y407V mutations.

33. The bispecific antibody of claims 16 and 32, wherein said first heavy chain comprises an S354C mutation and said second heavy chain comprises a Y349C mutation.

34. The antibody of any of claims 1-3, wherein at least one heavy chain of the antibody is attached to a therapeutic moiety.

35. The antibody of any of claims 1-3, wherein at least one heavy chain of the antibody is attached to an identifiable moiety.

36. The antibody of any of claims 1-3, being selected from the group consisting of a primate antibody, a porcine antibody, a murine antibody, a bovine antibody, a goat antibody and an equine antibody.

37. A pharmaceutical composition comprising the antibody of any one of claims 1-36 as the active agent and a pharmaceutically acceptable carrier.

38. A method of treating cancer in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of at least one antibody of any one of claims 1-36.

39. The method of claim 38, wherein said at least one antibody comprises a combination of the antibody of claim 1 and the antibody of claim 3.

40. The method of claim 38, wherein the cancer is a solid tumor or lymphoid tumor.

41. The method of claim 38, wherein said cancer is selected from the group consisting of colorectal cancer, lung cancer, breast cancer, renal cancer, ovarian cancer, gastric cancer, bladder cancer, liver cancer, ovarian cancer, fallopian cancer, glioblastoma, Mesothelioma and leukemia.

42. An isolated nucleic acid encoding a heavy chain of said first antigen binding site of the antibody of any one of claims 1-36.

43. An isolated nucleic acid encoding a light chain of said first antigen binding site of the antibody of any one of claims 1-36.

44. An isolated nucleic acid encoding a heavy chain of said second antigen binding site of the antibody any one of claims 1-36.

45. An isolated nucleic acid encoding a light chain of said second antigen binding site of the antibody of any one of claims 1-36.

46. An expression vector comprising the nucleic acid of any of claims 42-45.

47. A prokaryotic or eukaryotic host cell comprising the nucleic acid according to any of claims 42-45.

48. A method of preparing the antibody of claim 2, comprising:

(a) providing the nucleic acid of claim 42;

(b) providing the nucleic acid of claim 43;

(c) providing the nucleic acid of claim 44;

(d) providing the nucleic acid of claim 45;

(e) culturing host cells comprising said nucleic acids under conditions that permit expression of the nucleic acids; and

(f) recovering the antibody of claim 1.

49. The method of claim 48, wherein said host cells comprise bacterial cells.

50. The method of claim 48, wherein said host cells comprise mammalian cells.

51. The method of claim 49, wherein said expression takes place in inclusion bodies of said bacterial cells.

52. The method of claim 48, wherein each of said nucleic acid molecules are transfected into different host cells.

53. The method of claim 48, wherein each of said nucleic acid molecules are transfected into the same host cell.

54. The method of claim 49, wherein said bacterial cells comprise gram negative bacterial cells.

55. The antibody of any of claims 1-36 for treating cancer.

FIG. 1

GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAG
ACTCTCCTGTGCAGCCTCTGGATTCACCGTCAGGAGCCACTACATGAGTTGGGTCCG
CCAGGCTCCAGGGAAGTCTCTGGAGTGGGTCTCATCGATTACTAGTGGTGGCACTTA
TAGACACTACGCCGACTCAGTGACGGGCCGTTTCACCATCTCCAGAGACAATTCCAA
GAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATT
ACTGTGCAAGAGATAGCGCCTTAGATGCTTTTGATATCTGGGGCCAGGGCACCCTGG
TCACGGTCTCTTCAGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCT
CCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTC
CCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCACAC
CTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGT
GCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCA
GCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTGCGACAAAACTCACACA
TGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCC
CCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGT
GGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCG
TGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTA
CCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGT
ACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCC
AAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATGCCGGGA
GGAGATGACCAAGAACCAGGTCAGCCTGTGCCTGGTCAAAGGCTTCTATCCCA
GCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGAC
CACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTATAGCAAGCTCACCGT
GGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGG
CTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCCCCCGGGAAA

FIG. 2

GATATCGTGCTGACTCAGCCACCCTCAGCGTCTGGGACCCCCGGGCAGAGGGTCAC
CATCTCCTGCTCTGGAACCTCCTCCAACATTGGCTATAATTCTGTCTCCTGGTACCAG
CAGCTTCCAGGAACGGCCCCCAAACTCCTCATCTATGAGGCCACTAAGCGACCCTCA
GGGGTCCCTGACCGGTTCTCTGGCTCCAAGTCTGGCACCTCAGCCTCCCTGGCCATC
AGTGGGCTGCGGTCCGAGGATGAGGCTGATTATTACTGCGCAGCATGGGATAGCAG
TCTGAGCCAGTGGGTGTTCGGCTGTGGCACCCAGGTCACCGTCCTAGGTCAGCCCAA
GGCTGCCCCCTCGGTCACTCTGTTCCCGCCCTCCTCTGAGGAGCTTCAAGCCAACAA
GGCCACACTGGTGTGTCTCATAAGTGACTTCTACCCGGGAGCCGTGACAGTGGCCTG
GAAGGCAGATAGCAGCCCCGTCAAGGCGGGAGTGGAGACCACCACACCCTCCAAAC
AAAGCAACAACAAGTACGCGGCCAGCAGCTATCTGAGCCTGACGCCTGAGCAGTGG
AAGTCCCACAGAAGCTACAGCTGCCAGGTCACGCATGAAGGGAGCACCGTGGAGAA
GACAGTGGCCCCTGCAGAATGCTTCT

FIG. 3

GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAG
ACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGTAACTTTGGCATGAACTGGGTCCGC
CAGGCTCCAGGCAAGGGGCTGGAGTGGGTCTCAGGTCTTAACTGGAATGGTCGTAA
AAAAGCCTATGCGGACTCTGTGAAGGGCCGATTCACCATCTCCAGAGACAATTCCA
AGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTAT
TACTGTGCAAGAGAATCTGTAGATGGCTACAATTACTACTACCACTACGGAATGGAC
GTCTGGGGCCAGGGCACCCTGGTCACAGTCTCCTCAGCTAGCACCAAGGGCCCATC
GGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGG
CTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGC
CCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTC
CCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTG
CAACGTGAATCACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAAT
CTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGGA
CCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACC
CCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTT
CAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGG
AGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACT
GGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCC
ATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTGCAC
CCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTCAGCCTG[redacted]TGC[redacted]G
TCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCG
GAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTC
[redacted]AGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATG
CTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTC
CCCCGGGAAA

FIG. 4

CAGAGCGTGCTGACTCAGCCACCCTCAGCGTCTGGGACCCCCGGGCAGAGGGTCAC
CATCTCTTGTACTGGAAGCAGCTCCAACATCGGAAGTAACAGTGTGAACTGGTACCA
GCAGCTCCCAGGAAAGGCCCCCAAACTCCTCATTTATAGTAATGATCAGCGGCCCTC
AGGGGTCCCTGACCGGTTCTCTGGCTCCAAGTCTGGCACCTCAGCCTCCCTGGCCAT
CAGTGGGCTCCGGTCCGAGGATGAGGCTGATTATTACTGCAGCTCTTATACAAGCAG
CAATACTTGGGTGTTCGGCGGAgGCACCCAgGTCACCGTCCTAGGTCAGCCCAAGGC
TGCCCCCTCGGTCACTCTGTTCCCGCCCTCCTCTGAGGAGCTTCAAGCCAACAAGGC
CACACTGGTGTGTCTCATAAGTGACTTCTACCCGGGAGCCGTGACAGTGGCCTGGAA
GGCAGATAGCAGCCCCGTCAAGGCGGGAGTGGAGACCACCACACCCTCCAAACAAA
GCAACAACAAGTACGCGGCCAGCAGCTATCTGAGCCTGACGCCTGAGCAGTGGAAG
TCCCACAGAAGCTACAGCTGCCAGGTCACGCATGAAGGGAGCACCGTGGAGAAGAC
AGTGGCCCCTGCAGAATGTTCTTGA

FIG. 5A

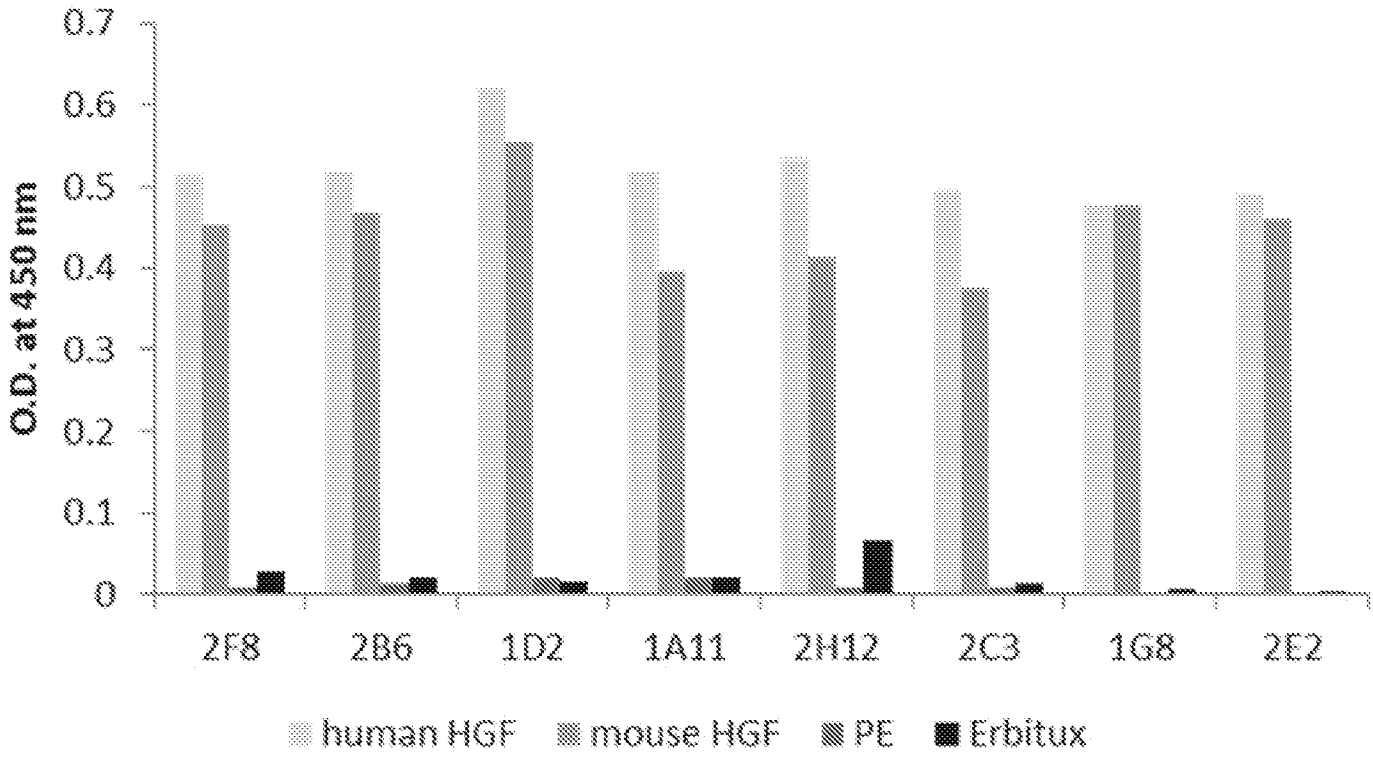
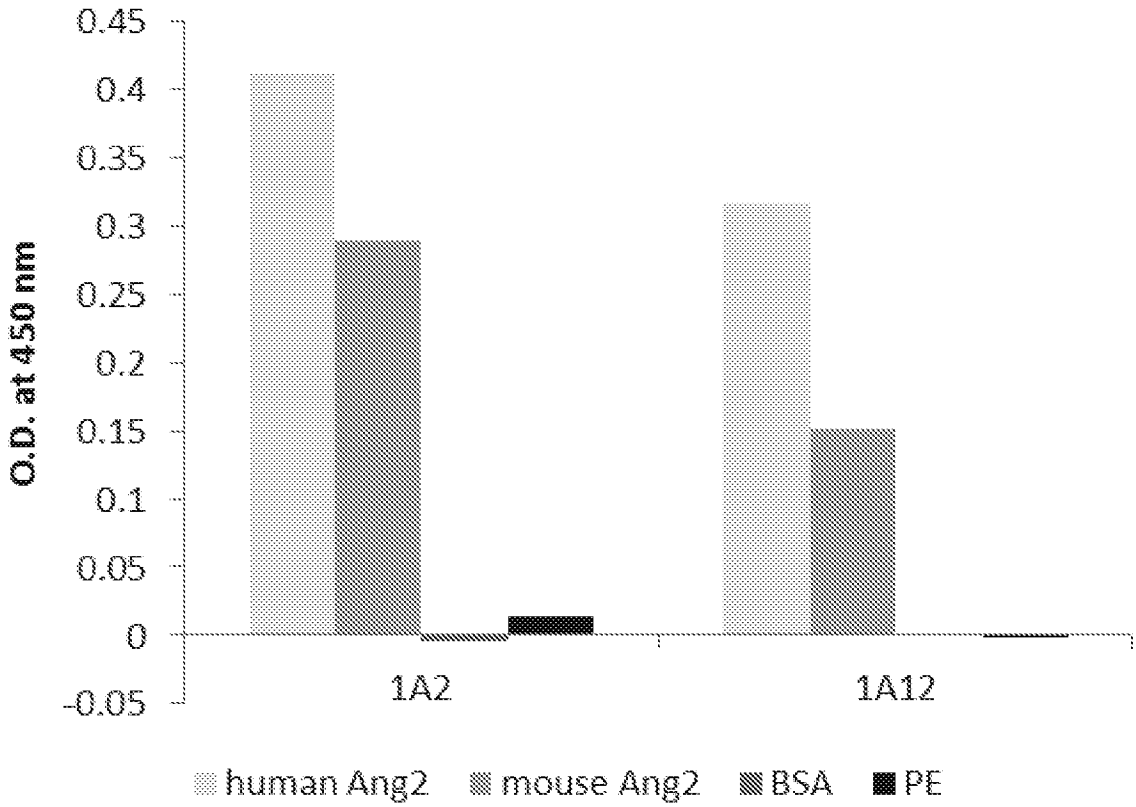

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
O.D. at 450 nm
2F8
2B6
1D2
1A11
2H12
2C3
1G8
2E2
human HGF
mouse HGF
PE
Erbitux

FIG. 5B


0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
O.D. at 450 nm
1A2
1A12
human Ang2
mouse Ang2
BSA
PE

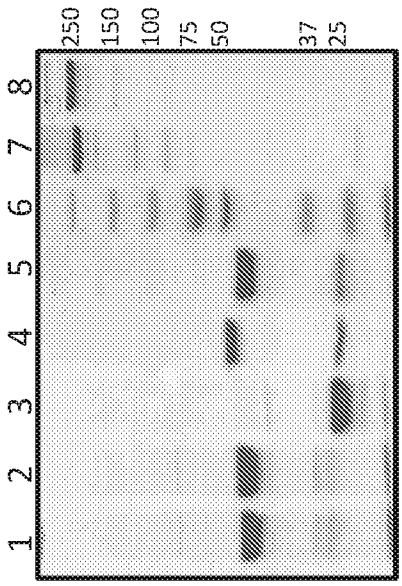


FIG. 6A

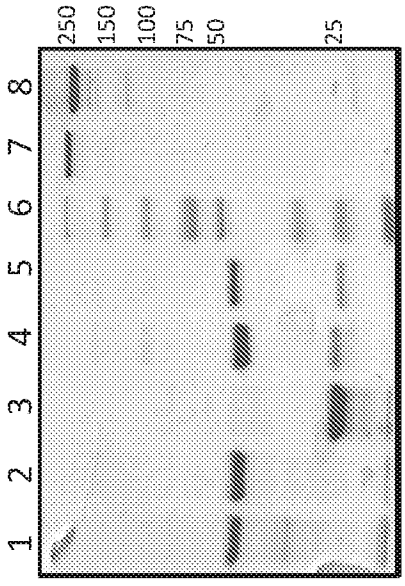


FIG. 6B

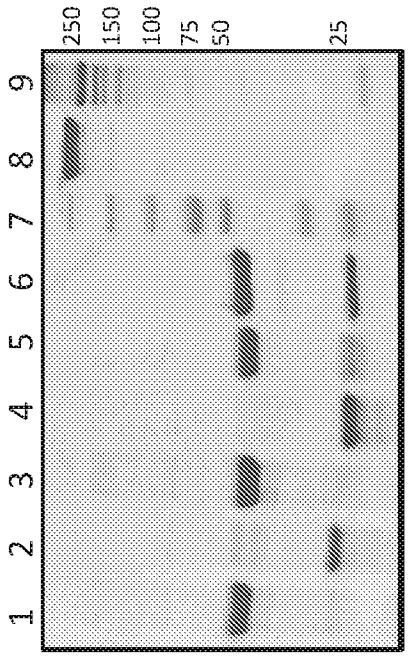


FIG. 6C

FIG. 7

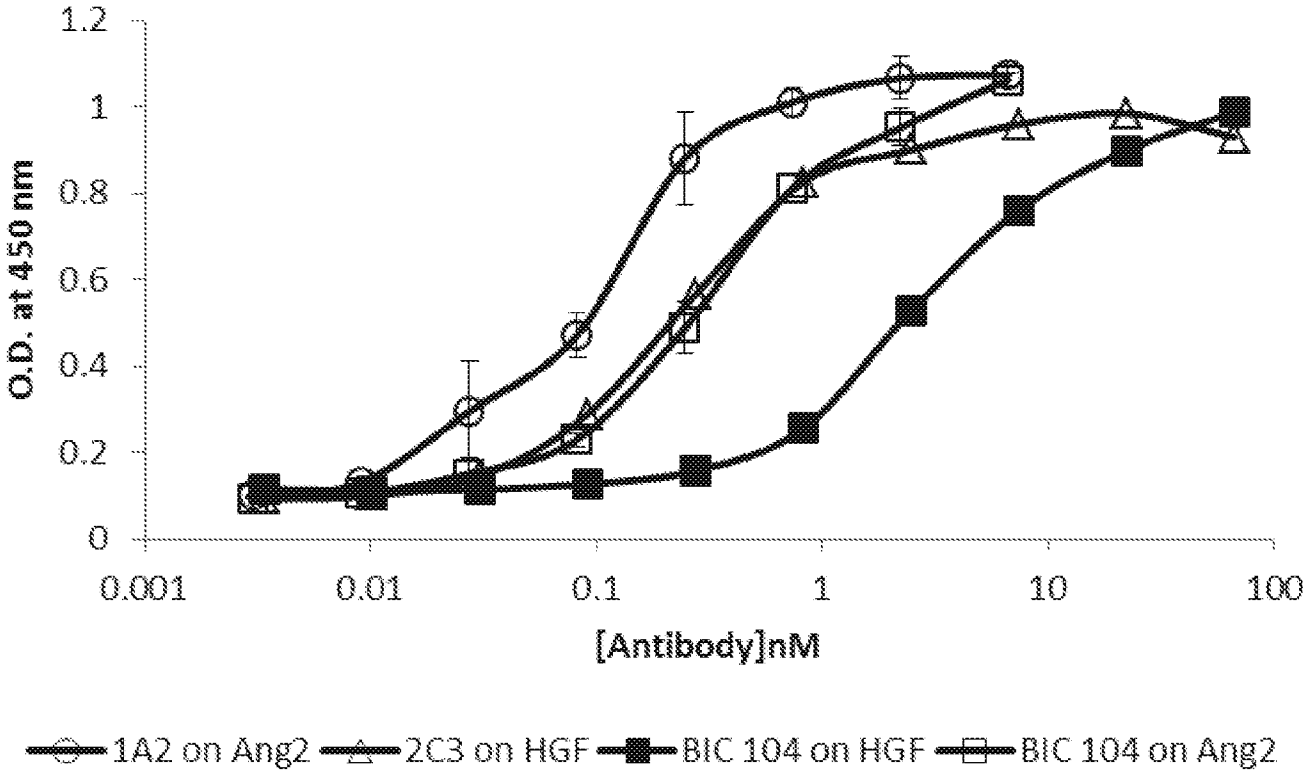

1.2
1
0.8
0.6
0.4
0.2
0
O.D. at 450 nm
0.001
0.01
0.1
1
10
100
[Antibody]nM
1A2 on Ang2
2C3 on HGF
BIC 104 on HGF
BIC 104 on Ang2

FIG. 8A

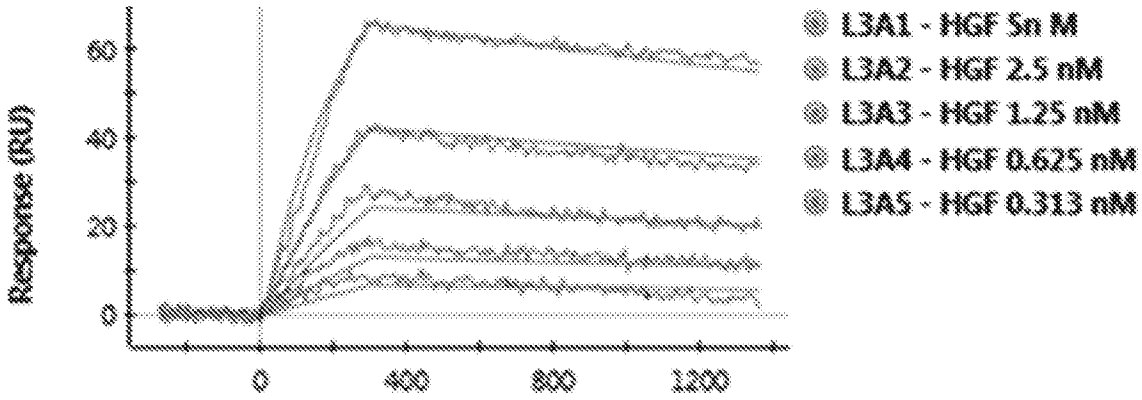


FIG. 8B

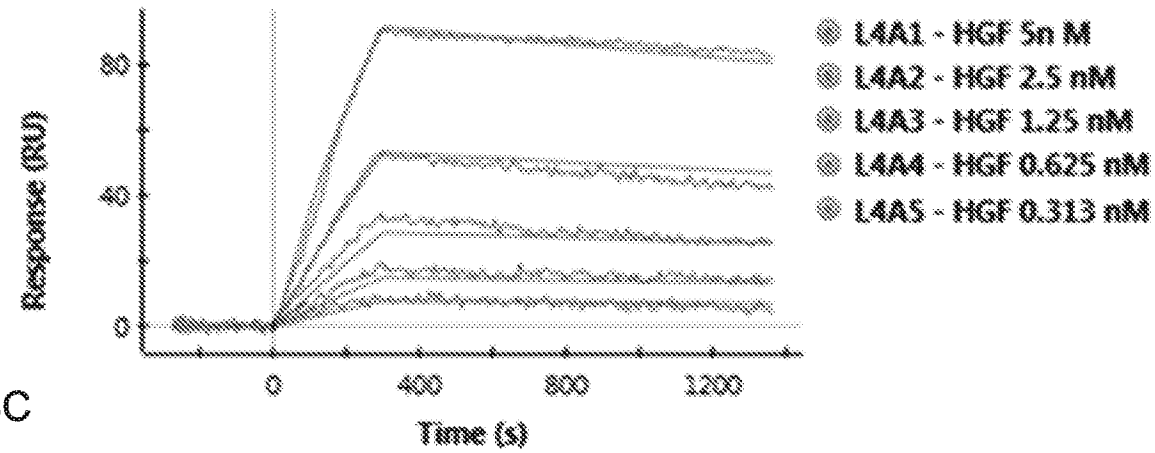


FIG. 8C

	Ka (1/Ms)	Kd (1/s)	KD (M)
BIC 104	8.13E+05	1.67E-04	2.05E-10
2C3 mono	4.43E+05	1.18E-04	2.65E-10

FIG. 9

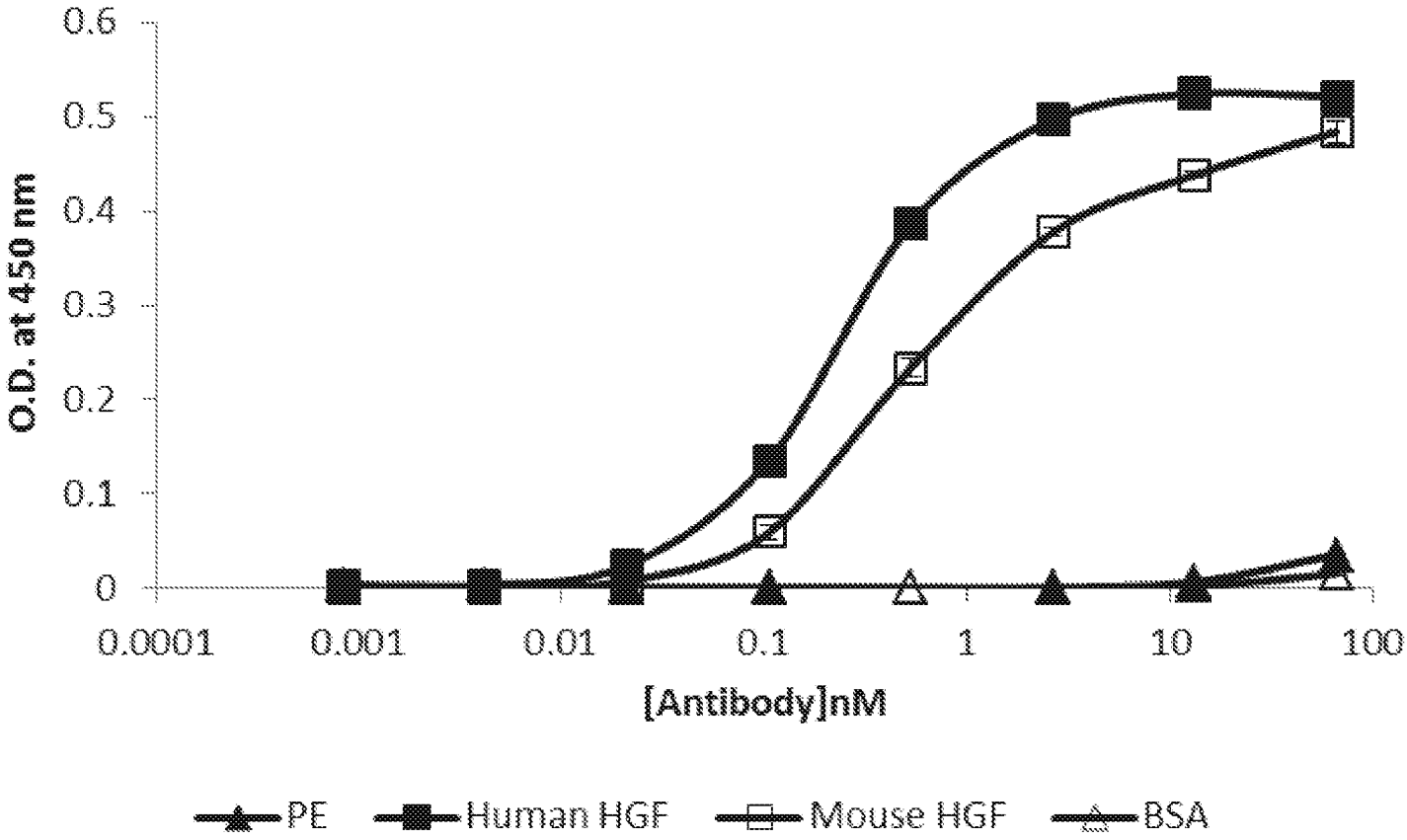

0.6
0.5
0.4
0.3
0.2
0.1
0
O.D. at 450 nm
0.0001
0.001
0.01
0.1
1
10
100
[Antibody]nM
PE
Human HGF
Mouse HGF
BSA

FIG. 10

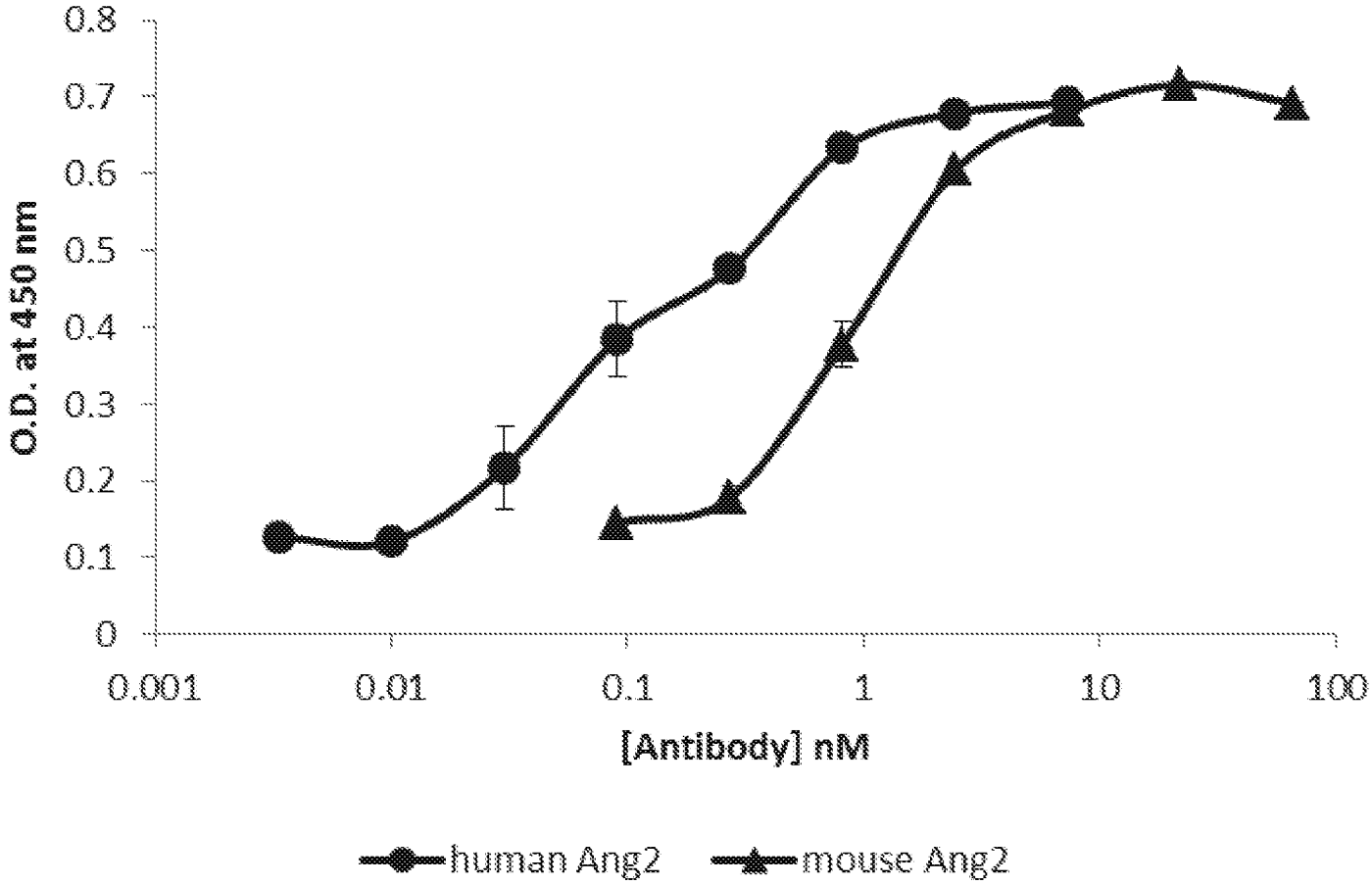

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
O.D. at 450 nm
0.001
0.01
0.1
1
10
100
[Antibody] nM
human Ang2
mouse Ang2

FIG. 11

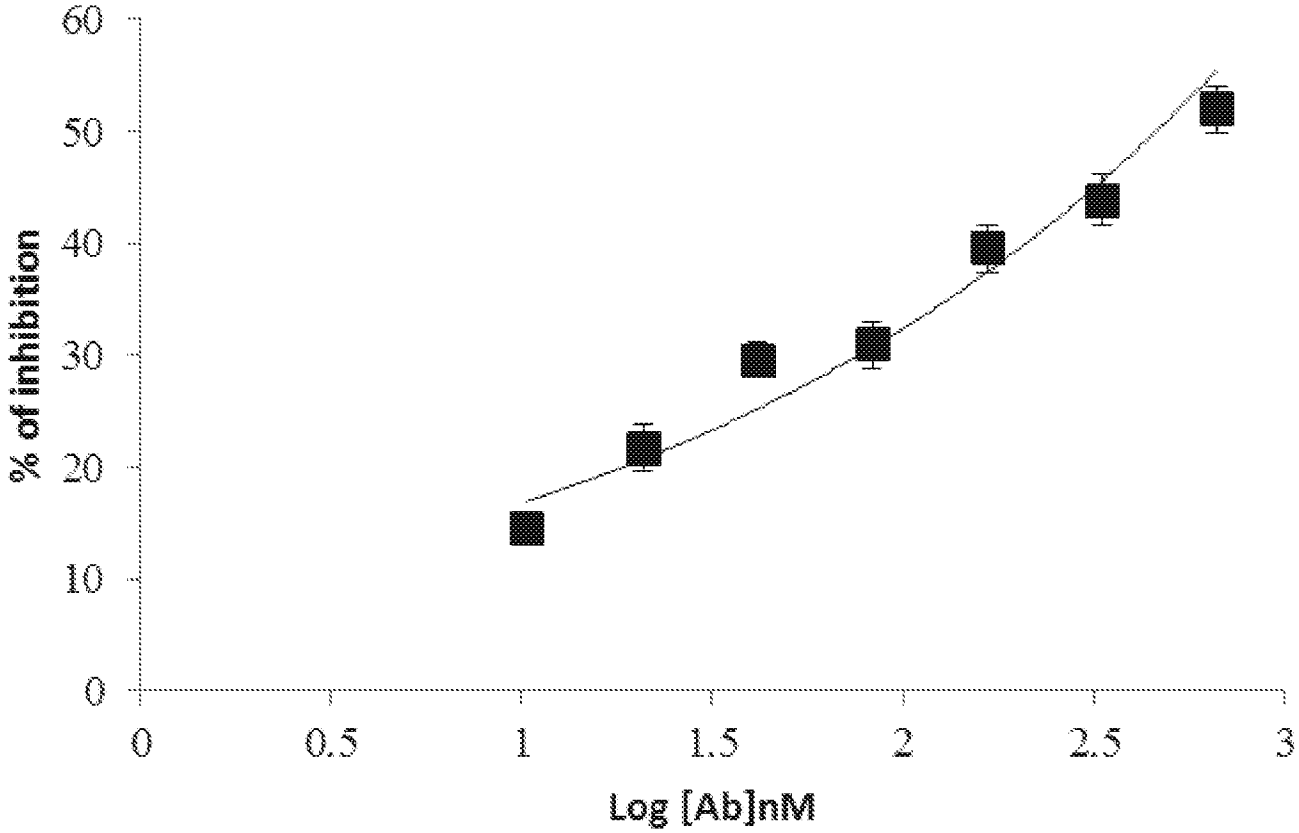


FIG. 12

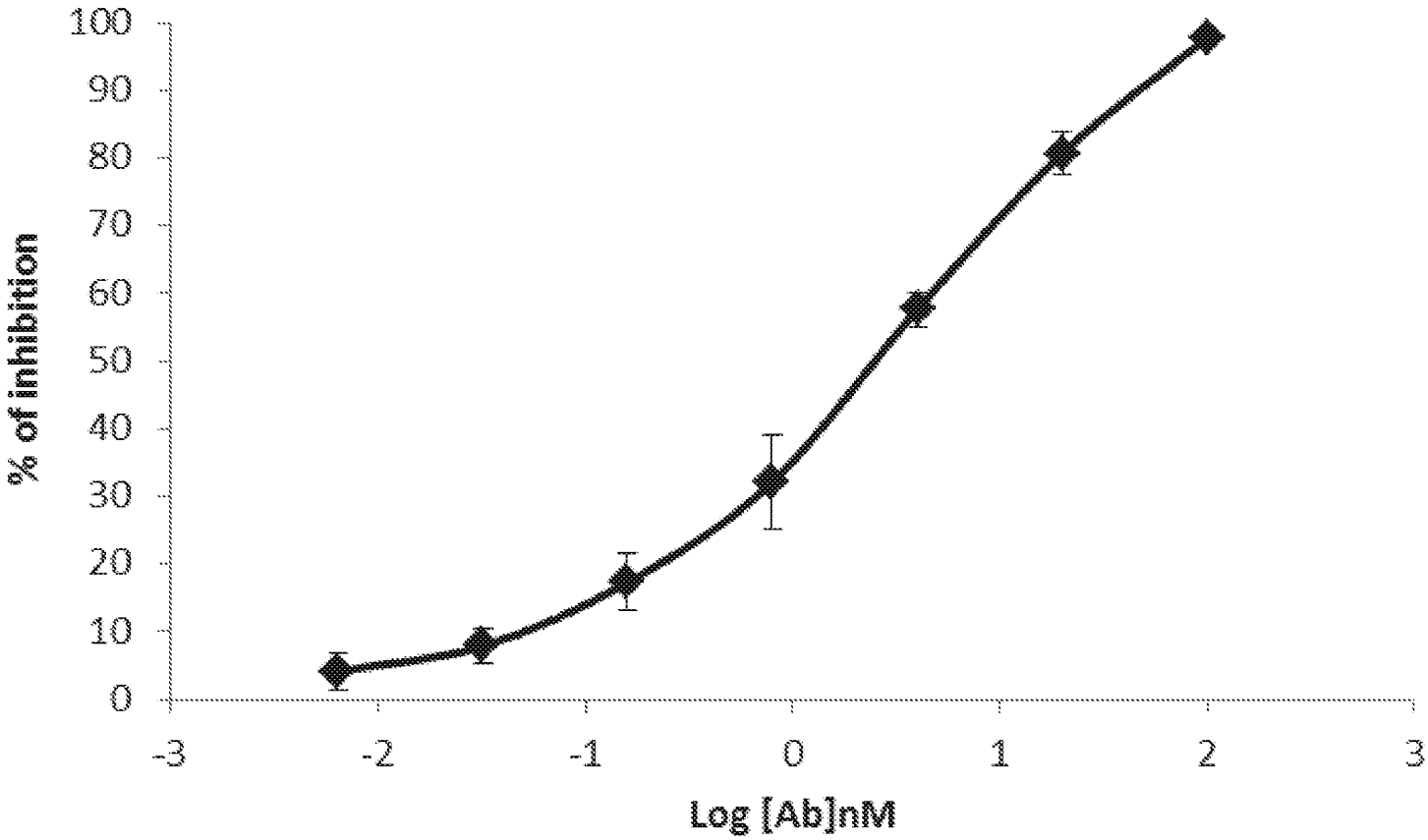


FIG. 13B

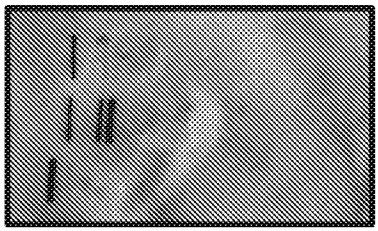


FIG. 13A

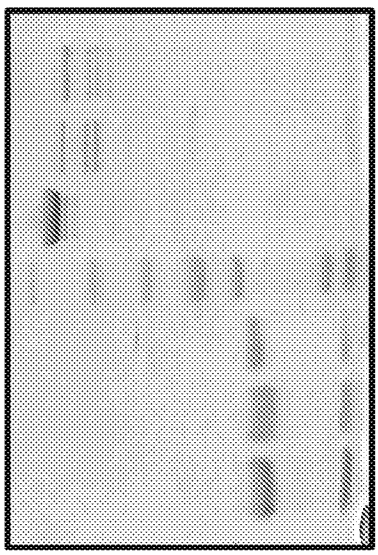


FIG. 14A

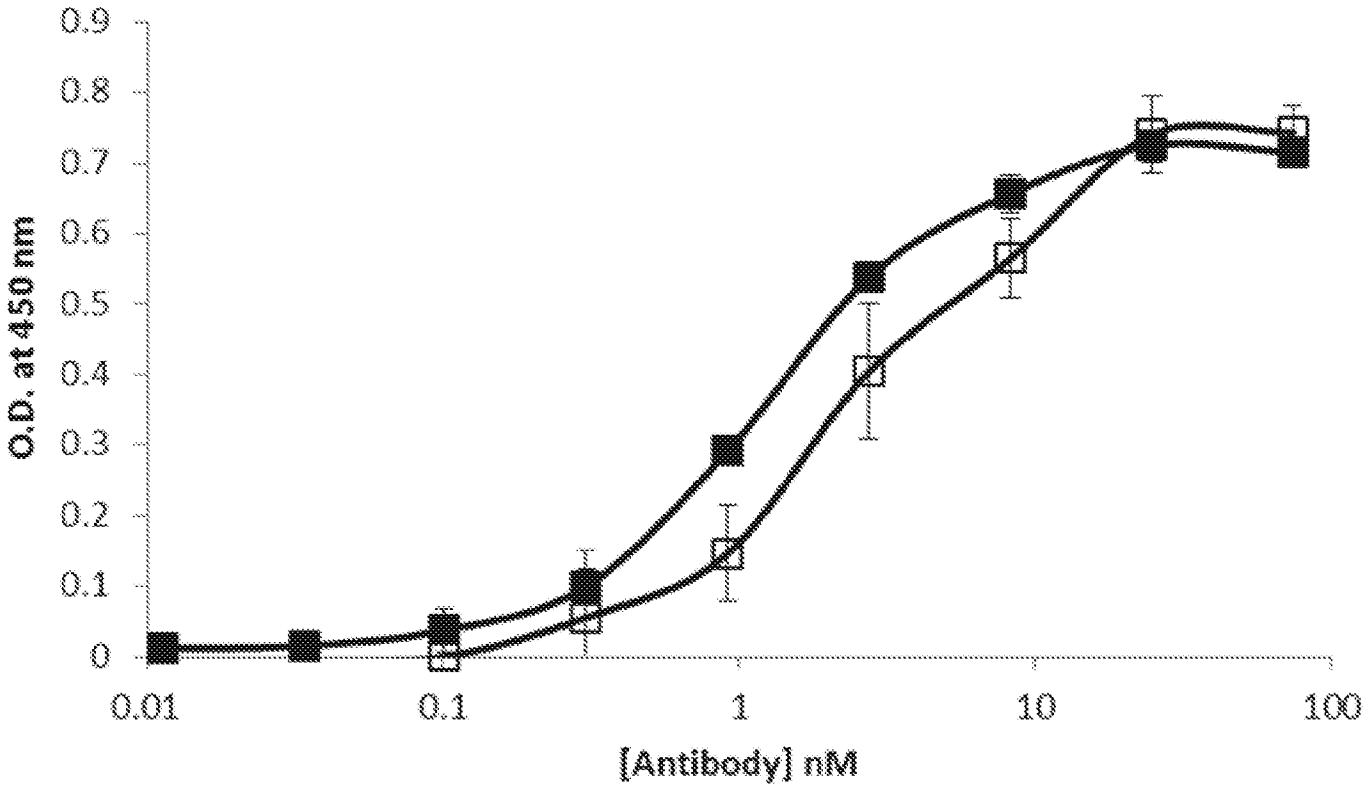


FIG. 14B

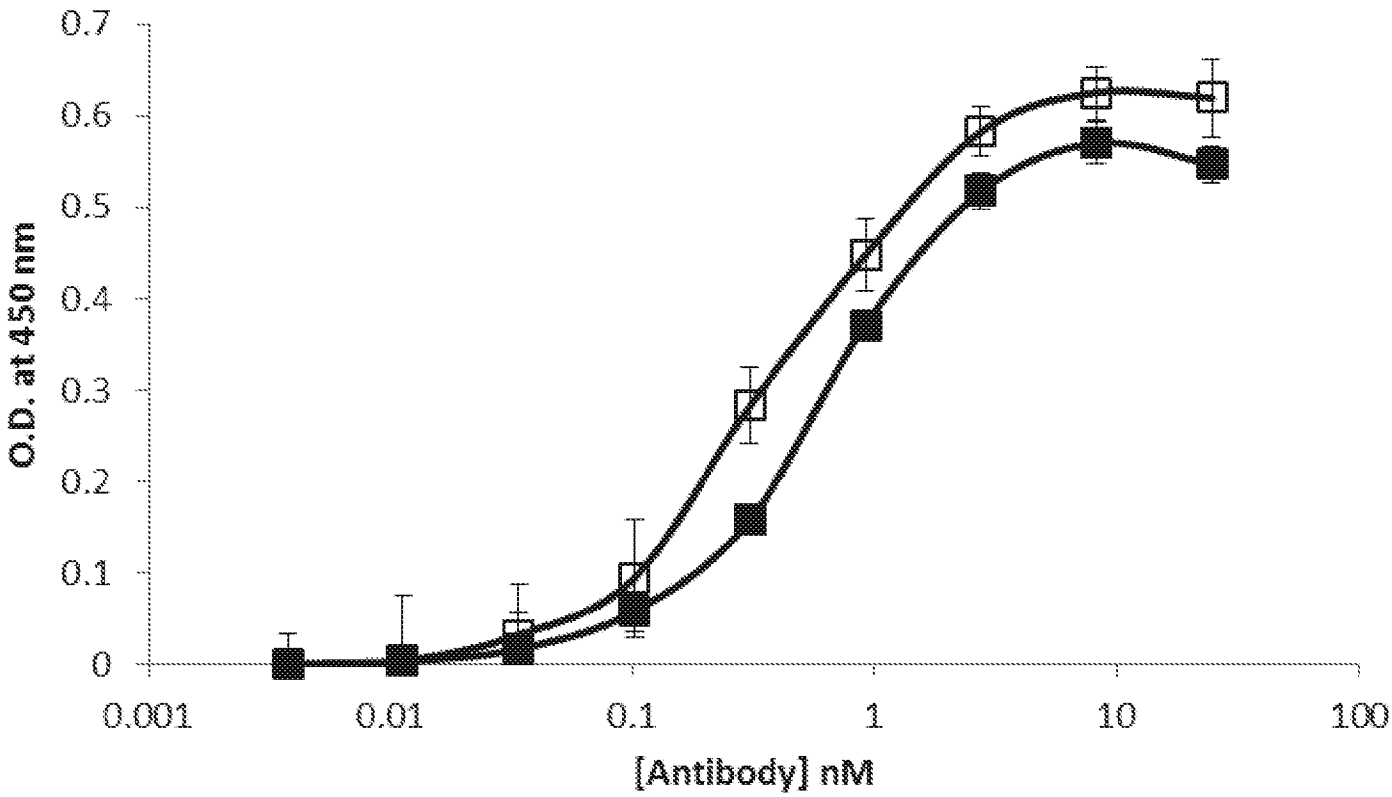


FIG. 15A

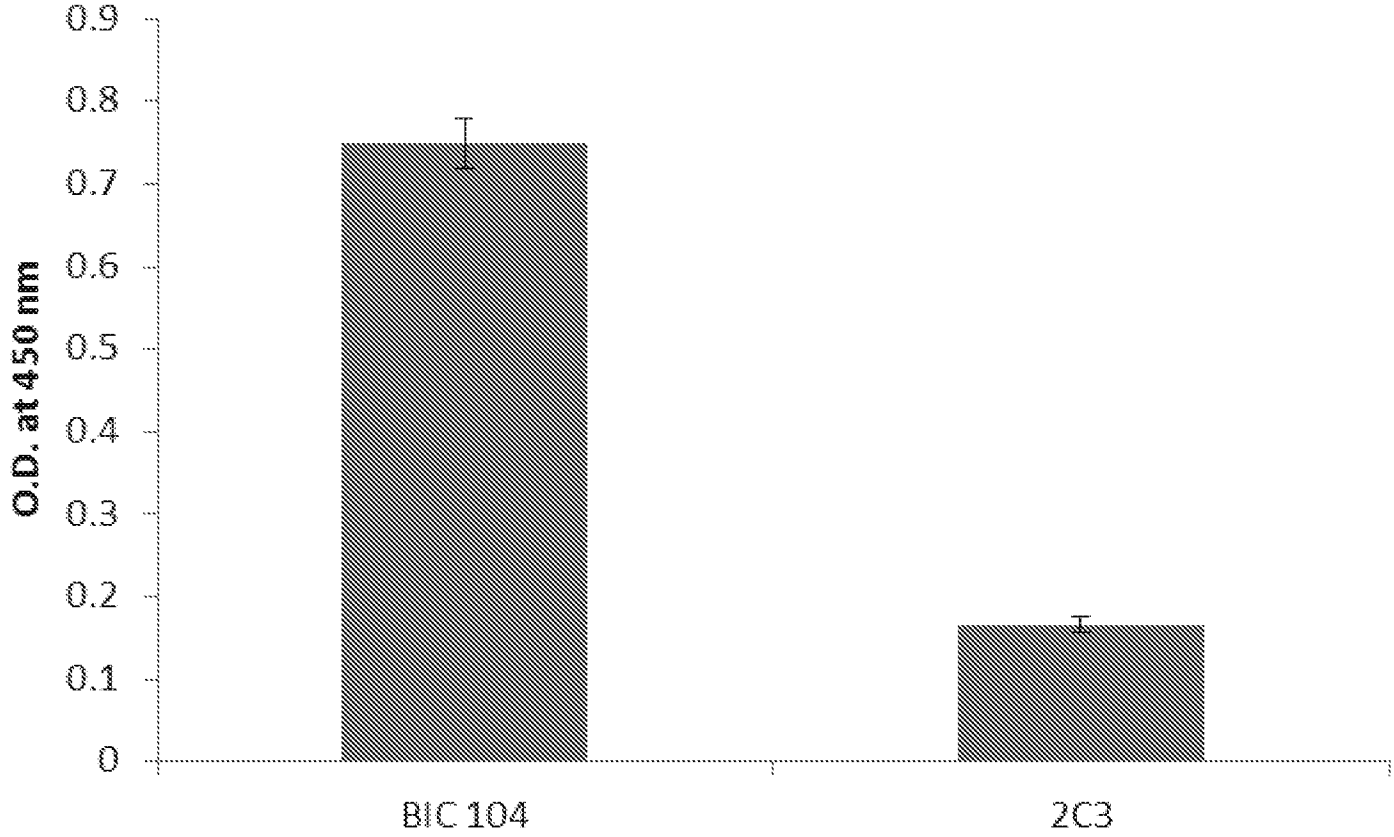


FIG. 15B

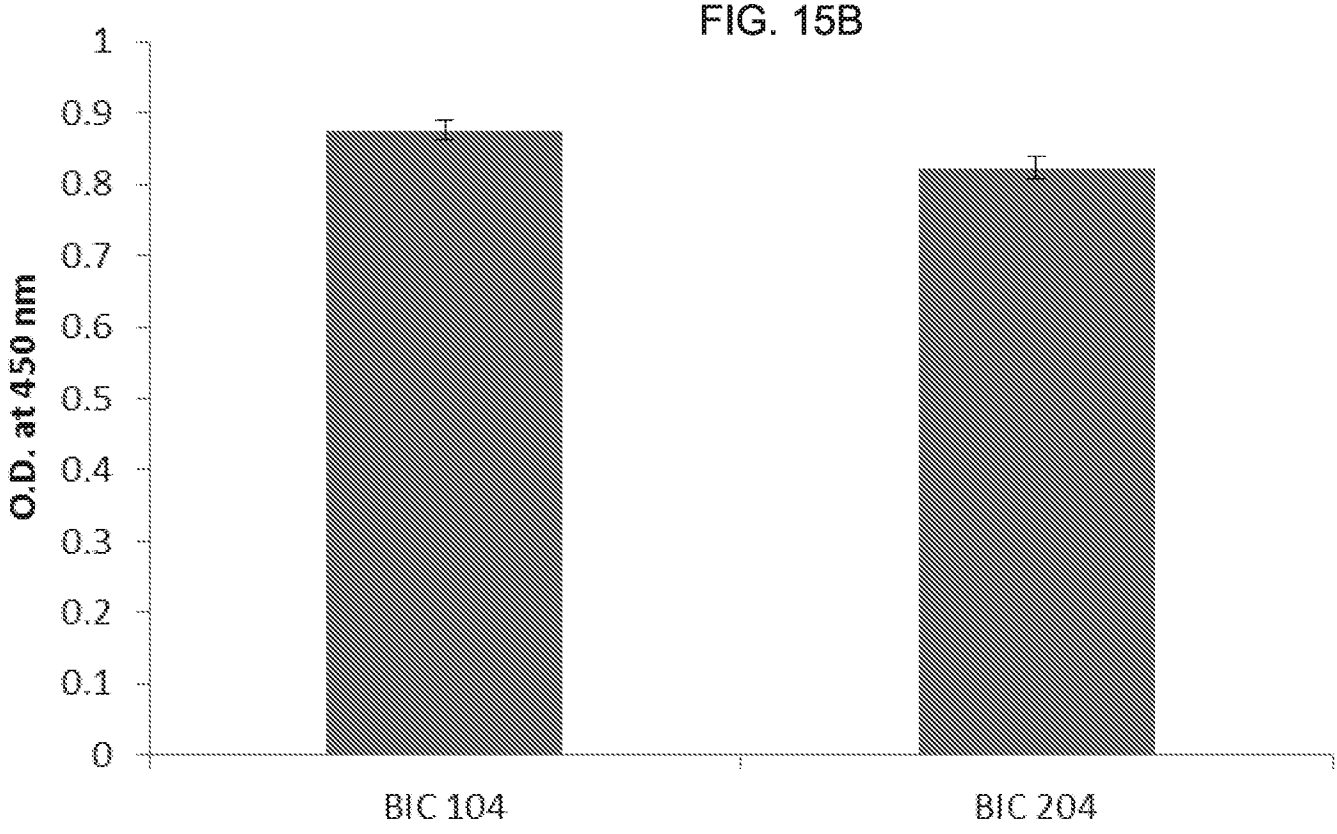


FIG. 16A

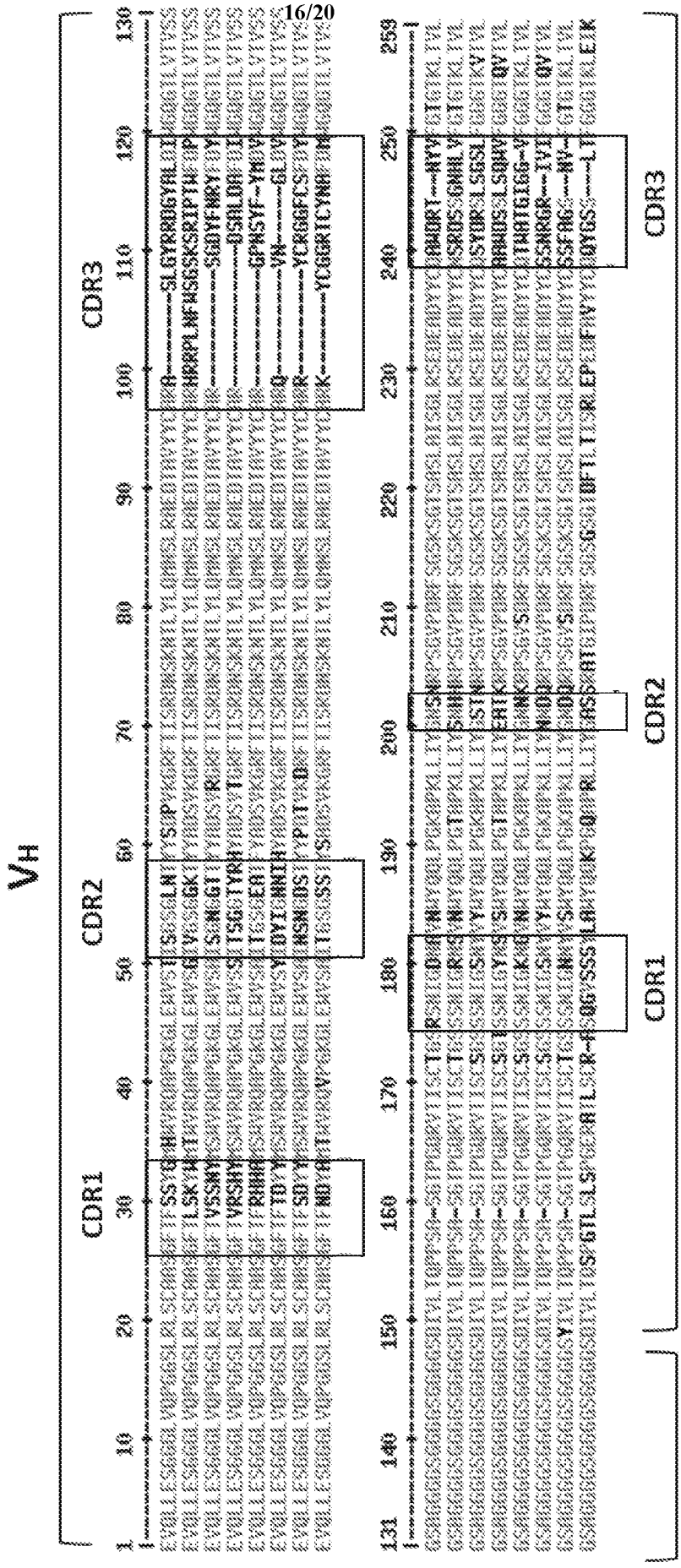


FIG. 16B

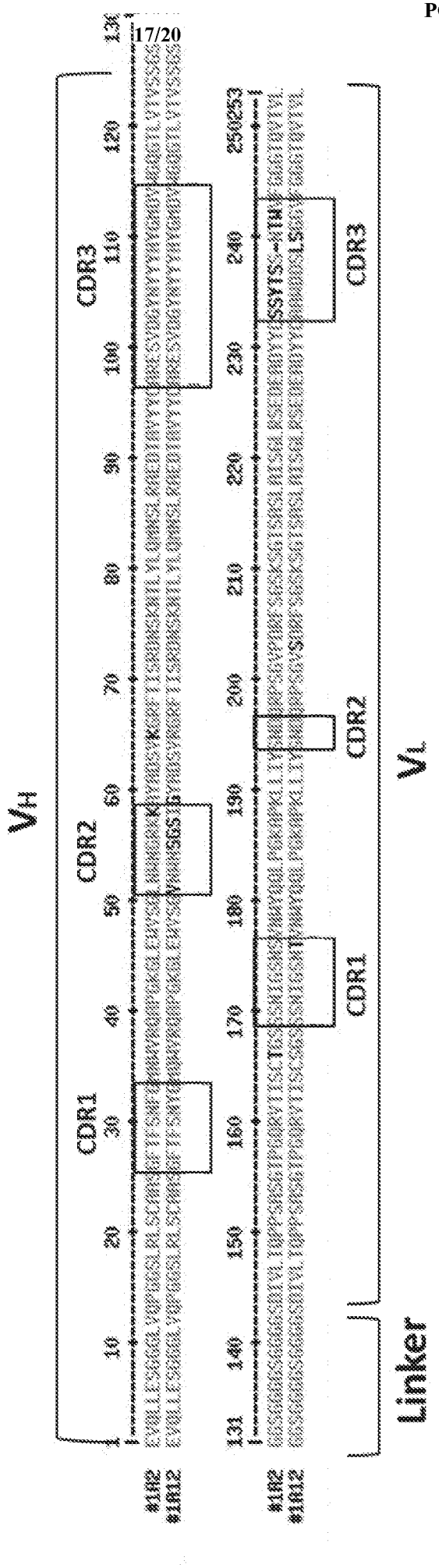


FIG. 17A

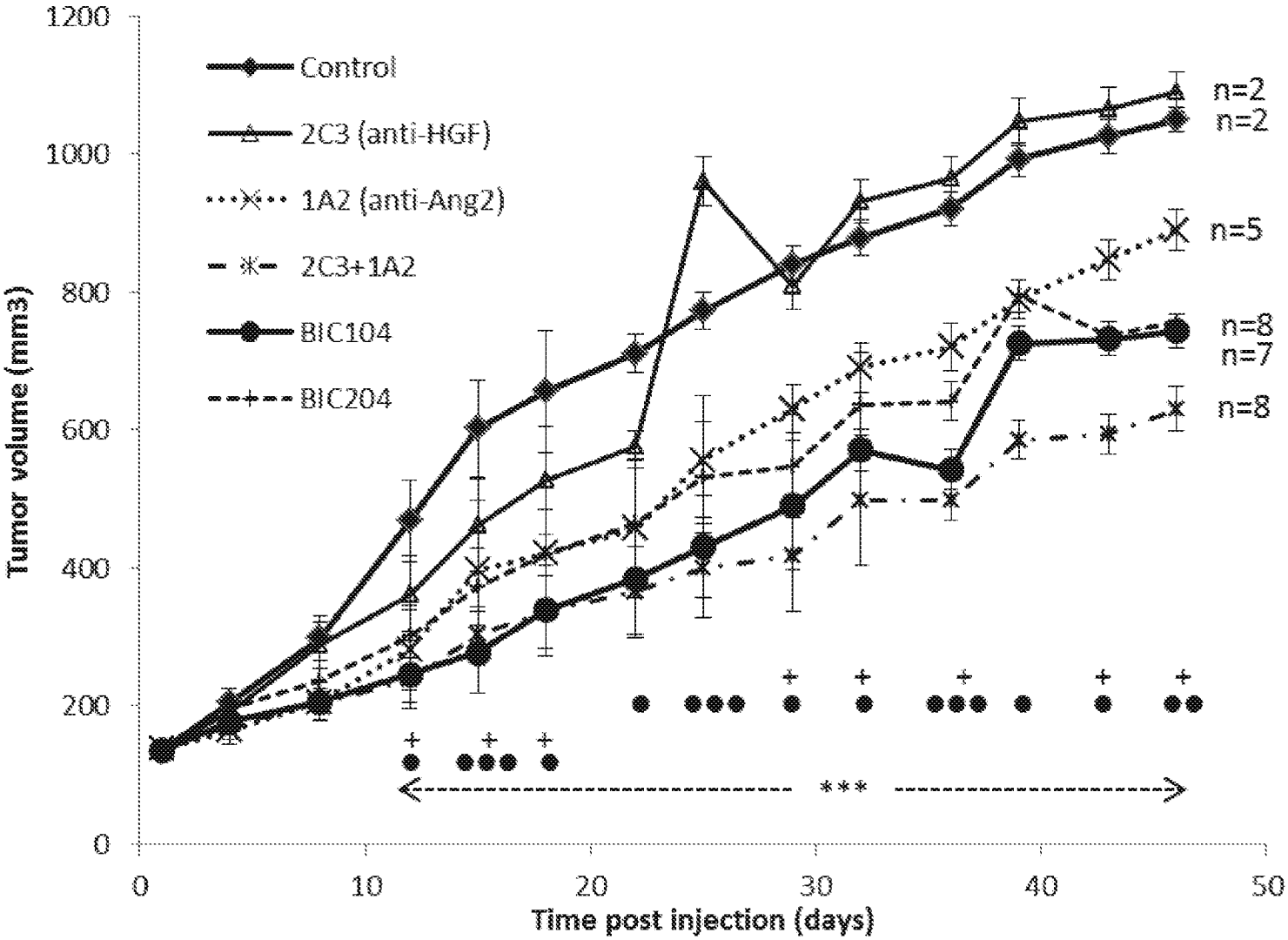

1200
1000
800
600
400
200
0
Tumor volume (mm3)
0
10
20
30
40
50
Time post injection (days)
Control
2C3 (anti-HGF)
1A2 (anti-Ang2)
2C3+1A2
BIC104
BIC204
n=2
n=2
n=5
n=8
n=7
n=8
***

FIG. 17B

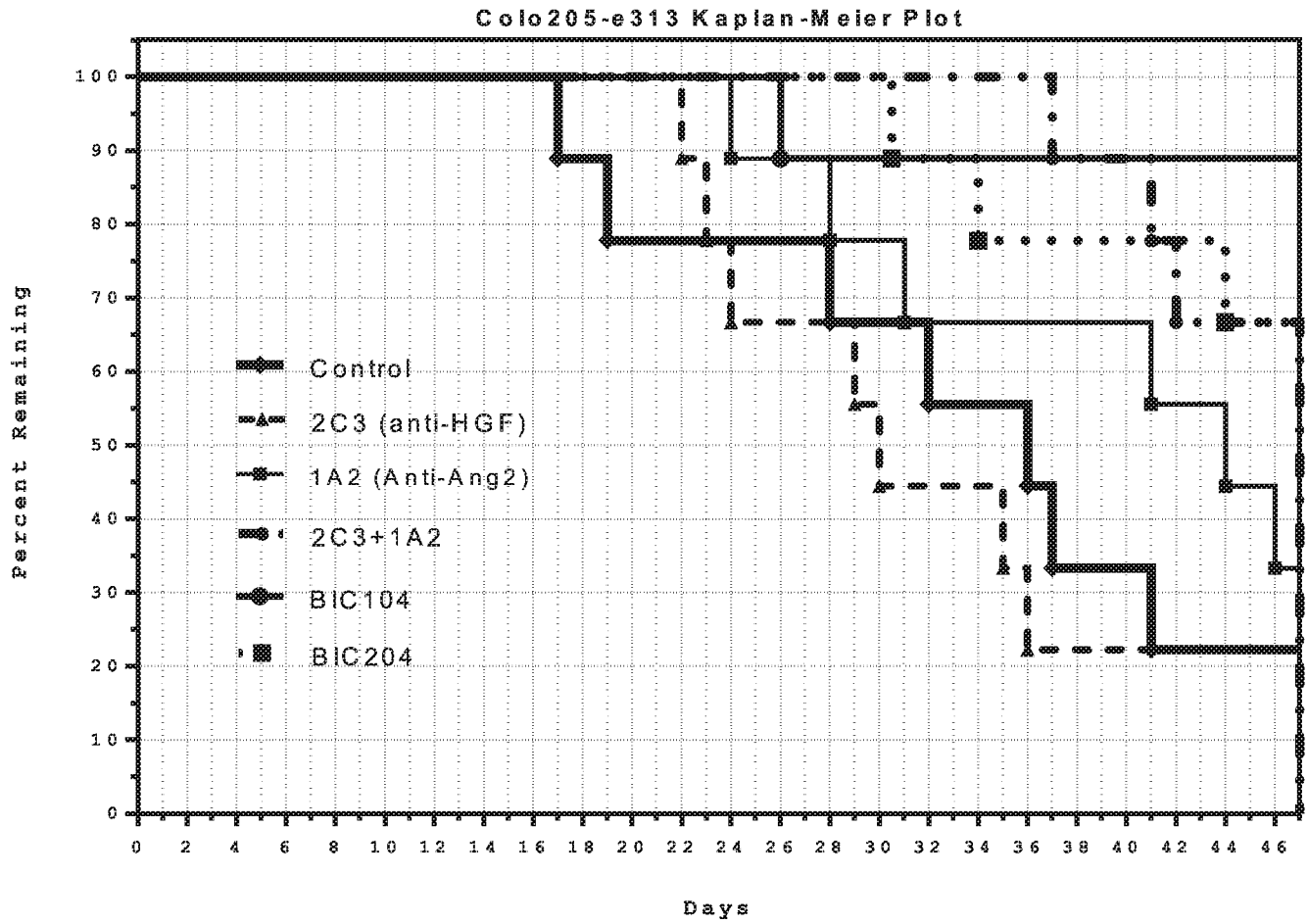
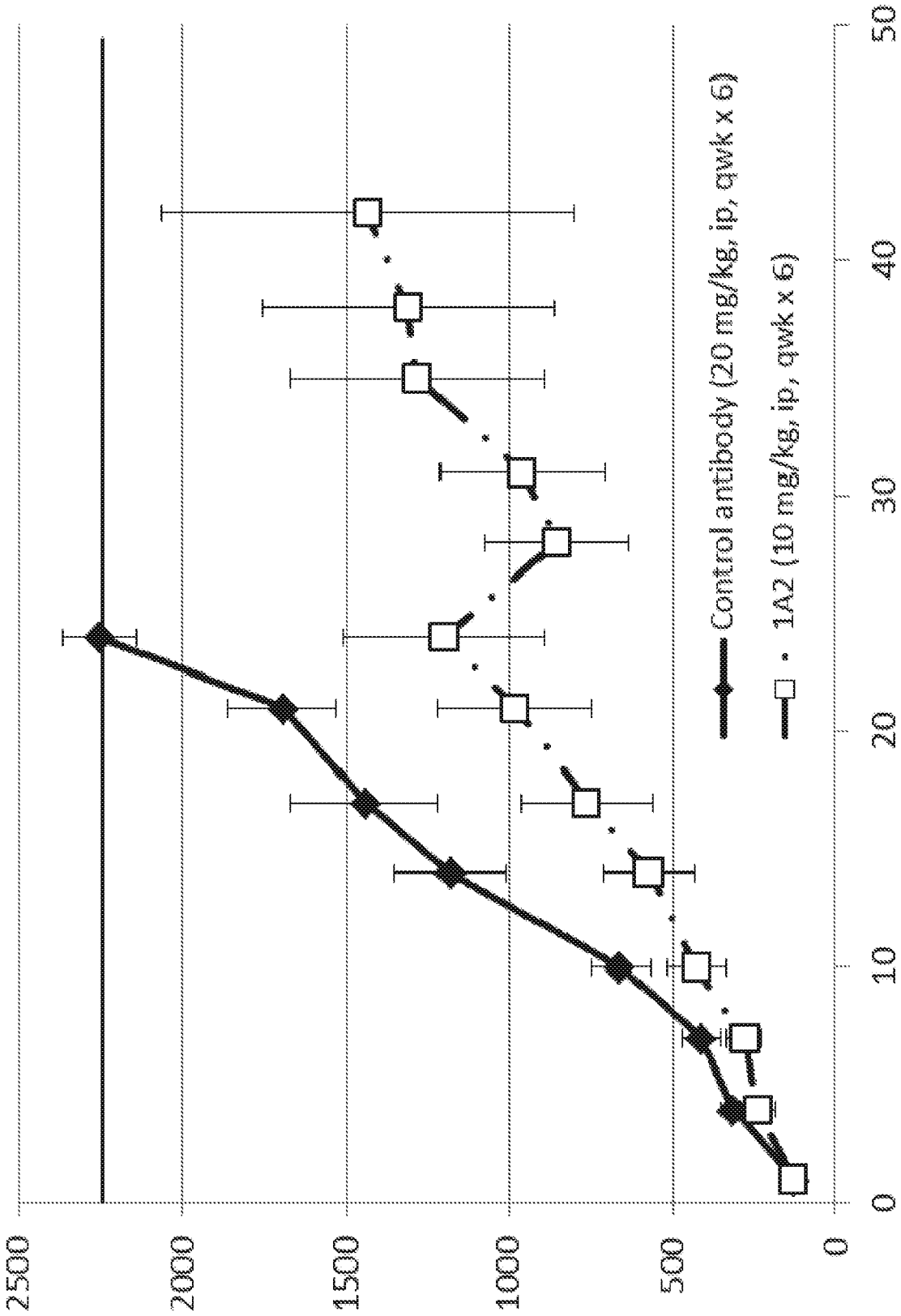

Colo205-e313 Kaplan-Meier Plot
Percent Remaining
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46
Days
Control
2C3 (anti-HGF)
1A2 (Anti-Ang2)
2C3+1A2
BIC104
BIC204

FIG. 18


2500
2000
1500
1000
500
0
0
10
20
30
40
50
Control antibody (20 mg/kg, ip, qwk x 6)
1A2 (10 mg/kg, ip, qwk x 6)

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2014/058159

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/22
ADD.

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/069532 A1 (HOFFMANN LA ROCHE [CH]; BRINKMANN ULRICH [DE]; GRIEP REMKO ALBERT [NO]) 24 June 2010 (2010-06-24)	1,4,5, 13,15, 26, 29-31, 34-38, 40-47,55
Y	page 42 - page 43 example 6 figure 6	39

-/--

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 March 2014	15/05/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bumb, Peter

2

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.
X	LEOW CHING CHING ET AL: "MEDI3617, a human anti-angiopoietin 2 monoclonal antibody, inhibits angiogenesis and tumor growth in human tumor xenograft models.", INTERNATIONAL JOURNAL OF ONCOLOGY MAY 2012, vol. 40, no. 5, May 2012 (2012-05), pages 1321-1330, XP002721374, ISSN: 1791-2423 figure 3	1,4,5, 13,15, 26, 29-31, 34-38, 40-47,55
X	THOMAS M ET AL: "489 LC06, a novel angiopoietin-2 selective human antibody with potent anti-tumoral and anti-angiogenic efficacy in different xenograft models", EUROPEAN JOURNAL OF CANCER. SUPPLEMENT, PERGAMON, OXFORD, GB, vol. 8, no. 7, 1 November 2010 (2010-11-01), pages 156-157, XP027498179, ISSN: 1359-6349, DOI: 10.1016/S1359-6349(10)72196-0 [retrieved on 2010-11-01] the whole document	1,4,5, 13,15, 26, 29-31, 34-38, 40-47,55
X	WO 2007/068895 A1 (ASTRAZENECA AB [SE]; ASTRAZENECA UK LTD [GB]; BROWN JEFFREY LESTER [US) 21 June 2007 (2007-06-21) page 29, line 6 table 1 page 38	1,4,5, 13,15, 26, 29-31, 34-38, 40-47,55
Y	WANG MING-HAI ET AL: "Potential therapeutics specific to c-MET/RON receptor tyrosine kinases for molecular targeting in cancer therapy", ACTA PHARMACOLOGICA SINICA,, vol. 31, no. 9, 1 September 2010 (2010-09-01), pages 1181-1188, XP009146790, table 1	39

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2014/058159

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

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

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

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

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

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an 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, 13, 15(completely); 4, 5, 26, 29-31, 34-47, 55(partially)

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 13, 15(completely); 4, 5, 26, 29-31, 34-47, 55(partially)

   An antibody comprising an ANG2 recognition region which comprises CDR amino acid sequences as set forth: (i) SEQ ID NOs: 7-12; or (ii) SEQ ID NOs: 107-112. Subsequent uses and nucleic acids.

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2. claims: 2, 6-12, 14, 16-25, 27, 28, 32, 33, 48-54(completely); 26, 29-31, 34-47, 55(partially)

   A bispecific antibody comprising a first antigen-binding site that specifically binds to human HGF and a second antigen-binding site that specifically binds to human ANG2. Subsequent uses and nucleic acids.

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3. claims: 3(completely); 4, 5, 26, 29-31, 34-47, 55(partially)

   An antibody comprising an HGF recognition region which comprises CDR sequences selected from the group consisting of: (i) SEQ ID NOs: 1-6 (ii) SEQ ID NOs: 17-22 (iii) SEQ ID NOs: 23-28 (iv) SEQ ID NOs: 29-34 (v) SEQ ID NOs: 35-40 (vi) SEQ ID NOs: 41-46 (vii) SEQ ID NOs: 47-52 and (viii) SEQ ID NOs: 53-58. Subsequent uses and nucleic acids.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2014/058159

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